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MOLECULAR ACTION OF DIURETICS

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Summary: This review will address several recent findings regarding the interaction of so called loop diuretics of the furosemide type with the $Na^+2Cl^-K^+$ cotransporter in the thick ascending limb of the loop of Henle (TAL): (i) The organ selectivity of these transport inhibitors is caused by their secretion by the proximal tubule leading to an increase in their luminal concentration. We have examined whether probenecid, a selective inhibitor of proximal organic anion secretion reduces the diuretic effect of known loop diuretics and we show that all tested compounds: furosemide (FUR), piretanide (PIR), bumetanide (BUM), azosemide (AZO) loose part of their diuretic effect when administered in the presence of probenecid. (ii) Previously we have shown that all loop diuretics of the furosemide type require an acidic group for the binding to the $Na^+2Cl^-K^+$ cotransporter. This is a carboxylate group in case of FUR, PIR and BUM, a tetrazolic acid group in case of AZO, and a sulfonylurea group in case of torasemide (TOR). Now we show that TOR-derivates with an even less acidic sulfonylurea group (pK_a >8) still are very potent inhibitors of the $Na^+2Cl^-K^+$ cotransporter. Experiments at various pH-values indicate that, even for these substances, it is only the anionic form which inhibits the $Na^+2Cl^-K^+$ cotransporter. (iii) Most of the loop diuretics with the exception of TOR are rather hydrophilic (FUR, PIR, BUM, AZO). Now we have examined whether one can design compounds which sustain their inhibitory effect on the $Na^+2Cl^-K^+$ cotransporter, but are highly lipophilic. We found that cyclo-alkyl substitutions at the tolyl- and sulfonylurea-moieties of TOR lead to highly lipophilic compounds with very high affinity to the $Na^+2Cl^-K^+$ cotransporter. (iv) Previous data suggested that loop diuretics bind to the $Na^+2Cl^-K^+$ cotransporter at the extracellular side of the luminal membrane. Now we have designed impermeable macromolecular derivates of

PIR and show that these macromolecular probes inhibit the $Na^+2Cl^-K^+$ cotransporter as well as PIR. (v) Previous data indicated that loop diuretics interrupt the macula densa feedback mechanism, and circumstantial evidence suggested that the uptake of Cl^- and/or Na^+ by macula densa cells may occur via the $Na^+2Cl^-K^+$ cotransporter. Now we show that macula densa cells sense the luminal NaCl concentration via FUR sensitive $Na^+2Cl^-K^+$ cotransport.

The mode of action of loop diuretics: Loop diuretics bind to the $Na^+2Cl^-K^+$ cotransporter of the thick ascending limb (TAL) of the loop of Henle (1,2) and inhibit the coupled uptake of all participating ions. This effect is instantaneous and easily reversible. The inhibition of the Na^+ influx reduces the requirement to remove Na^+ from the cell via the (Na^++K^+)-ATPase and thus reduces ATP- (3,4) and O_2-consumption of TAL cells (5) and hence protects this nephron segment against hypoxic or ischaemic damage (6). From studies with a large number of derivates (7-10) we have some understanding of the molecular requirements for the interaction of a compound with the $Na^+2Cl^-K^+$ cotransporter. Fig. 1 shows several loop diuretics: furosemide (FUR), piretanide (PIR), bumetanide (BUM), azosemide (AZO), torasemide (TOR), and gives the concentrations for the half maximal inhibition (IC_{50}) of active NaCl reabsorption in the rabbit cortical thick ascending limb of the loop of Henle (cTAL). It is important to note that all molecules share in common several properties: (i) All substances are more or less acidic. The pK_a-values of the -COOH group (FUR, PIR, BUM) are around 4, that for the tetrazolic group (AZO) is around 5, and that for sulfonylurea (TOR) is around 7. (ii) all substances except TOR possess a sulfonamide group. In case of TOR, the nitrogen of the pyrimidine ring probably serves the same

function (11). (iii) all substances possess a secondary or tertiary amine, and (iv) all have an apolar residue. Still most substances except TOR are rather hydrophilic.

In the following we will address, on the basis of recent findings from our laboratories, a few loosely related questions regarding (i) the secretion of loop diuretics by the proximal tubule and its inhibition by probenecid, (ii) the relevance of the acidic moiety of loop diuretics, (iii) the lipophilicity of loop diuretics, (iv) the site of interaction of loop diuretics with the TAL, and (v) the effect of FUR on macula densa cells.

Secretion by the proximal tubule, a prerequisite for the inhibitory effect of loop diuretics: It has been shown recently (12) that loop diuretics are secreted by the proximal tubule via basolateral uptake trough the so-called PAH-system. This prompted us to ask the question whether the secretion of all used loop diuretics is equally sensitive towards an inhibitor of basolateral anion uptake, namely probenecid (13). This question could be clinically relevant because it cannot be anticipated that proximal secretion of all loop diuretics would be equally affected by other organic acids such as probenecid. Fig. 2 summarizes the results from rat clearance experiments for PIR, BUM, FUR, and AZO. It is evident that comparable diuretic responses to any of these diuretics were almost equally attenuated by increasing doses of probenecid. These findings indicate: (i) The secretion of all of these loop diuretics is highly relevant for their effect in the TAL segment. The increase in luminal concentration by secretion and by water

reabsorption is the only cause for the organ selectivity of these drugs. In fact, the $Na^+2Cl^-K^+$ cotransporter is present in many cells of the body, and its sensitivity towards loop diuretics appears to be similar in all these cells (2,14). (ii) None of the tested diuretics has any detectable advantage over the others. I.e., a comparable diuretic effect is attenuated by probenecid comparably, irrespective of the used diuretic. This finding is surprising since the used doses of the diuretics were quite different: 17 μmol/kg PIR, 27 μmol/kg BUM, 90 μmol/kg FUR and AZO, and it may be explained by affinity sequences for the basolateral membrane organic anion transporter comparable to those on the $Na^+2Cl^-K^+$ cotransporter.

All loop diuretics inhibit in their anionic form: Previous data suggested that all loop diuretics act in their anionic form (7). For example, the pK_a of FUR of 3.6 predicts that some 99.9 % of the total luminal concentration of FUR is in the anionic form. On the other hand, we have found that TOR and its derivates are also strong inhibitors of the $Na^+2Cl^-K^+$ cotransporter (8,15), but the pK_a values of these compounds are much less acidic. Recently, we have examined even less acidic derivates (16) with pK_a values as high as 9.0, and found that these substances still were strong inhibitors of the $Na^+2Cl^-K^+$ cotransporter in the rabbit cTAL segment. In case of the compound BM 10 (c.f. Fig.4) with a pK_a value of 9.0, only 2.5 % would be anionic at the pH of 7.4, at which we carried out the experiments. Rabbit cTAL segments were perfused with solutions containing 0.5 μmol/l BM 10 at various pH-values. The results are shown in Fig. 3. It is evident that the inhibitory effect is enhanced with alkaline pH. This indicates that only the anionic moiety of this compound interacts with the $Na^+2Cl^-K^+$

cotransporter. In extension, this finding implies that the IC_{50}-value for this compound, which was determined at pH 7.4, would be almost two decades lower if expressed as the concentration of the anionic moiety of this compound (IC_{50} = 12 nmol/l instead of 0.5 μmol/l).

Inhibitors of the $Na^+2Cl^-K^+$ cotransporter can be designed as highly lipophilic compounds: Most loop diuretics are rather hydrophilic (FUR, PIR, BUM, AZO). Only TOR shows an octanol/water partition of around 3. It was tempting to examine whether one could design even more lipophilic compounds related to TOR which still would be inhibitors of the $Na^+2Cl^-K^+$ cotransporter (16). This effort was triggered by the hypothesis that such compounds would dissolve into a larger volume of distribution and, hence, be excreted more slowly. Furthermore, such compounds might be able to cross the blood brain barrier more easily and have direct effects on the volume of glial cells (BM, JD unpublished). Fig. 4 shows several derivates of TOR, and summarizes the pK_a values, the octanol/water partition (as log P = [octanol]/[water]), and the IC_{50} value in rabbit cTAL segments. It is evident that the tolyl- and the isopropyl-residues of TOR can be replaced by cyclo-alkyls. This leads to an increase in the octanol/water partition, an increase in pK_a value, and a reduction in the IC_{50} value, indicating that these compounds have a very high affinity to the $Na^+2Cl^-K^+$ cotransporter. An optimal structure is e.g. the compound BM 10 with apparent IC_{50} values of 0.5 μmol/l. The IC_{50} values would be even much smaller if one takes into account the above finding that the inhibition is caused only by the anionic moiety (IC_{50}^{cor} values in Fig. 4). We conclude that it is possible to design highly lipophilic loop diuretics which may offer some therapeutical advantages.

The interaction with the $Na^+2Cl^-K^+$ cotransporter in the luminal membrane of the cTAL segment occurs at an extracellular binding site: Several findings have suggested that drugs like FUR need not being incorporated into the cTAL cell to exert their effect. (i) We have noted that the effect of FUR (1) and PIR (4) was instananeous and rapidly reversible. The inhibition occured as rapidly as we were able to change the lumen perfusate (<< 1 sec), and the recovery after removal of the drugs was complete within a few seconds. These findings would not be expected for a compound entering the cell. (ii) We have measured the cytosolic Cl^--activity with ion selective microelectrodes in cTAL cells (17). The used ion exchanger is several hundred times more sensitive for FUR when compared to Cl^-. Still, we were able to show that the apparent cytosolic Cl^--activity signal fell rapidly after the addition of furosemide. Both arguments, even though strongly suggestive, do not prove that FUR interacts at the extracellular side. Therefore, we have designed macromolecular probes of piretanide with a molecular mass of 5300 Dalton and examined their effect in rabbit cTAL segments (18). We found that dextran- as well as polyethyleneglycol-macromolecules inhibited the $Na^+2Cl^-K^+$ cotransporter with IC_{50} values very similar to that for PIR. Hence, we conclude that the binding site for loop diuretics of the $Na^+2Cl^-K^+$ cotransporter is easily accessible at the extracellular side of the luminal membrane. These and other macromolecular probes of PIR are currently used to design antibodies against the $Na^+2Cl^-K^+$ carrier. When given systemically these macromolecular PIR derivatives show only a very moderate diuresis. This indicates that these substances cannot be secreted by the proximal tubule (c.f. above).

Macula densa cells sense luminal NaCl concentration via furosemide sensitive $Na^+2Cl^-K^+$ cotransport: It has been known for some time that loop diuretics inhibit the so called feedback response between macula densa cells and single nephron filtration rate (19). Furthermore, it has been shown that the feedback response is produced by the luminal Na^+ (20) or Cl^- concentration (21). This has prompted us to examine directly whether the macula densa cells possess the $Na^+2Cl^-K^+$ cotransporter. We have used an electrophysiological approach to examine this question. Rabbit cTAL segments with the glomerulus and the macula densa segment attached were perfused *in vitro* and the transepithelial voltages as well as the membrane voltage across the basolateral membrane of macula densa cells was examined (22). The key finding is shown in Fig. 5. The baolateral membrane voltage (V_{bl}) of macula densa cells hyperpolarized by some 14 mV when FUR was added to the luminal perfusate. Similarly, a hyperpolarization of V_{bl} was observed when the NaCl concentration in the lumen was reduced from 150 to 30 mmol/l. The examination of the conductance properties of these cells revealed that the luminal membrane is K^+- and the basolateral membrane Cl^--conductive (22). All these findings are compatible with the view that the luminal membrane of macula densa cells is equipped with the $Na^+2Cl^-K^+$ cotransporter, and that the general cell model of the macula densa cell is identical to that described for the TAL segment (2,23). It should be noted, however, that the transport rates across the macula densa cells are much lower than those across the TAL segment (22). Thus, it is likely that the $Na^+2Cl^-K^+$ carrier mediated uptake in macula densa cells serves to sense the concentrations in the tubule lumen and to trigger a thus far unknown transducing mechanism which translates the cytosolic signal in macula densa cells into the response of renin producing cells.

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Legends:

Fig.1: Chemical structures of diuretics and their concentrations required for half maximal inhibition of equivalent short circuit current (IC_{50} values) in isolated *in vitro* perfused rabbit thick ascending limbs of Henle's loop.

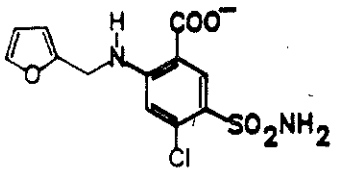
Fig.2: Attenuation of the diuretic response to FUR (= furosemide, 90 µmol/kg prime and same dose over one hour), PIR (= piretanide, 17 µmol/kg and same dose over one hour), BUM (= bumetanide, 27 µmol/kg and same dose over one hour), and AZO (= azosemide, 90 µmol/kg and same dose over one hour) by increasing doses of probenecid (0.07-0.28 mmol/kg).

Rat clearance experiments. The urinary Na^+ excretion is given for the control period, after the respective diuretic, and after additional application of probenecid. Note that probenecid attenuates the natriuretic response equally well for all diuretics used.

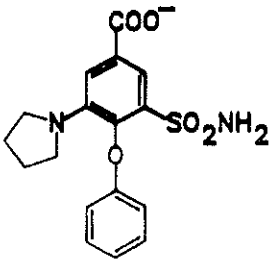
Fig. 3: pH dependence of the inhibitory effect of a sulfonylurea diuretic (BM 10, structural formula c.f. Fig. 4) in isolated perfused rabbit cTAL segments. The remaining equivalent short circuit current (in percent of control) is shown for different luminal pH values. The total concentration of BM 10 was 0.5 μmol/l at both pH values. The anionic form was 0.005 and the protonated form 0.495 μmol/l at pH 7.0 and 0.07 and 0.43 μmol/l respectively at pH 8.2. Note that the anionic form determines the inhibitory effect.

Fig. 4: Lipophilic sulfonylurea diuretic compounds. The chemical structure, the pK_a value, the log of the octanol/water partition (log P), the total luminal concentration for half maximal inhibition of equivalent short circuit current in isolated perfused rabbit cTAL segments (IC_{50} in μmol/l) and the concentration of the anionic form (IC_{50}^{cor}) are given for the individual compounds. Note that the highly lipophilic compound BM 10 has a very strong inhibitory effect.

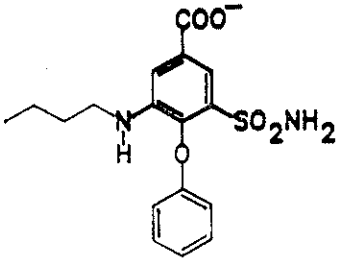
Fig. 5: Basolateral membrane voltage (V_{bl}) of macula densa cells of isolated *in vitro* perfused rabbit cTAL segments containing the macula densa region. Note that V_{bl} is hyperpolarized by luminal furosemide (10 μmol/l).



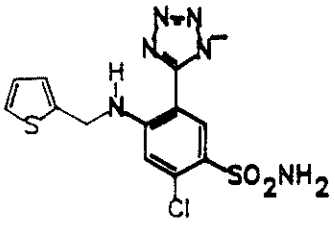
Furosemide
IC_{50}: $3 \cdot 10^{-6}$ mol/l



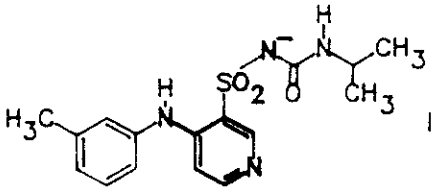
Piretanide
IC_{50}: $1 \cdot 10^{-6}$ mol/l



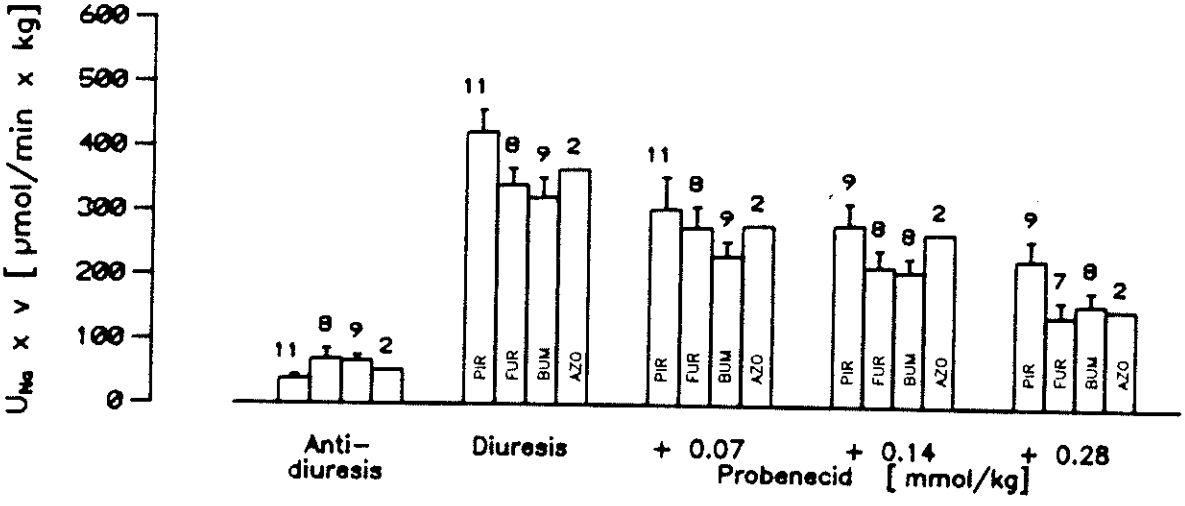
Bumetanide
IC_{50}: $2 \cdot 10^{-7}$ mol/l

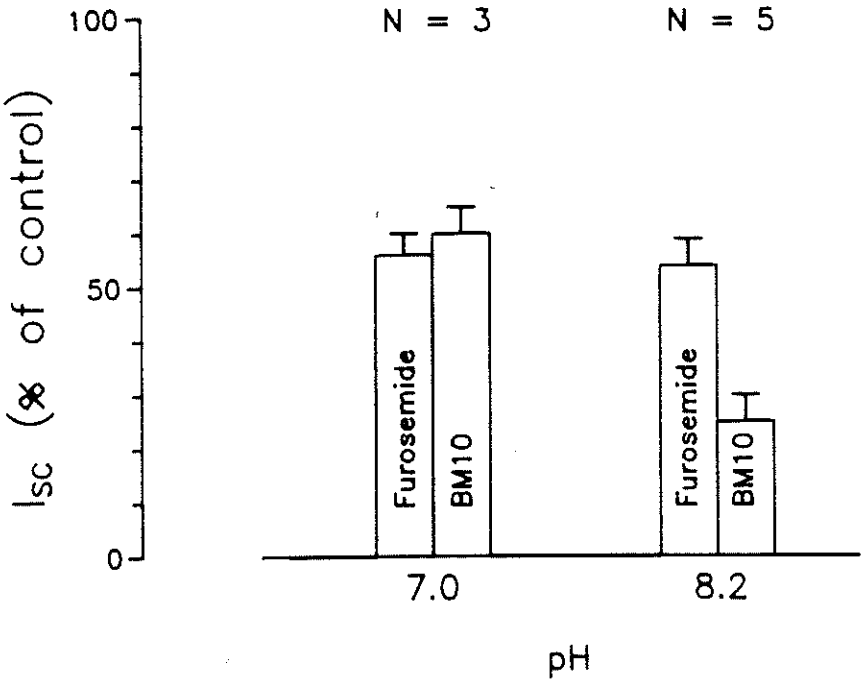


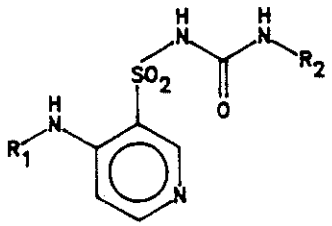
Azosemide
IC_{50}: $6 \cdot 10^{-6}$ mol/l

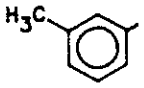


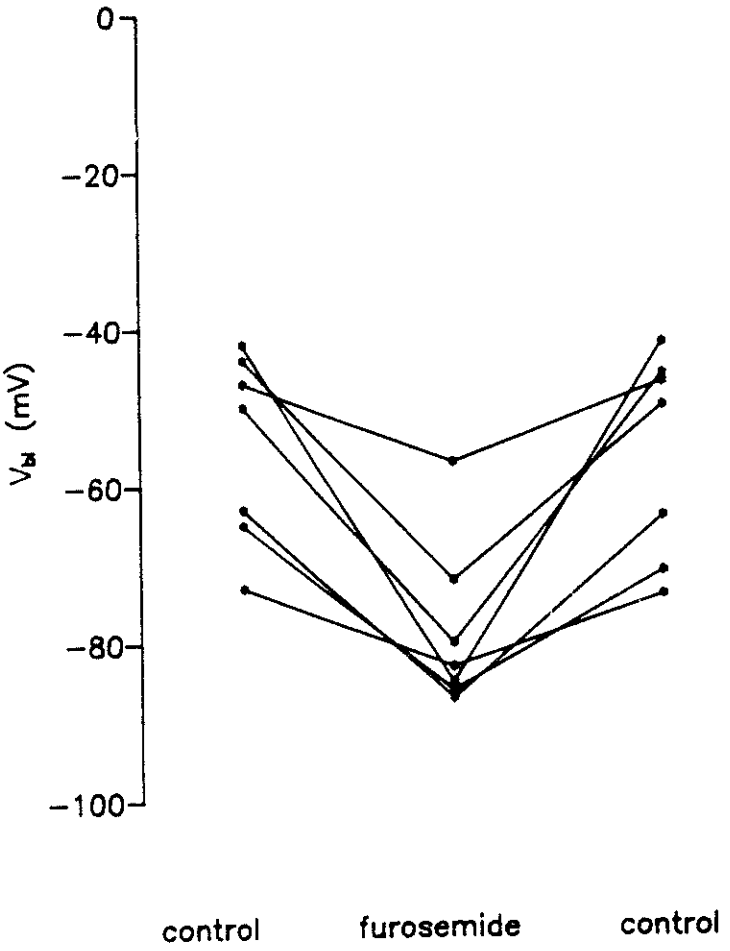
Torasemide
IC_{50}: $3 \cdot 10^{-7}$ mol/l







	R_1	R_2	pKa	log P	IC_{50} (umol/l)	IC_{50}^{cor}
TOR			6·82	0·449	0·30	0·24
BM2			9·03	1·331	19	0·43
BM8			9·39	1·717	9·6	0·097
BM4			9·15	2·074	14	0·24
BM3			9·30	1·665	3·5	0·043
BM27			n·t·	2·062	2·8	n·t·
BM10			8·98	2·063	0·47	0·012
BM6			9·13	2·449	2·0	0·036
BM9			7·70	2·704	0·56	0·18


0
−20
−40
−60
−80
−100
V_m (mV)
control
furosemide
control