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ACTION OF DIURETICS AT THE CELLULAR LEVEL

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Abstract: The present review will focus on loop diuretics and more specifically on the mode of action of sulfonylurea diuretics such as torasemide (TOR). TOR has two major sites of action in the thick ascending limb of the loop of Henle (TAL). It interacts with the $Na^+2Cl^-K^+$-cotransporter localized in the luminal membrane of the TAL segment, and it blocks Cl^--channels in the basolateral cell membrane of these TAL-cells. The former effect, with an IC_{50} of $3 \cdot 10^{-7}$ mol/l, requires very low and the latter 100 times larger concentrations. In the current study derivatives of TOR were designed in which the tolyl (R_1) and the isopropyl (R_2) moieties of TOR were replaced by cyclo-alkyl residues. From previous studies we knew that apolar substituents at these two sites of the sulfonylurea diuretic preserved the diuretic potency to some extent, but increased strongly the lipophilicity of the molecule. Both R_1 and R_2 were varied between cyclo-hexyl and cyclo-octyl. For each compound the pK_a, reflecting mostly the acidity of the sulfonylurea group, the octanol/water partition, and the inhibitory potency (IC_{50}) in isolated in vitro perfused rabbit cortical TAL segments (cTAL) were determined. The addition of the compound to the bath reflects its inhibitory potency on Cl^--channels, and the addition to the lumen perfusate reflects the inhibitory potency on the $Na^+2Cl^-K^+$-

cotransporter. The present data indicate that compounds with cyclo-hexyl as R_2, of the cyclo-octyl and cyclo-hexyl residues at R_1 had an inhibitory potency on the $Na^+2Cl^-K^+$-cotransporter comparable to TOR. These compounds are highly lipophilic, and their pK_a-values are between 7.7 and 9.0. The present data indicate that, on the basis of the TOR-structure, lipophilic specific inhibitors with very high affinity to the $Na^+2Cl^-K^+$-cotransporter can be designed.

Introduction: Diuretics are classified conviently by their site of action in the nephron. Recently, it has become evident that the different groups of diuretics represent rather specific inhibitors of different transport proteins. The proximal diuretics such as acetazolamide inhibit the membrane bound and cytosolic carbonic anhydrase. The loop diuretics such as furosemide, bumetanide, piretanide, azosemide and torasemide all interact with the $Na^+2Cl^-K^+$-cotransporter in the thick ascending limb of the loop of Henle (TAL). Thiazides, acting in the early distal tubule, interfere with a NaCl-cotransport system localized in the luminal membrane of this tubule segment. Potassium sparing diuretics such as triamterene and amiloride inhibit luminal membrane Na^+-channels in the late distal tubule and collecting duct (for review see: Greger and Heidland 1990, Greger and Schlatter 1983, Greger and Wangemann 1987).

One puzzling question which appeared immediately when the target sites of diuretics were recognized relates to their organ specificity. How should it happen, for instance, that an inhibitor of the widely distributed $Na^+2Cl^-K^+$-cotransporter exerted a diuretic effect but had little side effects? This is the more puzzling because this cotransporter, with an identical drug selectivity, is present in a variety of epithelia and apolar cells (Greger 1985). The answer to this question is rather simple. All diuretics act in the different tubule segments only from the luminal side, and all diuretics are accumulated in the tubule fluid such that the concentration of the diuretic is much higher in the luminal fluid than it is in the circulating plasma. Hence, the organ selectivity is related to the pharmacokinetics of these drugs. Along these lines it has been shown recently that an inhibitor of proximal tubular secretion of organic acids, namely probenecid, inhibited the diuretic effect of several loop diuretics (Braitsch et al. 1989).

The mechanism of action of loop diuretics is summarized in Fig. 1. This figure shows a simple scheme of NaCl-reabsorption in the TAL segment (Greger 1985). The entire transport process is energized by the basolaterally localized

$(Na^+ + K^+)$-ATPase. Cl^- is taken up into the cell by the $Na^+2Cl^-K^+$-cotransporter. The lumped electrochemical driving force for this cotransporter is approximately 50 mV favoring entry of all four ions into the cell. Cl^- leaves the cell mainly through Cl^- channels, and K^+ recycles across the luminal membrane into the lumen. The conductive ion movements (Cl^- and K^+) are responsible for the lumen positive voltage of 5-10 mV. This voltage drives Na^+ between the cells through the cation selective shunt pathway. Taken together, the TAL-segment can reabsorb NaCl most economically with a ratio of 6 moles NaCl for each mole ATP consumed.

After its initial description (Greger 1981), this concept has been proven correct in many subsequent studies (for review see Greger 1985, Schlatter 1989). The different transporters have been examined in some detail during the past few years. The $Na^+2Cl^-K^+$-cotransporter is currently purified (Forbush et al. 1988, Feit et al. 1988). The properties of the ion channels in the luminal and basolateral membrane in intact tubules have been examined by the patch clamp technique (Greger et al. 1990, Bleich et al. 1990). Also a large number of inhibitors of the $Na^+2Cl^-K^+$-cotransporter and of Cl^--channels have been examined (Schlatter et al. 1983, Wittner et al. 1987, Di Stefano et

al. 1985, Wangemann et al. 1986), and their mode of action has been studied in much detail.

It is very likely that Cl^--channel blockers bind to the outwardly facing mouth of the channel (Greger et al. 1989) and reduce the time which the channel spends in its open state. Hence they reduce the open state probability (Dreinhöfer et al. 1988). Cl^--channel blockers cannot be used as diuretics, since a concentration, sufficient to interfere with the TAL-function, would have a large number of side effects in many organs possessing the same type of chloride channels.

Loop diuretics such as furosemide and related substances bind to one of the two Cl^--binding sites of the $Na^+2Cl^-K^+$-cotransporter (Fig. 1). This interaction is entirely reversible. The different substances vary largely with respect to their affinity towards the $Na^+2Cl^-K^+$-cotransporter. For furosemide the IC_{50} is $3 \cdot 10^{-6}$ mol/l, whilst it is as low as $7 \cdot 10^{-8}$ mol/l for a related substance (#9 in: Schlatter et al. 1983). The potency sequence of bumetanide > torasemide > piretanide > furosemide ~ azosemide appears to be valid for several species including man. The loop diuretic induced inhibition of the coupled uptake of Na^+, $2Cl^-$ and K^+ reduces the work load and ATP-consumption of TAL-cells (Greger and

Wangemann 1988).

Torasemide, an inhibitor of the $Na^+2Cl^-K^+$-cotransporter and of Cl^--channels: Torasemide (TOR) shares in common with most loop diuretics some of its structure (Fig. 2). We have previously concluded that TOR has a somewhat intermediate position between loop diuretics such as furosemide (FUR) and chloride channel blockers such as diphenylamine-2-carboxylate (DPC) (Wittner et al. 1986). In fact, the chemical structure of TOR is very similar to DPC. The results in the rabbit TAL (Wittner et al. 1986) indicate that the affinity to the $Na^+2Cl^-K^+$-cotransporter is much higher (IC_{50} = $3 \cdot 10^{-7}$ mol/l) than the affinity to the chloride channel (IC_{50} = $3 \cdot 10^{-5}$ mol/l). It is not likely that this additional effect of TOR when compared to FUR or other loop diuretics is relevant for the diuretic effect. The circulating concentration of this compound will hardly ever reach a level sufficient to inhibit basolateral Cl^--channels in the TAL segment.

The diuretic effect of sulfonylurea diuretics has a longer half life than that of other loop diuretics. It has been noted in animal studies and in man that TOR has a longer lasting effect than furosemide and chemically related compounds. This can be explained by the pharmacokinetics of

TOR. TOR is largely metabolized in the liver and one of the metabolites shows biological activity (unpublished from the authors' laboratory). More relevant, probably is the fact that TOR is more lipophilic than other loop diuretics, and distributes into a larger volume of the body. Hence TOR is more slowly released and cleared.

Can one design even more lipophilic sulfonylurea diuretics? The structure of TOR lends itself to modifications at the two residues designated in Fig. 3. The tolyl residue (R_1) can be replaced by more lipophilic residues such as cyclo-alkyls and aliphates. In addition, the isopropyl residue (R_2) of TOR can also be replaced by cyclo-alkyls. The respective compounds have been synthesized and their structures are summarized in Fig. 3. These lipophilic compounds may be able to cross the blood brain barrier more easily and to reduce glial swelling as it may be caused by the stimulation of the astrocytic cotransporter as a result of the increase of the extracellular K^+-concentration. Hence these compounds might prevent some forms of brain edema (unpublished data from B. Masereel and J. Delarge)

The lipophilicity of these compounds was determined from the octanol/water partition. The logarithm of this ratio is given in Fig. 3. Notably the lipophilicity increases with

increasing size of the cyclo-alkyl residue in position R_1 and in position R_2. The most lipophilic compound is one with cyclo-octyl residues in both positions.

The overall pK of these compounds was also determined by titrimetric techniques. It was found that all tested compounds, unlike TOR, have pK-values higher than 7 up to 9. This is the more remarkable as these compounds are still rather potent inhibitors of the $Na^+2Cl^-K^+$- cotransporter (c.f. below).

The inhibitory effect of these compounds on the $Na^+2Cl^-K^+$-cotransporter in the luminal membrane and on the Cl^--channels in the basolateral membrane was examined in *in vitro* perfused cortical rabbit thick ascending limbs (cTAL). The equivalent short cicuit (I_{SC}) was measured under control conditions (Greger 1981). Then the drug under study was added to the luminal perfusate or to the bathing solution and I_{SC} was monitored continuously. The inhibitory effects were instantaneous, and they were entirely reversible. Dose-response curves were determined for each compound for the bath and the luminal applications. For each concentration the results of at least 3 tubules were pooled. Fig. 4 summarizes the dose-response curves for all compounds included in this study. The results are compared to those of

TOR. Like in a previous study (Wittner et al. 1987), the Hill coefficients for the luminal effect of these compounds were close to one. This is also in close agreement with previous studies on the effect of loop diuretics in the same preparation (Schlatter et al.1983). Data of this kind are compatible with the interpretation that one inhibitor molecule interacts with one $Na^+2Cl^-K^+$-cotransporter. The effect of the present lipophilic sulfonylurea diuretics on the Cl^--channel is rather modest. Even the most potent compound on the $Na^+2Cl^-K^+$-cotransporter had only a moderate effect on Cl^--channels. The concentration required for halfmaximal inhibition were read from the dose-response curves in Fig. 4 (IC_{50}-values) and are included in Fig. 3. It is evident that the potency for the inhibitory effect on the $Na^+2Cl^-K^+$-cotransporter increases with increasing size of the cyclo-alkyl residue at R_1. The most potent compounds possess a cyclo-hexyl or -octyl residue at R_2 and a cyclo-octyl residue at R_1. We have also tested even larger cyclo-alkyls. However, rings as large as C_{12} lead to a reduction in the inhibitory potency (data not shown). Also we know from a previous study (Wittner et al. 1987) that long aliphatic chains (C_7) as R_1 lead to a marked reduction of potency. Thus it appears that cyclo-octyl residues represent an optimized chemical structure for the inhibitory effect on the $Na^+2Cl^-K^+$-cotransporter for this kind of

sulfonylurea diuretic.

Amongst the 4 measured parameters in Fig. 3, the only reasonable correlation exists between the IC_{50} on the $Na^+2Cl^-K^+$-cotransporter and the log P-values. This correlation is shown in Fig. 5. It is clearly evident that TOR itself, with an IC_{50} of 0.3 μmol/l but with a much smaller octanol/water partition, falls out of this correlation. No correlation is evident between pK and IC_{50}-values. This might indicate that between certain limits the acidity of the amino-group close to the sulfonyl group is not very relevant for the inhibitory effect on the $Na^+2Cl^-K^+$-cotransporter. On the other hand, it might also be true that only the deprotonated form acts on the $Na^+2Cl^-K^+$-cotransporter and that its IC_{50}-value is even much lower than that determined for the sum of the deprotonated and protonated fractions at physiologic pH, as they have been used in this study. This question could, theoretically, be answered by measuring IC_{50}-values of a given substance at different pH-values of the luminal perfusate. Such experiments are, however, limited by the fact that large pH variations in the luminal perfusate lead to additional effects, such as inhibition of I_{SC} at low pH-values.

Conclusion: The present study indicates for the first time

that highly lipophilic derivatives of TOR are potent inhibitors of the $Na^+2Cl^-K^+$-cotransporter. These substances have little effect on the Cl^--channel. Substances of this kind might offer some advantage, inasmuch as they distribute into a larger apparent volume of distribution and ought to have markedly prolonged effectiveness after single dose application. Furthermore, these substances might be able to cross the blood brain barrier and hence reach other targets such as glial cells.

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Figure legends:

Fig. 1: NaCl reabsorption in the thick ascending limb. The symbols have the following meaning:  = primary active pump,  = carrier,  = ion channel. For further details see text.

Fig. 2: Chemical structures of furosemide, torasemide, and diphenylamine-2-carboxylate. Note that torasemide has simi-

larities to both furosemide and diphenylamine-2-carboxylate. All three molecules possess the secondary amine-"bridge", an acidic residue (carboxylate in case of furosemide and diphenylamine-2-carboxylate and sulfonylurea in case of torasemide, and anapolar residue (phenyl in case of diphenylamine-2-carboxylate, tolyl in case of torasemide, and furfuryl in case of furosemide).

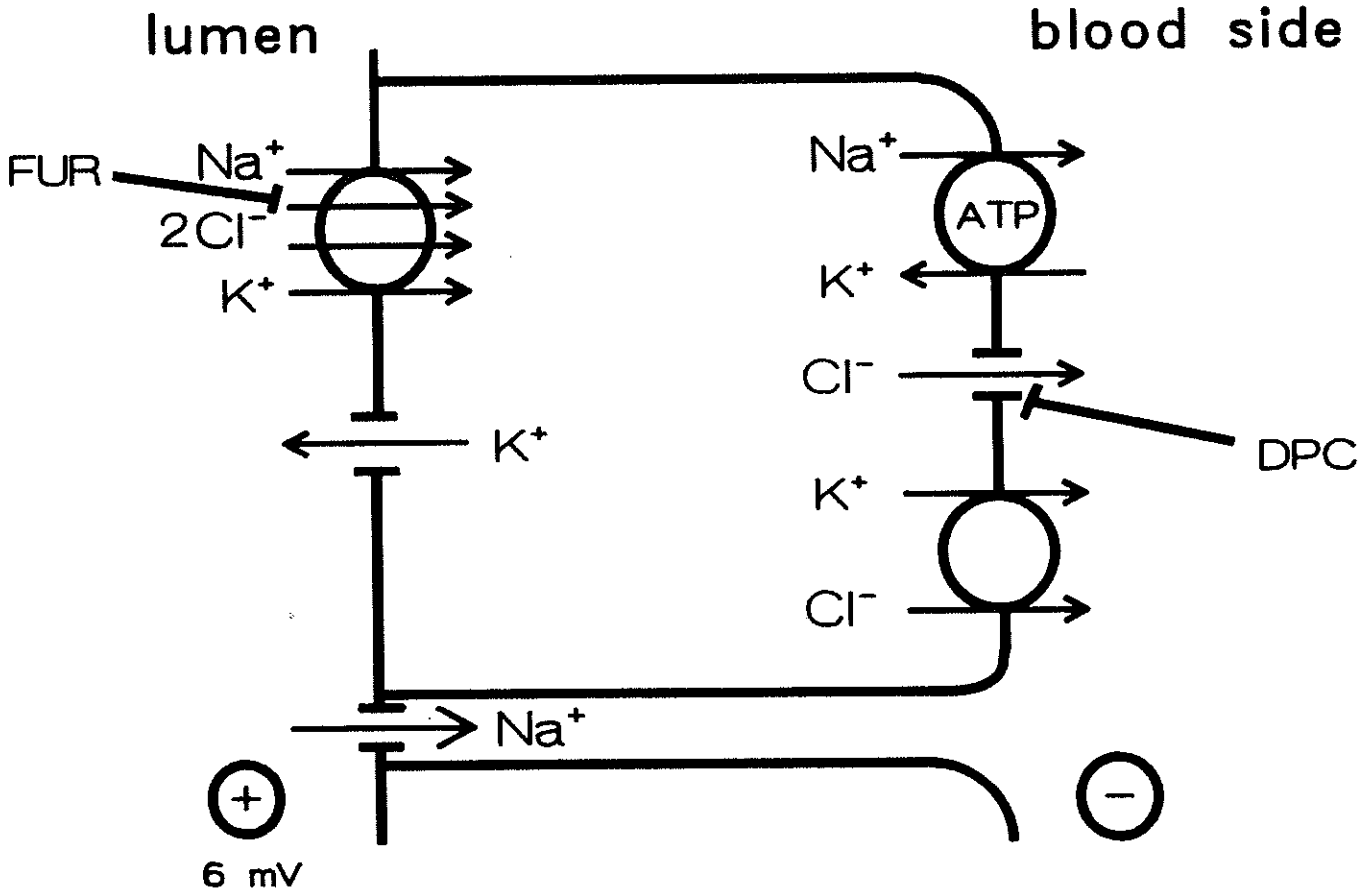
Fig. 3: Chemical structures of torasemide and lipophilic derivates. For each compound the following parameters are given: pK-value, logarithm of the n-octanol/water partititon (log P) and the IC_{50}-values (halfmaximal inhibition concentrations) for interaction with the $Na^+2Cl^-K^+$-cotransporter (lumen) and Cl^--channels (bath).

Fig. 4: Dose-response curves for torasemide derivatives. The equivalent short circuit current (I_{SC}) of in vitro perfused rabbit cTAL-segments in percent of control is plotted versus the log of the concentration. The different letters refer to the different derivates. The heavy curves are those for torasemide (TOR). The solid lines refer to the addition to the luminal perfusate (effect on the $Na^+2Cl^-K^+$- cotransporter), and the broken lines refer to the addition to the bath (effect on Cl^--channels). Note that the effects on the $Na^+2Cl^-K^+$-cotransporter is much stronger than that on the

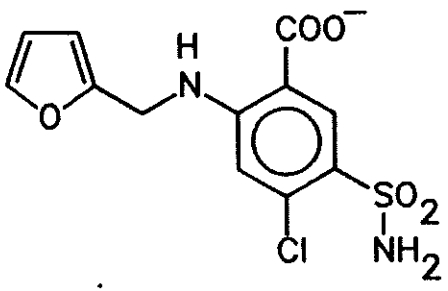
Cl^--channel. The compounds BM9 and BM10 have an inhibitory effect comparable to that of TOR on the $Na^+2Cl^-K^+$-cotransporter, but they have very little effect on the Cl^--channel. All other compounds are less potent.

Fig. 5: Correlation between the IC_{50}-value on the $Na^+2Cl^-K^+$-cotransporter and the log P (octanol/water partition). The correlation is statistically significant. Note that TOR falls cleary out of this correlation.

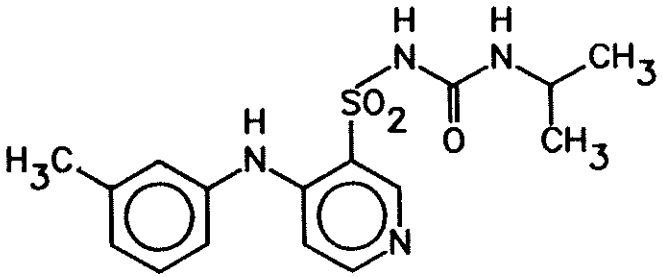
NaCl–Reabsorption in the TAL–segment



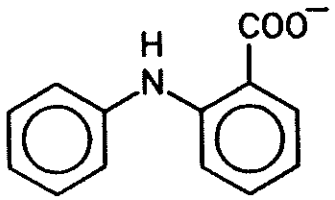
Furosemide

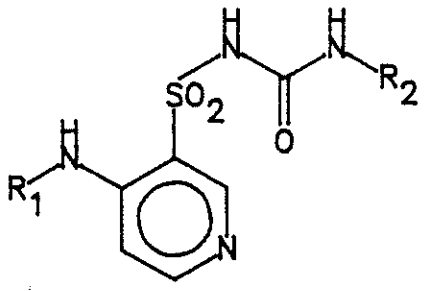


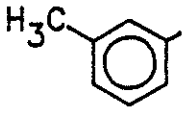
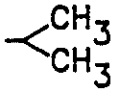
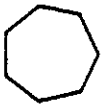
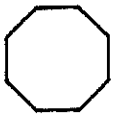
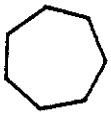
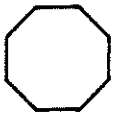
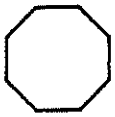
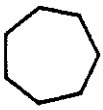
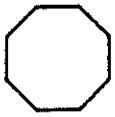
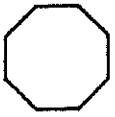
Torasemide



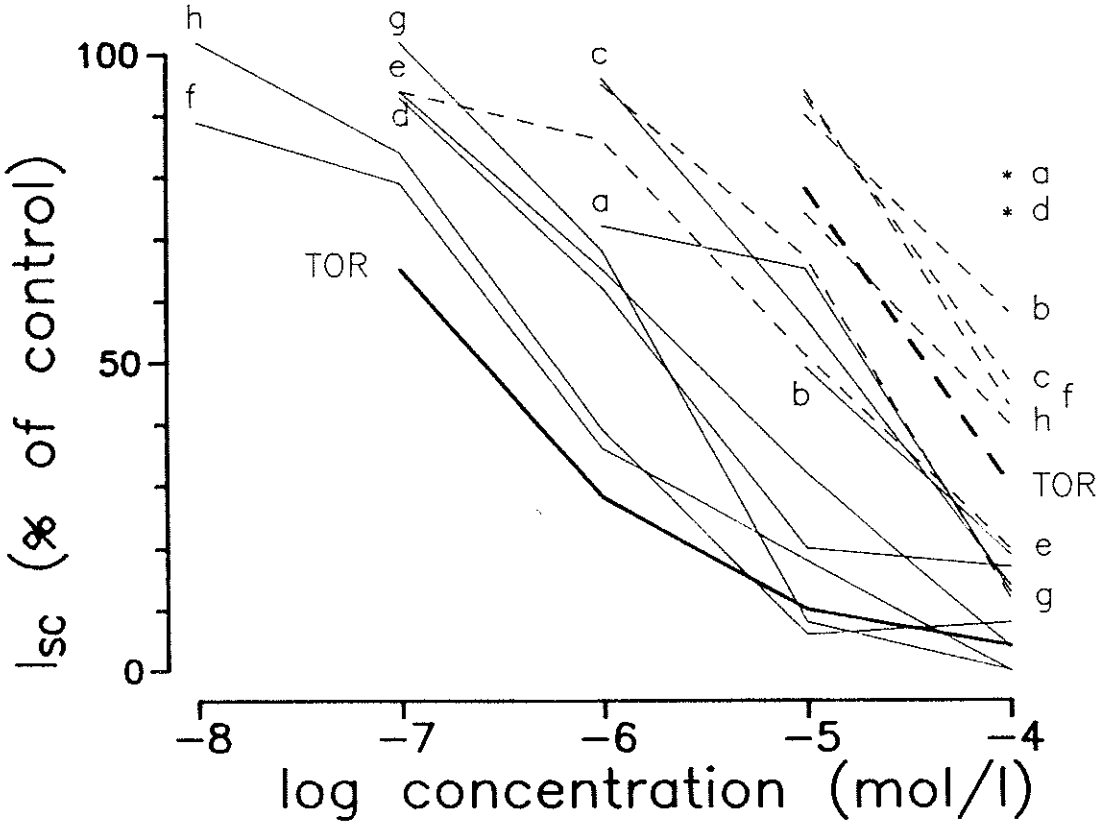
Diphenyl–amino–
2–carboxylate





	R_1	R_2	pKa	log P	IC_{50} (umol/l) lumen	bath
TOR			6·82	0·449	0·30	30
BM2			9·03	1·331	19	>100
BM8			9·39	1·717	9·6	>100
BM4			9·15	2·074	14	85
BM3			9·30	1·665	3·5	>100
BM27			n·t·	2·062	2·8	10
BM10			8·98	2·063	0·47	84
BM6			9·13	2·449	2·0	20
BM9			7·70	2·704	0·56	70

Dose–response curves for lipophilic sulfonylurea–compounds given in the luminal perfusate (solid line) or bath solution (dashed line)



R_1/R_2			
a	=	C_6/C_6	= BM2
b	=	C_6/C_7	= BM8
c	=	C_6/C_8	= BM4
d	=	C_7/C_6	= BM3
e	=	C_7/C_7	= BM27
f	=	C_8/C_6	= BM10
g	=	C_8/C_7	= BM6
h	=	C_8/C_8	= BM9
TOR	=	Torasemide	

Correlation between the inhibitory effect of Torasemide derivatives on $Na^+2Cl^-K^+$–cotransporter in isolated perfused cTAL and lipid–solubility (as logarithm of octanol/water partition)