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Thiazide diuretic drug receptors in rat kidney: Identification with [^{3}H]metolazone

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ABSTRACT Thiazides and related diuretics inhibit NaCl reabsorption in the distal tubule through an unknown mechanism. We report here that [^{3}H]metolazone, a diuretic with a thiazide-like mechanism of action, labels a site in rat kidney membranes that has characteristics of the thiazide-sensitive ion transporter. [^{3}H]Metolazone bound with high affinity (K_d = 4.27 nM) to a site with a density of 0.717 pmol/mg of protein in kidney membranes. The binding site was localized to the renal cortex, with little or no binding in other kidney regions and 11 other tissues. The affinities of thiazide-type diuretics for this binding site were significantly correlated with their clinical potency. Halide anions (Cl^-, Br^-, and I^-) specifically inhibited high-affinity binding of [^{3}H]metolazone to this site. [^{3}H]Metolazone also bound with lower affinity (K_d = 289 nM) to sites present in kidney as well as in liver, testis, lung, brain, heart, and other tissues. Calcium antagonists and certain smooth muscle relaxants had K_i values of 0.6–10 μM for these low-affinity sites, which were not inhibited by most of the thiazide diuretics tested. Properties of the high-affinity [^{3}H]metolazone binding site are consistent with its identity as the receptor for thiazide-type diuretics.

Thiazide diuretics are among the most widely prescribed drugs in clinical practice and are mainstays in the therapy of hypertension and the management of edema. In the 30 years since the development of chlorothiazide (1, 2), numerous other benzothiazide sulfonamides and pharmacologically related diuretics have been synthesized and have reached the clinic. Physiological studies have demonstrated that thiazides and closely related drugs act by inhibiting the reabsorption of NaCl in the distal tubule (3). However, despite their widespread use and extensive studies, the molecular entities through which thiazides exert their effects and the exact cellular locations of these sites have not yet been identified.

Diuretics having a thiazide-like mechanism of action—i.e., inhibition of distal tubular NaCl reabsorption—include sulfonamides with a benzothiadiazine-dioxide ring (thiazides) as well as pharmacologically related sulfonamide diuretics differing in the heterocyclic ring, such as metolazone, quinethazone, indapamide, and chlorthalidone. To identify the molecular site of action of the thiazide diuretics, we have used radioligand binding methods to study the interaction of [^{3}H]metolazone (7-chloro-1,2,3,4-tetrahydro-2-methyl-4-oxo-3-*o*-tolyl-6-quinazolinesulfonamide) with rat kidney membranes *in vitro*. We report here that [^{3}H]metolazone binds with high affinity to a site in the rat kidney that has characteristics consistent with its identity as the receptor for thiazide diuretics.

MATERIALS AND METHODS

Materials. [^{3}H]Metolazone was custom synthesized by Amersham. The compound was prepared by reduction of the unsaturated precursor, 7-chloro-3,4-dihydro-2-methyl-4-oxo-3-*o*-tolyl-6-quinazolinesulfonamide, using sodium boro[^{3}H]-hydride in aqueous ethanol (4). The product was purified by TLC to >98% purity and had a specific activity of 11.3 Ci/mmol (1 Ci = 37 GBq). The purity of the [^{3}H]metolazone was assessed by reverse-phase HPLC (5) and remained >98% throughout the course of the study.

Metolazone and its precursor were generously provided by Pennwalt (Rochester, NY). Diuretics were also donated by the following: bendroflumethiazide by Squibb, quinethazone by Lederle Laboratories (Pearl River, NY), polythiazide by Pfizer, benzthiazide by A. H. Robins (Richmond, VA), indapamide by Revlon Health Care (Tuckahoe, NY), chlorothiazide by Merck, trichlormethiazide by Schering, and methychlothiazide by Abbott. Ro 5-4864 was provided by Hoffmann-La Roche and PK 11195 was provided by Pharmuka (Gennevilliers, France). Other drugs were obtained from Sigma.

Tissue Preparation. Kidneys and other tissues were obtained from male Sprague–Dawley rats weighing 150–250 g. Tissues were homogenized with a Polytron (Brinkman) in ice-cold 50 mM Tris phosphate buffer (pH 7.4). Homogenates were centrifuged for 5 min at 120 × *g* and the supernatants were centrifuged for 20 min at 48,000 × *g*. The pellet was resuspended in fresh buffer and centrifuged again for 20 min at 48,000 × *g*. The resulting membrane pellet was resuspended in Tris phosphate buffer at a concentration of 0.8 mg of protein per ml for use in binding assays.

Binding Assays. Kidney membranes in 50 mM Tris phosphate buffer were incubated with [^{3}H]metolazone and unlabeled compounds in a volume of 1 ml in an ice bath. Incubations were carried out for 4 hr, by which time steady-state conditions were attained. An incubation time of 2 hr was used for experiments measuring low-affinity binding, which reached steady state more rapidly than high-affinity binding. Membranes were collected by filtration through glass fiber filters (GF/B, Whatman) using a cell harvester (Brandel Instruments, Gaithersburg, MD) and washed with 12 ml of ice-cold Tris phosphate buffer. Filters were soaked for at least 1 hr prior to use with 0.3% polyethyleneimine, a procedure that increased the amount of specific binding retained. Radioactivity on the filters was extracted into BetaPhase scintillation cocktail (WestChem Products, San Diego, CA) and counted at an efficiency of 52% in a liquid scintillation counter. There was no specific binding of [^{3}H]metolazone to the filters. Proteins were determined by the Bradford Coomassie blue method (Bio-Rad) with gamma globulin as the standard. Scatchard plots were analyzed with the LIGAND program of Munson and Rodbard (6) after data transformation with the EBDA program of McPherson (7). The EBDA program was used to analyze competition curves and obtain slope factors (apparent Hill coefficients) by iterative curve fitting. Inhibition constants (K_i values) were determined according to Cheng and Prusoff (8).

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RESULTS

Binding Constants. Unlabeled metolazone competed for the binding of [^{3}H]metolazone to membranes from kidneys and other tissues. Initial experiments addressed the potential binding of [^{3}H]metolazone to carbonic anhydrase, since metolazone and thiazide diuretics are chemically related to inhibitors of this ubiquitous enzyme. The carbonic anhydrase inhibitor acetazolamide at 100 μM blocked 12% of [^{3}H]metolazone binding to kidney membranes, 33% of binding to brain membranes, and 61% of binding to mandibular gland membranes. In all three tissues, inhibition was half-maximal at 10–100 nM and maximal at 10 μM acetazolamide. For all further experiments, 10 μM acetazolamide was added to the tissue homogenates to block the binding of [^{3}H]metolazone to carbonic anhydrase.

Scatchard plots of [^{3}H]metolazone binding to kidney membranes at steady state were biphasic, suggesting the presence of two binding sites (Fig. 1). Nonlinear regression analysis of Scatchard plots (n = 5) yielded a K_d of 4.27 ± 0.37 nM and B_{max} of 0.717 ± 0.058 pmol/mg of protein for the high-affinity binding site and a K_d of 289 ± 43 nM and B_{max} of 3.92 ± 0.36 pmol/mg of protein for the low-affinity site.

Initial screening of several drugs for their ability to inhibit [^{3}H]metolazone binding at high and low concentrations revealed that hydroflumethiazide selectively blocked the high-affinity binding site. As seen in Fig. 1, high-affinity binding is selectively blocked by 10 μM hydroflumethiazide, leaving only low-affinity binding in the presence of the drug.

Pharmacological Specificity. The ability of diuretics and other compounds to compete with [^{3}H]metolazone binding to kidney membranes was measured (Table 1). At the concentration of [^{3}H]metolazone (3 nM) used for studies of the high-affinity binding site, calculations based upon the previously described binding constants indicate that 92% of measured binding was to the high-affinity site and only 8% was to the low-affinity site. Diuretics of the benzothiadiazine (thiazide) class were potent inhibitors of the high-affinity metolazone binding site. The closely related quinazolinone diuretics, quinethazone and metolazone itself, also had high affinities for this site. In addition, indapamide and chlorthalidone, diuretic derivatives of 3-sulfamoyl-4-chlorobenzoic acid, competed for [^{3}H]metolazone binding to the high-affinity site. Slope factors (apparent Hill coefficients) for the competing ligands were significantly less than unity, varying from 0.5 to 0.8 (Table 1). Of the numerous other compounds tested, including diuretics of other classes, no compound inhibited the high-affinity [^{3}H]metolazone binding site by >25% at a concentration of 100 μM. These compounds include furosemide, bumetanide, diazoxide, triamterene, amiloride, ouabain, ethacrynic acid, acetazolamide, probenecid, theophylline, adenosine, dipyridamole, clonidine, phentolamine, atropine, verapamil, nitrendipine, nifedipine, diltiazem, papaverine, nicardipine, ethaverine, and sulfanilamide.

Table 1. Competition by diuretics for high-affinity [^{3}H]metolazone binding to kidney membranes

Diuretic	K_i, nM	pA$_2$	Slope factor	n
Methychlothiazide	0.43	9.32 ± 0.42	0.54 ± 0.08	5
Polythiazide	0.81	9.09 ± 0.35	0.55 ± 0.11	5
Bendroflumethiazide	5.0	8.30 ± 0.12	0.57 ± 0.09	4
Metolazone	5.1	8.29 ± 0.10	0.80 ± 0.09	4
Benzthiazide	6.7	8.17 ± 0.18	0.64 ± 0.03	5
Indapamide	29	7.54 ± 0.20	0.59 ± 0.06	4
Hydroflumethiazide	43	7.37 ± 0.10	0.66 ± 0.04	3
Chlorthalidone	48	7.32 ± 0.28	0.74 ± 0.04	4
Hydrochlorothiazide	98	7.01 ± 0.16	0.68 ± 0.12	4
Trichlormethiazide	100	7.00 ± 0.22	0.61 ± 0.11	3
Quinethazone	180	6.74 ± 0.12	0.50 ± 0.10	3
Chlorothiazide	3700	5.43 ± 0.23	0.62 ± 0.07	3

Values are the means ± SEMs for pA$_2$ ($-\log K_i$) and slope factors determined in three to five separate experiments.

The affinities of diuretics listed in Table 1 for the high-affinity binding site in rat kidney are positively correlated with the average daily dose of these drugs used clinically (Fig. 2). The correlation coefficient (r = 0.7513) for the pA$_2$ ($-\log K_i$) versus the logarithm of the average daily oral dose is significant at the $P < 0.01$ level.

The pharmacological profile of the low-affinity binding site in kidney membranes was determined by using [^{3}H]metolazone at a concentration of 125 nM and with 100 μM hy-

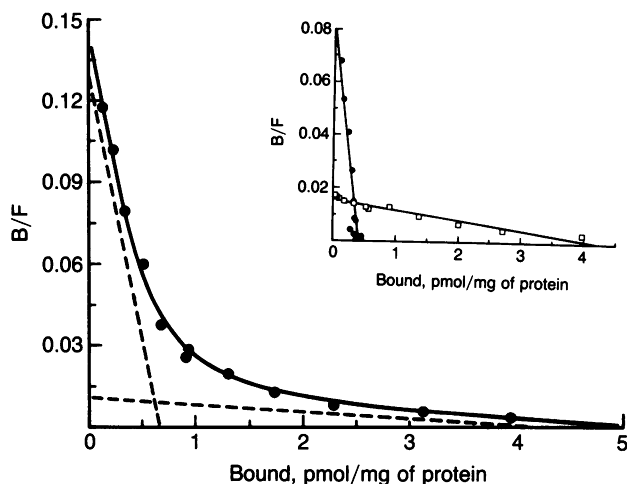

FIG. 1. Scatchard plots of [^{3}H]metolazone binding to kidney membranes. Biphasic curve of binding inhibited by 100 μM metolazone was resolved by the LIGAND program into the two sites indicated by the dashed lines. Values shown are from one of five separate experiments performed in duplicate. B/F, bound/free. High-affinity K_d = 4.27 ± 0.37 nM, B_{max} = 0.717 ± 0.058 pmol/mg of protein; low-affinity K_d = 289 ± 43 nM, B_{max} = 3.92 ± 0.36 pmol/mg of protein. (*Inset*) Use of hydroflumethiazide to define high- and low-affinity sites. Binding of [^{3}H]metolazone was measured over the same concentration range as in the main figure. ●, Binding inhibited by 10 μM hydroflumethiazide; □, binding in the presence of 10 μM hydroflumethiazide that was inhibited by 100 μM metolazone. Values are from one of two experiments performed in duplicate with similar results.

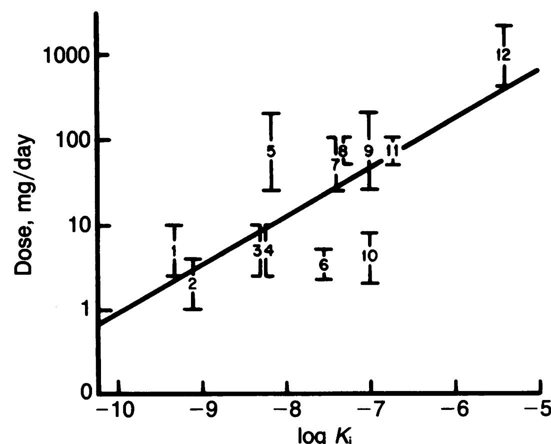

FIG. 2. Correlation of the daily clinical dose of thiazide-type diuretics with their affinity for high-affinity [^{3}H]metolazone binding sites in rat kidney. Daily dose range is from ref. 9; K_i values are from Table 1. Correlation coefficient, r = 0.7513. Methychlothiazide, 1; polythiazide, 2; bendroflumethiazide, 3; metolazone, 4; benzthiazide, 5; indapamide, 6; hydroflumethiazide, 7; chlorthalidone, 8; hydrochlorothiazide, 9; trichlormethiazide, 10; quinethazone, 11; chlorothiazide, 12.

droflumethiazide included in the incubation buffer to occupy high-affinity sites. Results from competition assays are listed in Table 2. Metolazone was the most potent inhibitor of low-affinity binding. Methychlothiazide and polythiazide were the only two thiazides tested that inhibited the low-affinity site and were also the only two thiazides with substituents at the 2-N position of the benzothiadiazine ring. Various smooth muscle relaxants, including dipyridamole, papaverine, and representative compounds from the three major classes of calcium antagonist drugs (dihydropyridine, phenylalkylamine, and benzothiazepine), inhibited low-affinity [^{3}H]metolazone binding with inhibitor affinities (K_i values) ranging from 0.6 to 7 μM. Benzodiazepine receptor ligands had K_i values of 10–60 μM. Thiazide diuretics and other compounds that produced <50% inhibition at 100 μM included trichloromethiazide, hydroflumethiazide, chlorothiazide, hydrochlorothiazide, quinethazone, ethacrynic acid, indacrinone, furosemide, bumetanide, amiloride, diazoxide, theophylline, adenosine, *p*-aminohippurate, l-methylnicotinamide, probenecid, quinazoline, sulfanilamide, atropine, clonidine, ouabain, indomethacin, haloperidol, and histamine.

Regional Distribution. The distribution of [^{3}H]metolazone binding was determined under conditions in which solely high- or low-affinity binding was detectable. High-affinity binding was measured at 15 nM [^{3}H]metolazone by using 100 μM hydroflumethiazide to define nonspecific binding. Low-affinity binding was measured at 250 nM [^{3}H]metolazone (near the low-affinity K_d) by using 100 μM metolazone to define nonspecific binding and with 10 μM hydroflumethiazide present to occupy high-affinity sites. The high-affinity binding site was ≈80% saturated, whereas the low-affinity binding site was 50% saturated under these respective conditions, as estimated from the binding constants obtained with kidney membranes. Of the 12 tissues studied, the kidney had by far the greatest density of high-affinity [^{3}H]metolazone binding sites (Table 3). Within the kidney, high-affinity sites were almost entirely limited to the renal cortex. Much lower levels of specific binding were detected in the renal medulla and papilla and in the testis, adrenal gland, lung, spleen, liver, and pancreas, whereas in 5 other tissues no specific binding was present. The kidney also contained the greatest density of low-affinity binding sites, which were more concentrated in the renal cortex than in the medulla or papilla (Table 3). Liver and testis contained half as great a density of low-affinity sites, whereas lung, brain, spleen, heart, and pancreas contained lower densities of these sites.

Effect of Ions. Since thiazide diuretics are postulated to block a NaCl cotransporter in the distal nephron, we determined whether these or other ions inhibited either the high- or low-affinity [^{3}H]metolazone binding sites. Ions were tested at 100 mM. Results are shown in Table 4. With fluoride, acetate, sulfate, or citrate as anion and sodium or potassium as counterion, high-affinity binding was only slightly inhibited or increased. With chloride, bromide, or iodide as anion, high-affinity binding was inhibited by >50%. When these ions were tested at 100 mM for inhibition of low-affinity binding (data not shown), only choline chloride produced >50% inhibition.

Table 2. Competition by drugs for low-affinity [^{3}H]metolazone binding to kidney membranes

Drug	K_i, μM	pA_2	Slope factor	*n*
Metolazone	0.26	6.59 ± 0.11	0.90 ± 0.09	4
Methoxyverapamil	0.62	6.21 ± 0.40	0.23 ± 0.04	3
Nicardipine	0.65	6.19 ± 0.26	0.58 ± 0.10	4
Nitrendipine	1.1	5.96 ± 0.15	0.46 ± 0.08	3
Verapamil	1.2	5.92 ± 0.28	0.38 ± 0.12	3
Dipyridamole	2.4	5.62 ± 0.22	0.44 ± 0.09	3
Diltiazem	5.8	5.24 ± 0.21	0.49 ± 0.07	5
Papaverine	6.9	5.16 ± 0.22	0.57 ± 0.05	4
Methychlothiazide	7.2	5.14 ± 0.15	0.81 ± 0.19	3
Ethaverine	8.0	5.10 ± 0.10	0.48 ± 0.07	4
Polythiazide	10	5.00 ± 0.02	0.90 ± 0.12	3
Ro 5-4864	12	4.92 ± 0.13	0.79 ± 0.07	4
Nifedipine	15	4.82 ± 0.10	0.76 ± 0.07	5
PK 11195	19	4.72 ± 0.12	0.57 ± 0.08	3
Diazepam	59	4.23 ± 0.02	0.90 ± 0.12	3

Values represent means ± SEMs for pA_2 ($-\log K_i$) and slope factors determined in three to five separate experiments.

Table 3. Relative distribution of [^{3}H]metolazone binding

Tissue	High-affinity binding, pmol/mg of protein	Low-affinity binding, pmol/mg of protein
Kidney	0.529 ± 0.087	1.397 ± 0.167
Cortex	0.808 ± 0.057	1.664 ± 0.074
Outer medulla	0.079 ± 0.006	0.332 ± 0.054
Inner medulla/papilla	0.065 ± 0.025	0.220 ± 0.154
Testis	0.072 ± 0.006	0.668 ± 0.094
Adrenal gland	0.051 ± 0.013	ND
Lung	0.041 ± 0.003	0.312 ± 0.034
Spleen	0.023 ± 0.006	0.183 ± 0.030
Liver	0.020 ± 0.006	0.672 ± 0.074
Pancreas	0.010 ± 0.001	0.062 ± 0.011
Brain	ND	0.208 ± 0.051
Heart	ND	0.163 ± 0.058
Salivary glands	ND	ND
Large intestine	ND	ND
Skeletal muscle	ND	ND

Values are means ± SEMs of five separate experiments performed in triplicate. High-affinity binding was measured at 15 nM [^{3}H]metolazone with or without 0.1 mM hydroflumethiazide. Low-affinity binding was measured at 250 nm [^{3}H]metolazone with or without 0.1 mM metolazone in the presence of hydroflumethiazide. ND, not detectable.

DISCUSSION

The high-affinity binding site for [^{3}H]metolazone described in this study has several properties expected of the receptor for thiazide diuretics. There exists a highly significant correlation ($P < 0.01$) between the affinity of several thiazides and thiazide-like diuretics for this binding site and their average daily clinical dose. These drugs include not only thiazides but also quinazolinones (metolazone itself and quinethazone), chlorthalidone, and indapamide, drugs that

Table 4. Inhibition of high-affinity [^{3}H]metholazone binding by ions

Ion	% control
NaF	143 ± 9
LiCl	4 ± 1
NaCl	20 ± 0.5
KCl	44 ± 2
Choline chloride	36 ± 7
NaBr	14 ± 2
NaI	25 ± 1
KI	12 ± 2
Sodium acetate	82 ± 5
Potassium acetate	95 ± 5
Disodium sulfate	152 ± 22
Dipotassium sulfate	118 ± 12
Trisodium citrate	112 ± 5

Specific binding of [^{3}H]metolazone at 3 nM to kidney membranes was measured in the presence of a 100 mM concentration of the indicated ions. Values are means ± SEMs of three experiments performed in triplicate.

share a thiazide-like mechanism of action (i.e., inhibition of distal tubular NaCl reabsorption) but that do not contain the benzothiadiazide ring. This correlation is especially significant when one considers that the data are obtained in different species and that biological efficacy is affected by the varying pharmacokinetic profiles of these drugs. In addition, some drugs may have other sites of action that contribute to their biological effects. In particular, indapamide is reported to have smooth muscle relaxant properties (10), which may produce a greater potency than would be obtained if interaction with thiazide receptors were its sole mechanism of action.

Much greater densities of high-affinity [^{3}H]metolazone binding sites are present in the kidney than in other tissues. Low levels of hydroflumethiazide-displaceable binding measured in six other tissues (Table 3) were near the limits of detection, with very low ratios of specific to nonspecific binding. Micropuncture studies indicate that thiazide-specific effects upon chloride transport are produced in the distal tubule of the rat nephron (11–13). The localization within the kidney of the high-affinity binding site, which is almost entirely limited to the cortex, is consistent with this site of action. A model epithelium that displays thiazide-inhibitable NaCl transport (the urinary bladder of the winter flounder) has only recently been reported (14), whereas ion transport in several other epithelial tissues is not affected by thiazides (15). The present results suggest that the thiazide receptor may be less widely distributed than other ion transporters such as the loop diuretic-sensitive Na/K/Cl cotransporter, which occurs in numerous cell types outside of the kidney (16, 17).

It is significant that chloride, iodide, and bromide at 100 mM greatly inhibited binding of [^{3}H]metolazone to its high-affinity site, whereas sodium, potassium, and several monovalent and divalent anions were ineffective. This finding may indicate that thiazide diuretics and halide anions compete for a transport site or channel, although noncompetitive actions are not excluded by current data. Competition between diuretics and halide anions for the thiazide receptor is consistent with the physiological effects of thiazide diuretics, which not only produce chloruresis but also increase excretion of bromide and iodide (18).

[^{3}H]Metolazone bound to at least two lower-affinity sites. One of these sites appeared to be a membrane-associated carbonic anhydrase, since acetazolamide blocked this binding site at concentrations below 1 μM. This acetazolamide-sensitive portion of [^{3}H]metolazone binding is of lower affinity than the two binding sites described here and is widely distributed, with especially high densities in salivary glands (unpublished data). Membrane-associated carbonic anhydrase isozymes have been identified but not yet completely characterized (19, 20). It has been proposed that proximal tubular effects of metolazone and chlorothiazide are secondary to inhibition of carbonic anhydrase in this tubule segment (21). With acetazolamide present to block carbonic anhydrase, [^{3}H]metolazone bound to a low-affinity site or sites most prevalent in the kidney but also present in significant quantities in liver, testis, lung, brain, heart, spleen, and pancreas. Most thiazide diuretics did not inhibit this site at concentrations below 100 μM, the exceptions being two thiazides (methychlothiazide and polythiazide), which are both substituted in the 2-N position of the benzothiadiazine ring. Metolazone is also N-substituted in the analogous position of the quinazoline ring. It is of interest that a chemically diverse group of calcium channel blockers and smooth muscle relaxants inhibited low-affinity binding with K_i values in a narrow range of 0.6–8 μM. Ligands for the peripheral-type benzodiazepine receptor, which is localized in the distal nephron (22) and inhibited by metolazone at micromolar concentrations (23), were relatively weak inhibitors of low-affinity binding. Biphasic dissociation of low-affinity binding (unpublished data) and the shallow competition curves obtained with inhibitors, which yielded slope factors significantly less than unity, indicate that this component of binding may be heterogeneous or involve cooperative interactions (24). Whether this component of metolazone binding may be significant for metolazone's reported effects in patients with limited renal function (25) remains to be determined.

In conclusion, [^{3}H]metolazone binds to a high-affinity site that is limited to kidney, blocked by halide anions, and inhibited by diuretics with thiazide-like mechanisms of action. This radiolabeled probe should be useful in the molecular characterization of the receptor for thiazide-type diuretics.

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