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Meta-iodobenzylguanidine derivatives containing a second guanidine moiety

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Abstract—Radioiodinated *meta*-iodobenzylguanidine (MIBG) is used in the diagnosis and therapy of various neuroendocrine tumors. To investigate whether an additional guanidine function in the structure of MIBG will yield analogues that may potentially enhance tumor-to-target ratios, two derivatives—one with a guanidine moiety and another with a guanidinomethyl group at the 4-position of MIBG—were prepared. In the absence of any uptake-1 inhibiting conditions, the uptake of 4-guanidinomethyl-3-[^{131}I]iodobenzylguanidine ([^{131}I]GMIBG) by SK-N-SH cells in vitro was 1.7±0.1% of input counts, compared to a value of 40.3±1.4% for [^{125}I[MIBG suggesting that guanidinomethyl group at the 4-position negated the biological properties of MIBG. On the other hand, 4-guanidino-3-[^{131}I]iodobenzylguanidine ([^{131}I]GIBG) had an uptake (5.6±0.3%) that was 12–13% that of [^{125}I]MIBG (46.1±2.7%), and the ratio of uptake by control over DMI-treated (nonspecific) cultures was higher for [^{131}I]GIBG (20.9±0.3) than [^{125}I]MIBG itself (15.0±2.7). The exocytosis of [^{131}I]GIBG and [^{125}I]MIBG from SK-N-SH cells was similar. The uptake of [^{131}I]GIBG in the mouse target tissues, heart and adrenals, as well as in a number of other tissues was about half that of [^{125}I]MIBG. These results suggest that substitution of guanidine functions, especially a guanidinomethyl group, in MIBG structure may not be advantageous.

1. Introduction

The guanethidine analogue *meta*-iodobenzylguanidine (MIBG) is avidly taken up by tissues rich in sympathetic neurons and neuroendocrine neoplasms via the norepinephrine transporter (NET).[1] MIBG, radiolabeled with $^{123/131}$I, is used in the scintigraphic evaluation of normal and malignant tissues of neuroadrenergic origin.[2,3] High specific activity [^{131}I]MIBG is used in the therapy of neuroblastoma, pheochromocytoma, and other neuroendocrine tumors.[4] Although radioiodinated MIBG has been used extensively in diagnostic applications with success, results from therapy with [^{131}I]MIBG are not very encouraging, even when combined with other therapeutic modalities.[5] Therapeutic outcome might be improved if higher tumor-to-background ratios could be achieved. One possibility is to add polar substituents to MIBG, creating analogues that may have lower retention in normal tissues.

In our efforts to improve the bioavailability of MIBG, we recently reported on the structure–activity studies of several analogues of MIBG.[6,7] It was concluded that introduction of substituents at the 5-position of the ring is detrimental to the affinity of MIBG binding to the NET. For example, while a MIBG derivative with a 4-amino substituent (AIBG) was taken up by the SK-N-SH human neuroblastoma cells to a degree similar to that for MIBG, the isomeric compound 5-amino-3-iodobenzylguanidine exhibited only nonspecific binding. There is not much latitude in altering the guanidine moiety either.[8] Thus to achieve our goal of developing an optimized MIBG analogue, it was deemed necessary to make alterations at the 4-position on the ring.

It has been reported that in the process of transporting its substrates, the NET binds to the protonated form of the amine substrate.[9] Because a guanidine moiety will remain almost exclusively in the protonated form at physiological pH, we wished to investigate whether the introduction of an additional guanidine group will have an additive, if not synergistic, effect on the uptake of MIBG, in addition to imparting hydrophilicity. It should be pointed out that a bis-guanidine derivative,

Keywords: *Meta*-iodobenzylguanidine; Neuroblastoma; Uptake-1; Norepinephrine transporter.
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formed by the substitution of a guanidine moiety on the guanidine side chain of MIBG, was not a good substrate for NET.[8]

In this study, we have prepared two MIBG derivatives with an additional guanidine substituent and evaluated their biological characteristics. While the derivative with the guanidine group directly attached to the 4-position of the ring showed some specificity to NET, the MIBG analogue with a 4-guanidinomethyl substituent was not a substrate for the transporter.

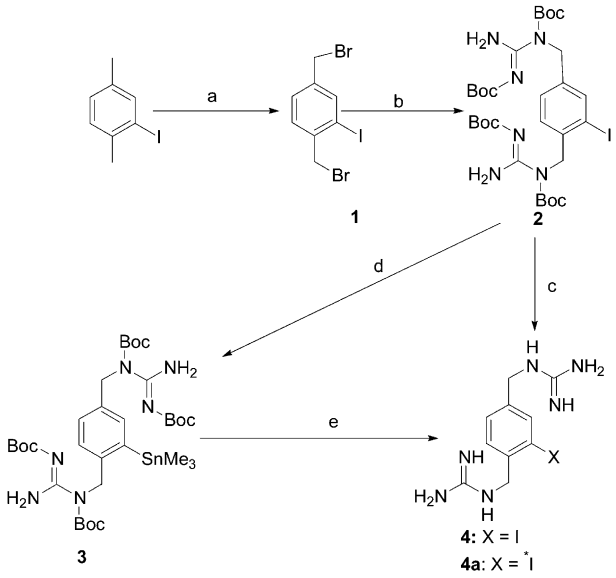
2. Results and discussion

2.1. Chemistry

In addition to the fact that NET transports NE and other amines in their protonated form, it has been proposed that ATP present in storage vesicles binds to the positively charged catecholamines via its four negative charges.[10] We hypothesized that, in addition to imparting added hydrophilicity, the presence of a second guanidine moiety in MIBG might augment its tumor binding and/or storage. The substitution of a guanidine group on the guanidine side chain, resulting in a bisguanidine, has been shown to be unfavorable[8] and our earlier studies have shown that the presence of a group or an atom other than hydrogen at the 5-position in the ring is also counterproductive. Thus, our target was a MIBG derivative with a guanidine function at the 4-position. From a retrosynthetic point of view, it appeared more facile to prepare a MIBG derivative with a guanidinomethyl substituent, instead of one in which the guanidine group was attached directly to the ring. We have investigated the effect of a methyl group itself at the 4-position of MIBG and have shown that 3-iodo-4-methylbenzylguanidine (MeIBG) retained the biological properties of MIBG (results will be published elsewhere). Because the introduction of a methyl group did not adversely affect the biological properties of MIBG, we chose to first prepare the guanidinomethyl derivative.

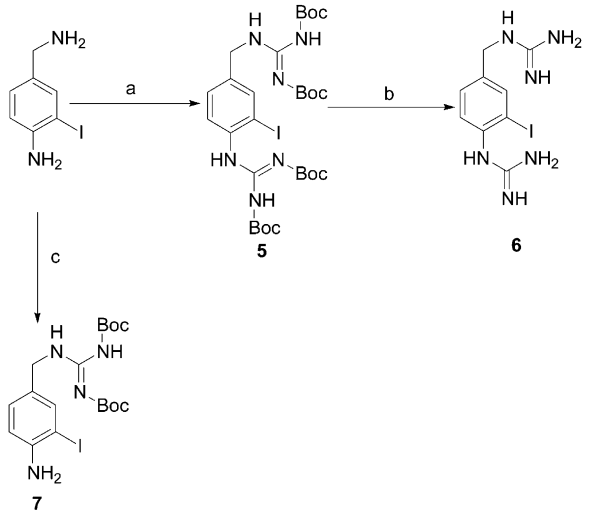
Scheme 1 shows the synthesis of 4-guanidinomethyl-3-iodobenzylguanidine (GMIBG). Benzylic bromination of 2-iodo-*p*-xylene yielded 1,4-bis(bromomethyl)-2-iodobenzene,[11] which was subjected to guanidinylation using a procedure reported earlier for the guanidinylation of benzyl bromides[12] to obtain the protected guanidine derivative **2**. The target bisguanidine was generated by the deprotection of **2** using a cocktail of TFA/water/triisopropylsilane. Palladium-catalyzed stannylation of **2** yielded the tin precursor **3** and the radiolabeled GMIBG was obtained from **3** by radioiodination and in situ deprotection in about 50% radiochemical yield.

GMIBG did not exhibit any significant uptake in SK-N-SH cells (see below). To investigate whether this might be due to steric constraints at the active site of the transporter as a result of the introduction of the relatively bulkier guanidinomethyl group at the 4-position of MIBG, we embarked on the synthesis of a MIBG derivative wherein the guanidine moiety is attached



Scheme 1. Reagents: (a) *N*-Bromosuccinimide, 1,1′-Azobis-(cyclohexanecarbonitrile), DCE; (b) *N*,*N*′-bis-tert-butyloxycarbonylguanidine, NaH, DMF; (c) Trifluoroacetic acid/water/triisopropylsilane (95/2.5/2.5), methylene chloride; (d) Hexamethyditin, Bis-(triphenylphosphine) palladium dichloride, dioxane; (e) i-Radioiodine, NCS ii-c.

directly to the ring (Scheme 2). The starting material, 4-amino-3-iodobenzylamine was prepared as reported.[13] This diamine was converted to the protected bisguanidine target **5** using *N*,*N*′-bis(*tert*-butyloxycarbonyl)-thiourea and Mukaiyama's reagent in methylene chloride following a method for guanidinylation reported by Yong et al.[14] The target, 4-guanidinomethyl-3-iodobenzylguanidine (GIBG), was obtained by the deprotection of **5** with TFA cocktail. Several attempts to prepare a tin precursor for use in the preparation of **6a** were not successful. Neither palladium-catalyzed stannylation of **5** nor electrophilic stannylation of the anion generated from reaction of **5** with butyl lithium

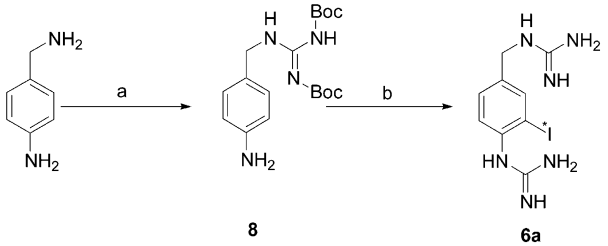


Scheme 2. Reagents: (a) *N*,*N*′-Bis-*tert*-butyloxycarbonylthiourea, Triethylamine, Mukaiyama reagent; (b) Trifluoroacetic acid/water/triisopropylsilane. (95/2.5/2.5); (c) *N*,*N*′-Bis-*tert*-butyloxycarbonyl-S-methylisothiourea, Triethylamine, DMF.

yielded the expected *N*,*N*′-bis(*tert*-butyloxycarbonyl)-4-[*N*,*N*′-bis(*tert*-butyloxycarbonylguanidino)]-3-(trimethylstannyl)benzylguanidine. Assuming that this might be due to steric hindrance from the bulky bis-Boc-guanidino group, *N*,*N*′-bis(*tert*-butyloxycarbonyl)-4-amino-3-iodobenzylguanidine **7** was prepared first, in the hope that this could be first stannylated and its 4-amino group converted subsequently to a protected guanidine moiety by the method of Yong et al.[14] Compound **7** itself was prepared initially from 4-amino-3-iodobenzylamine following the method of Yong et al.[14] and using DMF as the solvent. However, the yields were meager and the chromatographic isolation was challenging. Subsequently, **7** was prepared in excellent yields, by following a literature procedure for guanidinylation,[15] by the treatment of 4-amino-3-iodobenzylamine dihydrochloride with the commercially available *N*, *N*′-bis(*tert*-butyloxycarbonyl)]-*S*-methylisothiourea. Attempts to convert **7** to *N*,*N*′-bis(*tert*-butyloxycarbonyl)-4-amino-3-(trimethylstannyl)benzylguanidine using both of the approaches described above also were futile. Finally, radioiodinated GIBG was prepared at a no-carrier-added level in a three-step approach starting from *N*,*N*′-bis(*tert*-butyloxycarbonyl)-4-aminobenzylguanidine **8**, which itself was prepared from the commercially available 4-aminobenzylamine following Yong et al.[14] and conducting the reaction in DMF (Scheme 3). Compound **8** was first radioiodinated to yield [^{131}I]**7**. For reasons not clear, [^{131}I]**7** could not be converted to [^{131}I]**5** by treatment with *N*,*N*′-bis(*tert*-butyloxycarbonyl)thiourea and Mukaiyama's reagent in methylene chloride, even though this reaction was facile in the conversion of unlabeled **7** to **5** (experimental details not given). However, treatment of [^{131}I]**7** with cyanamide and subsequent deprotection by in situ treatment with TFA cocktail yielded [^{131}I]GIBG in about 55% overall radiochemical yield.

3. Biological evaluation

To investigate the effects of additional guanidine substitution on the biological behavior of these MIBG analogues, they were subjected to a standard set of assays utilized in our laboratories. In these assays, the uptake of the new tracers and that of MIBG by SK-N-SH human neuroblastoma cells in the presence and absence of various uptake-1[16] blocking agents was determined in a paired-label format. In the absence of any uptake-1 inhibiting conditions, the uptake of [^{131}I]GMIBG was 1.7±0.1% of input counts (Fig. 1A) a value considerably lower ($p<0.05$) than that for [^{125}I]MIBG (40.3±1.4%). In fact, it was even less than that for [^{125}I]MIBG (2.1±0.1%) in cells pretreated with DMI, a tricyclic antidepressant inhibitor of the uptake-1 pump. We speculate that this loss in NET specificity may be due to either the charge of the guanidine moiety and/or the steric bulk of the guanidinomethyl group.



Scheme 3. Reagents: (a) *N*,*N*′-Bis-tert-butyloxycarbonylthiourea, Triethylamine, Mukaiyama reagent; (b) (i)Radioiodine, NCS (ii) cyanamide; (iii) Trifluoroacetic acid/water/triisopropylsilane (95/2.5/2.5).

To probe the role of the intervening methylene spacer of the guanidinomethyl moiety in the loss of NET specificity, we investigated the effect of a guanidine group attached directly to the 4-position of MIBG. As shown in Figure 1B, [^{131}I]GIBG had an uptake of 5.6±0.3% of input counts compared to 46.1±2.7% for [^{125}I]MIBG in SK-N-SH cells. Although the uptake of [^{131}I]GIBG was only about 12–13% of [^{125}I]MIBG, it was encouraging to note that the ratio of uptake by control over DMI-treated (nonspecific uptake) cultures was higher for [^{131}I]GIBG (20.9±0.3) compared to that for [^{125}I]MIBG (15.0±2.7; $p<0.05$). A value of 20 for this ratio for MIBG in SK-N-SH cells has been reported previously.[17] In addition, other than ouabain, all uptake-1 blocking conditions reduced the uptake of [^{131}I]GIBG to a degree similar to that observed with [^{125}I]MIBG. Thus, it appears that, although the absolute uptake of [^{131}I]GIBG is only about a sixth that of

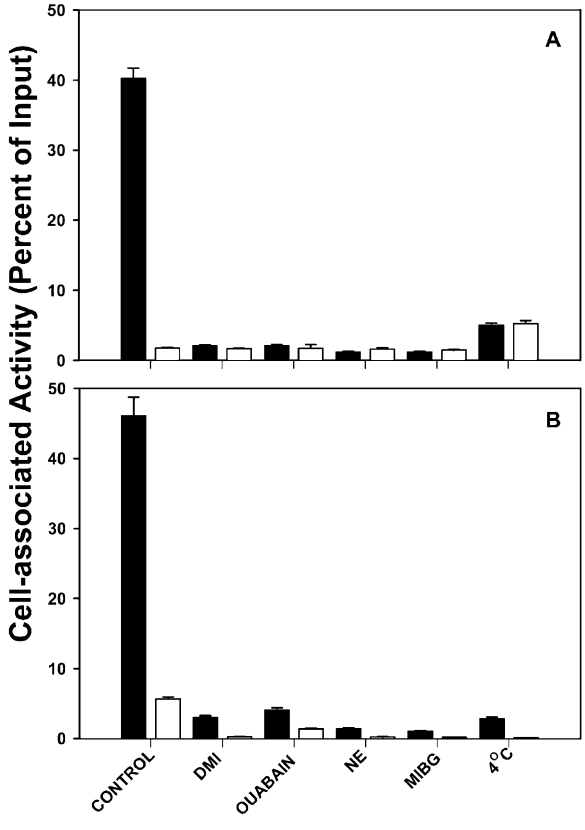


Figure 1. Figure 1. Uptake of [^{131}I]GMIBG (A) and [^{131}I]GIBG (B) by SK-N-SH human neuroblastoma cells in vitro (open bars). Results obtained for [^{125}I]MIBG from respective paired-label assays are also shown (filled bars). The effect of various uptake-1-blocking conditions is also presented. Cells were incubated as described in the text in the absence or presence of various uptake-1 inhibitors or at 4 °C, and the cell-associated radioactivity was determined as a percent of input radioactivity.

[^{125}I]MIBG, the specificity of its uptake via NET is higher.

Before undertaking studies in SK-N-SH xenograft models, we wanted to evaluate [^{131}I]GIBG further in vitro to investigate whether it held advantages over MIBG. To augment their intracellular retention, monoclonal antibodies which are internalized after binding to antigens have been labeled with templates containing positive charges.[18,19] In these cases, the ligands were localized in the lysosomal compartment, the pH of which is distinctly acidic (~5). This ensured that the ligands containing basic moieties will be predominantly charged, causing them to remain trapped within the lysosome. In neuroblastomas, MIBG is known to be stored in the cytoplasm.[20,21] Nevertheless, we were interested in studying if the added guanidine group in GIBG enhanced retention by SK-N-SH cells in vitro compared to that seen for MIBG. It has been proposed that the intracellular levels of MIBG are maintained by SK-N-SH cells by a dynamic equilibrium, generated by a rapid reuptake of drug diffusing from the cells.[17] If GIBG remains completely trapped within the tumor cells due to the presence of an additional guanidine group in its structure, then one would expect no change over a period of time in the cell-associated amount of radioactivity (after accounting for decay) that was taken up initially. Since it is unlikely that DMI will have a depleting effect on the drug stored intracellularly,[17] the cell-associated radioactivity should not be affected by the presence of DMI. As shown in Figure 2, the cell-associated radioactivity normalized to the initially bound for the two tracers was comparable at different time points indicating their similar rate of efflux. Besides, DMI reduced the retained radioactivity for both tracers at different time points to a similar degree suggesting that, like MIBG, GIBG is retained in SK-N-SH cells by a reuptake pathway. The observation that the additional positive charge did not augment the cell retention may point to the fact that the efflux of MIBG and its analogues by neuroblastoma cells occurs most likely via an active carrier-mediated mechanism as proposed by Servidei et al.,[22] rather than by a diffusion pathway. Taken together, GIBG has biological characteristics similar to MIBG albeit with a lower uptake capacity and/or affinity.

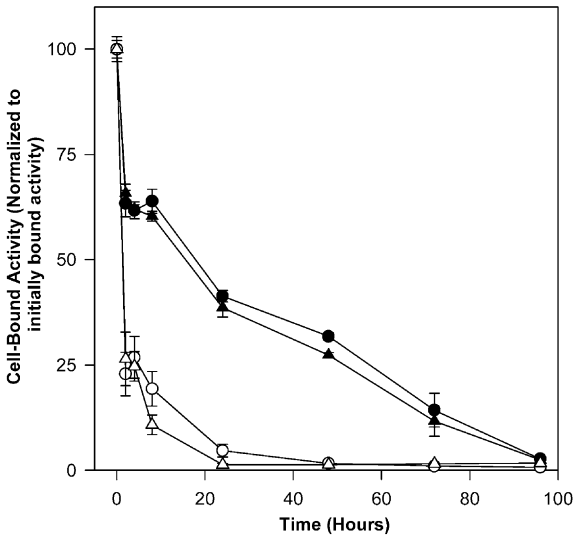


Figure 2. Paired-label exocytosis of [^{131}I]GIBG (●, without DMI; ○, with DMI) and [^{125}I]MIBG (▲, without DMI; △, with DMI) as a function of time. Cells were allowed to take up the tracers at 37 °C for 2 h. The medium containing the tracers was replaced with fresh medium without or with DMI, and the cell-associated radioactivity was determined periodically.

Finally, a tissue distribution of GIBG was performed in normal mice in a paired-label format with MIBG to investigate whether the normal tissue uptake of GIBG was lower than that of MIBG, which may warrant its further evaluation in xenograft models. The data presented in Table 1 shows that the uptake of [^{131}I]GIBG in the uptake-1-mediated target tissues, heart and adrenals, was about 50% of that of [^{125}I]MIBG. Although not dramatic, the ratio of uptake of [^{131}I]GIBG to [^{125}I]MIBG in these tissues increased with time. The uptake of [^{131}I]GIBG in these tissues, like that of [^{125}I]MIBG, was specific as demonstrated by reduction in the uptake by DMI pretreatment of the mice. The reduction in heart uptake, about 26% of control for both tracers, is less than that reported earlier for [^{125}I]MIBG (40–50%).[23] However, the current study was performed in a paired-label format and therefore it can be concluded that the degree of specificity of GIBG uptake in mouse heart is same as that of MIBG. Compared to [^{125}I]MIBG, the uptake of [^{131}I]GIBG was less in most of the tissues, with the differences generally being about a factor of two. A notable difference was in blood where the uptake of [^{131}I]GIBG was 2- to 4-fold higher than that of [^{125}I]MIBG. These normal mice tissue distribution results in concert with the observation that [^{131}I]GIBG uptake in vitro was only one sixth of MIBG uptake, suggest that achieving higher tumor-to-tissue ratios with GIBG is not likely. This may indicate that additional positive charge in MIBG, while not enhancing, may actually decrease the transport of MIBG derivatives by NET.

In summary, methods were designed to synthesize MIBG derivatives containing guanidine and guanidinomethyl substituents at the 4-position. While the introduction of the 4-guanidinomethyl moiety diminished the affinity of MIBG to, and its transport by, NET, direct attachment of the guanidine function at the 4-position yielded a compound that partly retained MIBG characteristics. The results from an in vitro retention assay and tissue distribution in normal mice suggest that GIBG may not deserve further consideration as a radiopharmaceutical for treating tumors that overexpress NET.

4. Experimental

4.1. General

All chemicals were purchased from Aldrich unless otherwise noted. Sodium [^{125}I]iodide and sodium [^{131}I]iodide, with specific activities of 2200 Ci/mmol and

Table 1. Paired-label tissue distribution of [^{125}I]MIBG and [^{131}I]GIBG in normal mice

Tissue	Percent Injected Dose per Gram[a]							
	1 h Control		1 h DMI		4 h		24 h	
	[^{125}I]MIBG	[^{131}I]GIBG	[^{125}I]MIBG	[^{131}I]GIBG	[^{125}I]MIBG	[^{131}I]GIBG	[^{125}I]MIBG	[^{131}I]GIBG
Liver	5.63±1.05	3.01±0.40	6.30±0.52	3.11±0.46	2.86±0.28	1.53±0.12	0.70±0.07	0.50±0.05
Spleen	3.08±0.36	4.22±0.64	3.15±0.36	5.13±0.65	2.58±0.25	3.45±0.57	1.04±0.17	1.27±0.16
Lungs	4.32±1.39	2.71±0.41	4.04±0.38	2.67±0.19	2.61±0.28	2.07±0.61[b]	0.82±0.10	0.59±0.14
Heart	11.10±3.06	5.01±1.05	8.24±0.87	3.71±0.50	8.35±1.30	4.22±0.53	3.39±0.41	2.13±0.21
Kidneys	2.18±0.22	1.86±0.20	2.35±0.21	2.05±0.22	1.43±0.22	0.87±0.07	0.52±0.05	0.38±0.02
Stomach	2.86±0.48	2.18±0.34	2.39±0.66	1.54±0.37	1.54±0.66	1.03±0.54	1.36±0.18	0.98±0.14
Sm. Int.	4.51±0.65	4.08±0.53	5.28±0.36	4.21±0.31	3.28±0.52	1.78±0.29	1.33±0.07	0.89±0.03
Lg. Int.	2.68±0.63	2.90±0.67[b]	2.46±0.29	1.92±0.15	3.80±0.73	3.09±0.80	1.44±0.19	1.03±0.14
Thyroid[c]	0.08±0.07	0.13±0.05	0.09±0.04	0.07±0.01[b]	0.21±0.08	0.12±0.03	0.22±0.05	0.06±0.02
Muscle	1.46±0.17	0.84±0.07	1.64±0.26	0.99±0.13	1.16±0.66	0.57±0.05	0.36±0.07	0.21±0.04
Blood	0.62±0.17	1.56±0.42[b]	0.69±0.10	1.74±0.20	0.33±0.04	1.07±0.19	0.08±0.01	0.26±0.02
Adrenals	7.78±3.56	4.03±1.43	5.72±1.59	3.33±1.14	6.43±1.61	3.34±1.02	5.35±1.45	3.20±0.78
Bone	0.62±0.18	0.57±0.13[b]	0.77±0.21	0.83±0.28[b]	0.57±0.08	0.45±0.06	0.15±0.02	0.12±0.02
Brain	0.09±0.02	0.08±0.01	0.08±0.01	0.05±0.00	0.06±0.01	0.04±0.01[b]	0.01±0.00	0.01±0.00

[a] Mean±SD ($n=5$).
[b] Except for these tissues, the difference in uptake between two preparations statistically significant ($p<0.05$).
[c] %ID/organ.

1200 Ci/mmol, respectively, were obtained from Perkin Elmer Life Sciences (Boston, MA). Unlabeled MIBG was obtained from Sigma or prepared in house using a literature protocol.[1] The dihydrochloride salt of 4-amino-3-iodobenzyl amine was prepared as reported.[13]

Melting points were determined on a Haake Buchler apparatus and were uncorrected. High pressure liquid chromatography was performed using a Beckman System Gold HPLC equipped with a Model 126 programmable solvent module, a Model 168 diode array detector, a Model 170 radioisotope detector, and a Model 406 analogue interface module. For reversed-phase chromatography, a Waters XTerra C18 column (4.6×250 mm, 5 μ) or a Waters Bondapak C18 column (10 μ, 3.9×300 mm) was used. Normal-phase HPLC was performed using a 4.6×250 mm Partisil (10 μ) silica column (Alltech, Deerfield, IL). Analytical TLC was performed on aluminum-backed sheets (Silica gel 60 F_{254}), and normal-phase column chromatography was performed using Silica gel 60, both obtained from EM Science (Gibbstown, NJ). Column chromatographic fractions were collected using a Gilson model 203 micro fraction collector (Middleton, WI) or an ISCO Foxy 200 fraction collector (Lincoln, NE), and products identified by TLC. In some cases, an ISCO UA-6 UV–vis detector was placed between the column outlet and the fraction collector to identify fractions. Preparative thick layer chromatography was performed using 20 × 20 cm, 1000 μ plates (Whatman, Clifton, NJ). Before applying the sample, the plates were run in ethyl acetate to clean the plates of any adsorbed impurities. Radioactivity was measured using a dose calibrator (Capintec, CRC-7R, USA) for higher amounts and an automated gamma counter (LKB 1282, Wallac, Finland) for lower count rates. Proton NMR spectra (300 MHz) were obtained on a Varian Mercury 300 spectrometer or a General Electric Midfield GN-300 spectrometer. Chemical shifts are reported in δ units; solvent peaks were referenced appropriately. Mass spectra were obtained on a Hewlett-Packard GC/MS/DS Model HP-5988A instrument, or on a JEOL SX-102 high resolution mass spectrometer.

4.2. Cells and culture conditions

The human neuroblastoma cell line SK-N-SH (uptake-1 positive)[24] was purchased from the American Type Culture Collection (Rockville, MD). The incubation medium (JRH Biosciences, Lenexa, KS) was made by mixing 440 mL of RPMI 1640, 50 mL of Serum Plus, 5 mL of penicillin-G/streptomycin (5000 U of penicillin and 5000 μg of streptomycin in 1 mL of 0.85% saline), and 5 mL of glutamine (200 mM in saline). The cells were grown at 37 °C in a humidified incubator containing 5% CO_2. Cell viability was evaluated prior to each binding experiment by trypan blue dye,[25] and was 95–98% for all studies.

4.3. Syntheses of standards and precursors

4.3.1. 1,4-Bis-bromomethyl-2-iodobenzene (1). The title compound was prepared by modification of a procedure reported for benzylic bromination.[26] The radical initiator 1,1′-aza-biscyclohexanenitrile[27] (ABCN) was used in lieu of the traditional aza-bisisobutyronitrile (AIBN). Thus, a mixture of 2-iodo-*p*-xylene (233 mg, 1 mmol; Lancaster, Windham, NH), *N*-bromosuccinimide (356 mg, 2 mmol), and ABCN (50 mg, 0.2 mmol) in dichloroethane (10 mL) was heated at reflux in the presence of an incandescent lamp. After 3–4 h, dichloroethane was evaporated from the reaction mixture and the residue was taken in ethyl acetate. The ethyl acetate solution was washed with saturated sodium metabisulfite and brine, dried with sodium sulfate, and evaporated. The crude mixture was purified by silica gel chromatography using 10% ethyl acetate in hexane to obtain 106 mg (27%) of a white solid: mp 111–113 °C (lit.[11] 109–110.5 °C). ^{1}H NMR ($CDCl_3$) δ 4.37 (s, 2H), 4.59 (s, 2H), 7.40 (m, 2H), 7.88 (d, 1H). MS (EI^+) *m*/*z*: 389 (M^+).

HRMS (EI$^+$) calcd for $C_8H_7{}^{79}Br^{81}BrI$ (M^+): 389.7937. Found: 389.7939 ± 0.0009 ($n = 4$).

4.3.2. 1,4-Bis-[(*N*,*N*′-bis-*tert*-butyloxycarbonyl)guanidinomethyl]-2-iodobenzene (2). To a slurry of sodium hydride (60% dispersion in mineral oil; 88 mg, 2.2 mmol), in DMF (10 mL) was added 627 mg (2.4 mmol) of *N*,*N*′-bis-*tert*-butyloxycarbonylguanidine.[28] The mixture became homogeneous upon stirring for 5 min. To this solution was added 390 mg (1 mmol) of **1** in one portion, and the reaction mixture was stirred for an additional 10 min. The mixture was partitioned between ethyl acetate and water, and the aqueous layer was extracted twice with ethyl acetate. The combined ethyl acetate solution was washed with brine, dried, and evaporated. The crude mixture was subjected to silica gel chromatography using 15% ethyl acetate in hexane to obtain 440 mg (59%) of a waxy solid: mp 161–162 °C. ^{1}H NMR ($CDCl_3$) δ 1.28 (s, 9H), 1.42 (s, 9H), 1.46 (s, 9H), 1.50 (s, 9H), 5.08 (s, 2H), 5.16 (s, 2H), 6.86 (dd, 1H), 7.22 (dd, 1H), 7.78 (d, 1H), 9.42 (br m, 4H). MS (FAB$^+$) *m*/*z*: 747 (MH^+), 647. HRMS (FAB$^+$) calcd for $C_{30}H_{48}IN_6O_8$ (MH^+): 747.2578. Found: 747.2608 ± 0.0007 ($n = 2$).

4.3.3. 1,4-Bis-[(*N*, *N*′-bis-*tert*-butyloxycarbonyl)guanidinomethyl]-2-(trimethylstannyl)benzene (3). A mixture of **2** (44 mg, 0.06 mmol), hexamethylditin (163 mg, 0.5 mmol), and bis(triphenylphosphine)palladium dichloride (16 mg, 0.02 mmol) in 2 mL of dioxane was refluxed for 1 h. The mixture was filtered through a bed of Celite, which was washed with ethyl acetate. The combined filtrate was concentrated and chromatographed using 15% ethyl acetate in hexane to yield 15 mg (32%) of an oil. An analytically pure sample was obtained by the preparative TLC of the above oil: ^{1}H NMR ($CDCl_3$) δ 0.34 (s, 9H [^{119}Sn-H, d]), 1.26 (s, 9H), 1.37 (s, 9H), 1.42 (s, 9H), 1.48 (s, 9H), 5.14 (s, 2H), 5.20 (s, 2H), 6.86 (dd, 1H), 7.16 (dd, 1H), 7.40 (d, 1H), 9.42 (br m, 4H). MS (FAB$^+$) *m*/*z*: cluster peaks around 785 (MH^+), 769, 685. HRMS (FAB$^+$) calcd for $C_{33}H_{57}N_6O_8{}^{116}Sn$ (MH^+): 781.3255. Found: 781.3246 ± 0.0020 ($n = 3$).

4.3.4. 1,4-Bis-(guanidinomethyl)-2-iodobenzene (4). To 34 mg (0.04 mmol) of **2** in a 1/2-dram vial was added a 95/2.5/2.5 (v/v/v) cocktail of TFA/water/triisopropylsilane (1 mL) and the mixture was stirred at room temperature for 3–4 h. The solvents were evaporated and any residual solvent was removed by coevaporating three times each with 1 mL methanol and methylene chloride. The residue was dried to obtain **4** as a film in almost quantitative yield. ^{1}H NMR (CD_3OD) δ 4.40 (s, 2H), 4.41 (s, 2H), 7.44 (m, 2H), 7.96 (d, 1H). MS (FAB$^+$) *m*/*z*: 347 (MH^+). HRMS (FAB$^+$) calcd for $C_{10}H_{16}IN_6$ (MH^+): 347.0481. Found: 347.0482 ± 0.0001 ($n = 2$).

4.3.5. (*N*,*N*′-bis-*tert*-butyloxycarbonyl)-4-(*N*,*N*′-bis-*tert*-butyloxycarbonylguanidino)-3-iodobenzylguanidine (5). To a mixture of free base of 4-amino-3-iodobenzylamine (87 mg, 0.35 mmol), *N*,*N*′-bis(*tert*-butyloxycarbonyl)-thiourea[29] (213 mg, 0.77 mmol), and triethylamine (150 mg, 1.48 mmol) in 10 mL methylene chloride was added Mukaiyama's reagent (218 mg, 0.85 mmol). The reaction was allowed to stir at room temperature until completion, as judged by TLC. The methylene chloride was evaporated, and the residue partitioned between ethyl acetate and water. The organic layer was dried with anhydrous sodium sulfate, the solvent was removed on a rotary evaporator, and the product purified by silica gel chromatography using 15% ethyl acetate in hexane to obtain 67 mg (28%) of an oil: ^{1}H NMR ($CDCl_3$) δ 1.5 (s, 18H), 1.55 (s, 18H), 4.62 (br s, 2H), 7.34 (dd, 1H), 7.76 (d, 1H), 8.22 (d, 1H). MS (FAB$^+$) *m*/*z*: 733.2 (MH^+). HRMS (FAB$^+$) calcd for $C_{29}H_{46}IN_6O_8$ (MH^+): 733.2422. Found: 733.2422 ± 0.0011 ($n = 2$).

4.3.6. 4-Guanidino-3-iodobenzylguanidine (6). *N*,*N*′-(bis-*tert*-butyloxycarbonyl)-4-(*N*,*N*′-bis-*tert*-butyloxycarbonylguanidino)-3-iodobenzylguanidine (**5**; 20.6 mg, 0.03 mmol) was treated with a 95:2.5:2.5 (v/v/v) mixture of trifluoroacetic acid:water:triisopropylsilane (685 μL). After stirring at room temperature for 70 min, the solvents were evaporated using an argon stream. The residual solvents were removed by co-evaporating once with methanol and thrice with ethyl acetate (1 mL each). The residue was dried under high vacuum to obtain **6** as a film in almost quantitative yield. ^{1}H NMR (CD_3OD) δ 4.46 (s, 2H), 7.44 (m, 2H), 7.96 (dd, 1H). MS (FAB$^+$) *m*/*z*: 333 (MH^+), 289, 274. HRMS (FAB$^+$) calcd for $C_9H_{14}IN_6$ (MH^+): 333.0325. Found: 333.0317 ± 0.0001 ($n = 2$).

4.3.7. (*N*,*N*′-bis-*tert*-butyloxycarbonyl)-4-amino-3-iodobenzylguanidine (7). *Method A*. To a mixture of 4-amino-3-iodobenzylamine dihydrochloride (80 mg, 0.25 mmol) and triethylamine (240 μL) in DMF (120 μL) was added 1, 3-bis-(*tert*-butyloxycarbonyl)-2-methyl-2-thiopseudourea (88 mg, 0.3 mmol), and the mixture was stirred at room temperature for 14 h. The mixture was partitioned between ethyl acetate and water, and the combined organic layer was dried and concentrated. The crude mixture was chromatographed using 15% ethyl acetate in hexane to obtain 101 mg (82%) of a foam: ^{1}H NMR ($CDCl_3$) δ 1.47 (s, 9H), 1.53 (s, 9H), 4.50 (d, 2H), 6.70 (d, 1H), 7.10 (dd, 1H), 7.59 (d, 1H). MS (FAB$^+$) *m*/*z*: 491 (MH^+). HRMS (FAB$^+$) calcd for $C_{18}H_{28}IN_4O_4$ (MH^+): 491.1155. Found: 491.1155 ± 0.0006 ($n = 2$). *Method B*. The title compound **7** was isolated in 52% yield by following a procedure similar to that described above for the preparation of **5** with the following changes: one equivalent of Mukaiyama's reagent and DMF, in lieu of methylene chloride as the solvent, were used.

4.3.8. (*N*,*N*′-bis-*tert*-butyloxycarbonyl)-4-aminobenzylguanidine (8). To a solution of 4-aminobenzyl amine (61 mg, 0.5 mmol) in anhydrous DMF (Pierce, Rockford, IL; 165 μL) was added *N*,*N*′-bis(*tert*-butyloxycarbonyl)thiourea (165 mg, 0.6 mmol) and triethylamine (154 μL, 1.1 mmol). A suspension of Mukaiyama's reagent (153 mg, 0.5 mmol) in DMF (330 μL) was added dropwise to the above mixture. An additional 430 μL of DMF was used to transfer the sedimented Mukaiyama's reagent to the reaction flask. The reaction mixture was stirred at room temperature for 1.5 h and then partitioned between water and ethyl acetate. The

combined organic layer was washed with brine, dried, and concentrated. The product was isolated by silica gel chromatography using a stepwise gradient of 10% through 50% ethyl acetate in hexane to obtain 108 mg (60%) of a yellow oil. An analytical sample was obtained as a foamy solid by further preparative TLC: mp 125–128 °C. ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9H), 1.50 (s, 9H), 3.65 (br s, 2H), 4.45 (s, 2H), 6.60 (dd, 2H), 7.08 (dd, 2H), 8.4 (s, 1H), 11.45 (br s, 1H). MS (FAB^+) *m/z*: 365 (MH^+). HRMS (FAB^+) calcd for $C_{18}H_{29}N_6O_4$ (MH^+): 365.2189. Found: 365.2204 ± 0.0004 ($n = 2$).

4.4. Radiochemistry

4.4.1. 1,4-Bis-(guanidinomethyl)-2-[^{131}I]iodobenzene (4a). To a 1/2-dram vial containing 100 μg of **3** was added 1–2 μL of ^{131}I (about 1 mCi) in 0.1N NaOH followed by 5 μL of a 3:1 (v/v) mixture of acetic acid and 30% (w/v) H_2O_2. The contents of the vial were sonicated for 30 s and the solvents were evaporated with a stream of argon. To the residual radioactivity was added 100 μL of a 95/2.5/2.5 mixture of TFA/water/triisopropylsilane. The vial was vortexed and left at room temperature for 10 min. Most of the solvents were evaporated under a flow of argon, and any residual solvents were removed by coevaporating with chloroform and methanol in that order (each 3×25 μL). The radioactivity was reconstituted in 25 μL methanol and injected onto a reversed-phase column (Bondapak) that was eluted isocratically with 99:1 0.2 M ammonium dihydrogen phosphate, pH 7:THF at a flow rate of 1 mL/min. The product **4a** ($t_R = 14$ min) was isolated in an overall radiochemical yield of 50%.

4.4.2. 4-Guanidino-3-[^{131}I]iodobenzylguanidine (6a). To a solution of **8** in methanol containing 1% acetic acid (0.18 mg per mL; 50 μL) was added a solution of *N*-chlorosuccinimide in methanol (1 mg/mL; 10 μL) followed by 1–3 μL of ^{131}I in 0.1 N NaOH (1–2 mCi). The vial was vortexed and the reaction was allowed to proceed at room temperature for 30 min. The reaction mixture was injected onto a normal phase HPLC column eluted at 1 mL/min with hexane (A) and ethyl acetate (B), each containing 0.2% acetic acid. The column was initially eluted isocratically with 93:7 A:B for 20 min, then the percent composition of solvent B increased linearly to 90 over 15 min. The fractions corresponding to [^{131}I]**7** ($t_R = 15$ min) were collected, and a radiochemical yield greater than 90% was obtained. The solvents from these fractions were evaporated and any residual acetic acid was removed by coevaporating with ethyl acetate (3 × 100 μL). To the residual radioactivity was added 1-5 mg of cyanamide in 20 μL methanol. The vial was vortexed, and the methanol was evaporated with a stream of argon. The vial was heated at 100 °C in oil bath for 10 min. Trifluoroacetic acid (100 μL) was added to the reaction vial and the vial was heated for another 5 min. Trifluoroacetic acid was removed with an argon stream, and any residual acid present was removed by coevaporating with 3×25 μL of methanol. Finally, the radioactivity was dissolved in 25 μL of methanol and injected onto a reversed-phase HPLC column (XTerra) and eluted isocratically at 1 mL/min with 5% THF in 5 mM ammonium bicarbonate, pH 10.3. The fractions corresponding to **6a** ($t_R = 14$ min) were isolated in 60% radiochemical yield (for this step). Radioactivity from reversed-phase HPLC fractions in all cases was concentrated using a C18 solid-phase cartridge.

4.5. Paired-label in vitro uptake of MIBG and analogues by SK-N-SH cells

SK-N-SH cells were plated in 6-well plates at an initial density of 5×10^5 cells per well in 3 mL of medium, and incubated for 24 h. The medium was removed by aspiration, 100 nCi each of [^{125}I]MIBG and either [^{131}I]GMIBG or [^{131}I]GIBG were added to each well in 3 mL of medium, and the cells were allowed to take up the tracers for 2 h at 37 °C. The specificity and energy dependence of tracer uptake was determined using DMI and ouabain, respectively. For this, the initial medium was removed and the cells were incubated with 3 mL each of either 1.5 μM DMI or 1 mM ouabain for 30 min. The medium was removed and tracers were added as above and incubated for 2 h at 37 °C. The energy dependence of tracer uptake also was ascertained by determining the effect of temperature. For this, the original medium was removed and fresh medium that has been cooled to 4 °C was added. Then tracers (100 nCi each) in 10 μL of the medium were added and the cells were incubated for 2 h at 4 °C. Finally, the effect of norepinephrine and MIBG was ascertained by removing the original medium and co-incubating the cells at 37 °C for 2 h with the tracers (100 nCi in 10 μL each) and 3 mL each of either 50 μM norepinephrine or 10 μM MIBG. At the end of the 2 h incubation period, the cells were solubilized by incubation with 500 μL of 0.5 N NaOH for 30 min at room temperature and then removed with cotton swabs. The cell-bound radioactivity was counted along with input standards using a dual-channel gamma counter. Three to six replicates were performed for each of the uptake-1-blocking conditions.

4.6. Paired-label exocytosis of [^{131}I]GIBG and [^{125}I]MIBG from SK-N-SH cells

Cells were added to 6-well plates at a density of $\sim4\times10^5$ cells per well per 3 mL of medium and incubated at 37 °C for 24 h. The medium was then replaced with fresh medium containing ~1 μCi of each tracer in a total volume of 3 mL per well. After incubating the cells with the radioactivity for 2 h at 37 °C, the medium was removed and 3 mL/well of either fresh medium or 1.5 μM desipramine in medium was added. The cell-bound radioactivity was determined at 0, 2, 4, 8, 24, 48, 72, and 96 h after the initial uptake. The medium was aspirated at the end of each period and the cells were washed twice with 0.5 mL each of PBS. The cells were solubilized by incubating with 500 μL of 0.5 N NaOH for 30 min and then removed with cotton swabs. The cell-bound radioactivity was counted using a dual-channel gamma counter. The assay was performed in quadruplicate for each time point.

4.7. Biodistribution in normal mice

The study utilized male BALB/c mice weighing about 25 g with groups of five mice for each time point. About 5 μCi each of [^{125}I]MIBG and [^{131}I]GIBG in 100 μL of PBS was injected via the tail vein to three groups of five animals and the animals were killed at 1, 4, and 24 h post-injection. To determine the specificity of uptake, another group of 5 animals was pretreated with DMI (10 mg/kg; ip) 30 min before injecting the tracers and the tissue distribution was performed 1 h later. The mice were killed by an overdose of halothane and tissues of interest were isolated, washed, blot-dried, and weighed. The tissues, along with 5% dose standards, were counted for ^{131}I and ^{125}I using a dual-label program in an automated gamma counter. The tissue radioactivity levels were expressed as percent injected dose per gram of tissue (%ID/g) unless otherwise specified. The statistical significance in the difference between the uptake of ^{125}I and ^{131}I in each tissue was calculated by the Students paired *t*-test using the Excel computer program (Microsoft).

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