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Biological Evaluation of Ring- and Side-Chain-Substituted *m*-Iodobenzylguanidine Analogues

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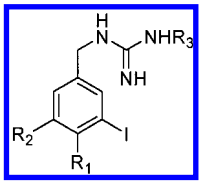
A number of ring- and side-chain-substituted *m*-iodobenzylguanidine analogues were evaluated for their lipophilicity, in vitro stability, uptake by SK-N-SH human neuroblastoma cells in vitro, and biodistribution in normal mice. As expected, the lipophilicity of *m*-iodobenzylguanidine increased when a halogen was introduced onto the ring and decreased with the addition of polar hydroxyl, amino, and nitro substitutents. Most of the derivatives showed reasonable stability up to 24 h in PBS at 37 °C. While N^1-hydroxy-N^3-3-[^{131}I]iodobenzylguanidine and 3,4-dihydroxy-5-[^{131}I]iodobenzylguanidine generated a more nonpolar product in addition to the free iodide, 3-[^{131}I]iodo-4-nitrobenzylguanidine decomposed to a product more polar than the parent compound. The specific uptake of 4-chloro-3-[^{131}I]iodobenzylguanidine, 3-[^{131}I]iodo-4-nitrobenzylguanidine, and N^1-hydroxy-N^3-3-[^{131}I]iodobenzylguanidine by SK-N-SH human neuroblastoma cells in vitro, relative to that of *m*-[^{125}I]-iodobenzylguanidine, was 117 ± 10%, 50 ± 4%, and 12 ± 2%, respectively. The specific uptake of the known *m*-iodobenzylguanidine analogues 4-hydroxy-3-[^{131}I]iodobenzylguanidine and 4-amino-3-[^{131}I]-iodobenzylguanidine was 80 ± 4% and 66 ± 4%, respectively. None of the other *m*-iodobenzylguanidine derivatives showed any significant specific uptake by SK-N-SH cells. Heart uptake of 4-chloro-3-[^{131}I]-iodobenzylguanidine in normal mice was higher than that of *m*-[^{125}I]iodobenzylguanidine at later time points (11 ± 1% ID/g versus 3 ± 1% ID/g at 24 h; $p < 0.05$) while uptake of 3-[^{131}I]iodo-4-nitrobenzylguanidine and of N^1-hydroxy-N^3-3-[^{131}I]iodobenzylguanidine in the heart was lower than that of *m*-iodobenzylguanidine at all time points. In accordance with the in vitro results, none of the other novel *m*-iodobenzylguanidine derivatives showed any significant myocardial or adrenal uptake in vivo.

INTRODUCTION

Since its development about 2 decades ago, radioiodinated MIBG[1] (Table 1) has been used extensively in a number of oncologic and cardiologic applications; however, its properties are far from ideal for these applications (Wafelman et al., 1994a). As described by these authors, there are a number of compounding factors responsible for the sub-optimal performance of MIBG. Our laboratory has been investigating radiochemical variables with the goal of improving the clinical efficacy of MIBG. For example, we developed a no-carrier-added synthesis of radioiodinated MIBG (Vaidyanthan and Zalutksy, 1993, 1995), and a few centers around the world are evaluating the clinical utility of nca [^{123}I]MIBG (Farahati et al., 1997; Samnick et al., 1999; Owens et al., 2000). We also have evaluated an analogue of MIBG labeled with the alpha particle emitter ^{211}At which might be useful for the treatment of metastatic diseases (Vaidyanathan and Zalutsky, 1992). Currently we are looking at the possibility of developing an MIBG analogue that will yield higher tumor retention and tumor-to-normal tissue ratios.

Because polar compounds are generally eliminated rapidly via the urine, MIBG derivatives containing polar substituents may yield higher tumor-to-normal tissue ratios, provided that such analogues are taken up efficiently by the tumor. Although many compounds with diverse structural features are substrates for the norepinephrine transporter, subtle changes in the structure

Table 1. Structures of MIBG and Analogues



abbreviation	R_1	R_2	R_3
MIBG	H	H	H
CIBG	Cl	H	H
FIBG	F	H	H
*p*AIBG	NH_2	H	H
HIBG	OH	H	H
*m*INBG	H	NO_2	H
*p*INBG	NO_2	H	H
NHIBG	H	H	OH
HIMBG	OH	OCH_3	H
DHIBG	OH	OH	H
*m*AIBG	H	NH_2	H

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[1] Abbreviations: ABG, 4-aminobenzylguanidine; CIBG, 4-chloro-3-iodobenzylguanidine; DHIBG, 3,4-dihydroxy-5-iodobenzylguanidine; DMI, desipramine; FIBG, 4-fluoro-3-iodobenzylguanidine; HIBG, 4-hydroxy-3-iodobenzylguanidine; HIMBG, 4-hydroxy-3-iodo-5-methoxybenzylguanidine; *m*AIBG, 5-amino-3-iodobenzylguanidine; MIBG, *m*-iodobenzylguanidine; *m*INBG, 5-iodo-3-nitrobenzylguanidine; nca, no-carrier-added; NET, norepinephrine transporter; NHIBG, N^1-hydroxy-N^3-3-iodobenzylguanidine; *p*AIBG, 4-amino-3-iodobenzylguanidine; *p*INBG, 3-iodo-4-nitrobenzylguanidine.

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of aralkylguanidines can result in significant alterations in their accumulation in adrenergic-rich tissues (Wieland, 1986; Wieland et al., 1984). Because polar substituents such as OH and NH_2 were not included in earlier structure−activity studies of aralkylguanidines (Short and Darby, 1967; Fielden et al., 1965), it was speculated that such substituents may be counterproductive (Lee et al., 1986). Indeed, our attempt to make MIBG more polar by replacing its benzene ring with a pyridine has yielded a compound that was not taken up by human neuroblastoma cells in vitro (Vaidyanathan et al., 1998). In contrast, while substituting a hydroxyl group at the 4-position of MIBG (HIBG) did not affect its adrenomedullary uptake in dogs significantly, the 4-amino derivative (*p*AIBG) had a higher uptake (Lee et al., 1986) than MIBG. Also, in patients, AIBG sequestered in pheochromocytoma tumor to a degree similar to that of MIBG (Shulkin et al., 1986).

HIBG and *p*AIBG have not been evaluated in tumor cells or xenografts containing upregulated norepinephrine transporter. It should be pointed out that there are differences in the norepinephrine uptake and storage systems not only between tumors and sympathetically innervated tissues, but also within different nontumor target tissues such as heart and salivary gland. While MIBG is stored in vesicles in tissues such as adrenals and heart, and in pheochromocytoma, SK-N-SH human neuroblastoma cells lack storage vesicles; the storage in this case seems to be cytoplasmic (Clerc et al., 1993; Sisson and Sulkin, 1999). The uptake of [^{131}I]MIBG in heart is diminished by endogenous norepinephrine; in contrast, its uptake in parotid glands was little affected (Sisson and Wieland, 1986). Thus, the aim of the present study was to evaluate a number of newly developed MIBG analogues, as well as HIBG and *p*AIBG, in the SK-N-SH neuroblastoma cell line.

As described in the preceding paper (Vaidyanathan et al., 2001), the synthesis of several new analogues of MIBG has been developed. In the current study, these compounds have been evaluated with respect to their stability, lipophilicity, and uptake in SK-N-SH human neuroblastoma cells in vitro and in normal mouse tissues in vivo. Our results show that a number of these novel compounds completely lost their affinity for NET. Unfortunately, the others which retained the affinity do not seem to offer better in vivo properties than MIBG itself.

MATERIALS AND METHODS

General. All chemicals and reagents are obtained from Aldrich or Sigma unless otherwise noted. The details of the preparation of all new tracers used in this study are described in the preceding publication (Vaidyanathan et al., 2001). Except for *p*[^{131}I]INBG, which was prepared by an exchange radioiodination, all radioiodinated MIBG analogues used in this study were nca preparations. Previously reported protocols were used for the syntheses of 4-hydroxybenzylguanidine hemisulfate and 4-hydroxy-3-iodobenzylguanidine hemisulfate (HIBG) (Lee et al., 1986). No-carrier-added (nca) [^{125}I]MIBG and radioiodinated FIBG were prepared as reported before (Vaidyanathan and Zalutsky, 1993; Vaidyanathan et al., 1997a). Details concerning the various analytical methods such as NMR, HPLC, etc., used to chemically characterize the compounds reported in this paper, are described in the preceding article (Vaidyanathan et al., 2001).

4-Aminobenzylguanidine Nitrate (ABG). A mixture of 4-aminobenzylamine (244 mg, 2 mmol) and 3,5-dimethylpyrazole-1-carboxamidine nitrate (403 mg, 2 mmol) in ethanol (10 mL) was refluxed for 3 h. The solvent was evaporated, and the residual solid was washed with ethyl acetate. Recrystallization of the solid from ethanol/ether afforded 318 mg (70%) of a crystalline solid: mp 139−141 °C. ^{1}H NMR (CD_3OD) 4.50 (s, 2H), 7.4−7.58 (m, 3H). MS (FAB^+) *m*/*z*: 165 (MH^+).

4-Amino-3-iodobenzylguanidine (*p*AIBG). An HPLC standard of the title compound was prepared as follows: A solution of Iodogen (150 μg) in chloroform (30 μL) was taken in a half-dram vial, and the solvent was evaporated. To this vial was added a solution of ABG (0.5 mg) in acetate buffer, pH 4.5 (1 mL), followed by a solution of sodium iodide (0.45 mg) in the same buffer (420 μL). The mixture was incubated at room temperature for 30 min, and the product was isolated by semipreparative HPLC (t_R = 7.5 min). For HPLC, a C18 column (μ Bondapak) was eluted with 99.5:0.5 mixture of water/acetonitrile containing 0.1% (w/v) TFA. MS (ESI) *m*/*z*: 291 (MH^+).

[^{131}I]HIBG and *p*[^{131}I]AIBG. The title compounds were prepared following protocols reported in the literature (Lee et al., 1986) and isolated by reversed-phase HPLC. For HPLC, a C18 column (μ Bondapak) was eluted with 99.7:0.3 water/acetonitrile containing 0.1% (w/v) TFA at a flow rate of 1 mL/min. The retention times of *p*[^{131}I]AIBG and [^{131}I]HIBG under these HPLC conditions were 9 and 15 min, respectively. Concentration of the tracers was performed by solid-phase extraction using bigger (tC18 ENV; Waters) cartridges by a method similar to that described in the preceding paper (Vaidyanathan et al., 2001). After eluting the initial solvent from the HPLC fractions, the cartridge was washed with 5 mL of water and 0.5 mL of 5 mM NaOAc, pH 4.5, in that order before eluting the tracer with methanol or ethanol.

Octanol−Saline Partition Coefficient. The lipophilicity of the MIBG analogues was compared to that of MIBG by determining their octanol−PBS (pH 7.4) partition coefficients. The assay was performed in a paired-label format, in most cases directly comparing the analogue with MIBG. Both octanol and PBS were presaturated with each other. About 500 000 counts of each tracer in 10 μL of PBS were added in triplicate to glass tubes containing a mixture of 2 mL each of octanol and PBS. The contents of the tubes were thoroughly mixed by vortexing, and the layers were separated by centrifugation using a microcentrifuge. Duplicate samples (50 μL) of each layer from each tube were withdrawn and counted for radioactivity. The aqueous layer was discarded, and fresh PBS (1.9 mL) was added and the process repeated. The assay was repeated a third time with 1.8 mL of fresh PBS. The partition coefficient was calculated as the ratio of counts in octanol to that in PBS. The mean and standard deviation from 18 measurements for each tracer were calculated.

In Vitro Stability. To determine the influence of various substituents on the degree of dehalogenation, the stability of some of the radioiodinated derivatives was determined. About 100−200 μCi of activity was taken in PBS (100 μL) and incubated at 37 °C. Aliquots were taken periodically over a period of about 24 h, and the percent of total activity that was associated with the intact tracer was determined by reversed-phase HPLC. The HPLC system used for each tracer was the same as reported earlier for the purification and isolation of the compound (Vaidyanathan et al., 2001).

Cells and Culture Conditions. The human neuroblastoma cell line SK-N-SH (Uptake-1 positive; Biedler et al., 1973) 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 units 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 (Kaltenbach et al., 1958), and was 95−98% for all studies.

Paired-Label in Vitro Uptake of MIBG Analogues by SK-N-SH Cells in Comparison to That of MIBG. 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, and 100 nCi each of radioiodinated MIBG and one of the MIBG analogues[2] in 3 mL of medium was added to each well and incubated for 2 h at 37 °C. The specificity and energy dependence of tracer uptake were determined using DMI and ouabain, respectively. The initial medium was removed, and the cells were incubated with 3 mL each of 1.5 μM DMI and 1 mM ouabain, respectively, 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 was ascertained by determining the effect of temperature as well. For this, the original medium was removed, and fresh medium that was cooled to 4 °C was added followed by tracers (100 nCi each) in 10 μL of the medium, 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 with the tracers (100 nCi in 10 μL each) and 3 mL each of 50 μM norepinephrine and 10 μM MIBG, respectively, at 37 °C for 2 h. 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 activity was counted along with input standards using a dual-channel gamma counter. Each set of experiments was performed 3−6 times. The entire experiment was conducted twice in some cases.

Paired-Label Exocytosis of [^{131}I]FIBG and [^{125}I]-CIBG from SK-N-SH Cells. In a previous study, we demonstrated that washout of FIBG from SK-N-SH cells in vitro was considerably slower than that of MIBG (Vaidyanthan et al., 1997a). To investigate whether a chlorine substituent yielded a similar effect, a paired-label study was performed comparing the washout of [^{131}I]FIBG and [^{125}I]CIBG from SK-N-SH cells. Cells were added to 6-well plates at a density of $\sim 4 \times 10^5$ cells per well per 3 mL of medium and incubated at 37 °C for 24 h. After the 24 h period, the medium was replaced with fresh medium containing $\sim$1 μCi of each tracer in a total volume of 3 mL per well. After incubating the cells with the activity for 2 h at 37 °C, the medium was removed and supplemented with 3 mL of either fresh medium or 1.5 μM desipramine in medium. The cell-bound activity 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 removed with cotton swabs. The cell-bound activity was counted using a dual-channel gamma counter. For each time point, the assay was performed in quadruplicate.

[2] If MIBG was labeled with ^{131}I, the analogue was labeled with ^{125}I, and vice versa.

Table 2. Octanol−Saline Partition Coefficients of MIBG and Analogues

tracer	partition coefficient (mean ± SD)
MIBG	2.61 ± 0.07
4-chloro-3-iodobenzylguanidine	8.36 ± 1.43
4-fluoro-3-iodobenzylguanidine	3.01 ± 0.20
4-amino-3-iodobenzylguanidine	0.32 ± 0.02[a]
4-hydroxy-3-iodobenzylguanidine	0.60 ± 0.03[a]
3-iodo-5-nitrobenzylguanidine	2.08 ± 0.14
3-iodo-4-nitrobenzylguanidine	1.13 ± 0.03
N-hydroxy-3-iodobenzylguanidine	3.82 ± 0.17
4-hydroxy-3-iodo-5-methoxybenzyl-guanidine	0.60 ± 0.04
3,5-dihydroxy-5-iodobenzylguanidine	0.38 ± 0.02[a]
3-amino-5-iodobenzylguanidine	0.68 ± 0.03[a]

[a] In these cases, the partition coefficient values of these compounds increased substantially with each equilibration step, while that of MIBG from the paired-label format remained fairly constant; values shown are from the first equilibration.

Biodistribution Studies in Normal Mice. These studies were performed for all tracers except [^{131}I]HIBG and *p*[^{131}I]AIBG in a paired-label format comparing the new tracer directly to MIBG. The study utilized male BALB/c mice weighing about 25 g with groups of five mice for each time point. Tracers (1−5 μCi) in 100 μL of PBS were injected via the tail vein, and groups of animals were killed at 1, 4, and 24 h postinjection. The specificity of uptake was determined by pretreating a group of mice with DMI 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 uptakes 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 paired Student's *t*-test using the Excel computer program (Microsoft).

RESULTS

Syntheses of ABG and *p*AIBG. ABG was prepared from readily available 4-aminobenzylamine in more than 70% yield. An HPLC sample of *p*AIBG was obtained by the microscale iodination of ABG. Both were characterized by standard spectral techniques.

Octanol−Saline Partition Coefficient. The relative lipophilicities of the MIBG analogues were obtained by measuring their octanol−saline partition coefficients using their radioiodinated analogues (Table 2). As expected, halogen susbtitution increased lipophilicity. While the effect was only minimal with fluorine (3.01 ± 0.20 for FIBG vs 2.61 ± 0.07 for MIBG), substituting chlorine at the 4-position resulted in an almost 4-fold increase in the partition coefficient. The only other derivatve with a higher partition coefficient than that of [^{125}I]MIBG was [^{131}I]NHIBG. This corroborates the earlier claim that conversion of a guanidine to *N*-hydroxy-substituted guanidine results in increased lipophilicity (Balley and DeGrazia, 1973). While a nitro group at the para position decreased the lipophilicity by more than a factor of 2, meta substitution of the nitro group did not yield a similar effect. Partition coefficients for *p*[^{131}I]AIBG, *m*[^{131}I]-AIBG, [^{131}I]HIBG, and [^{131}I]DHIBG increased to a considerable extent with each equilibration step, while values for [^{125}I]MIBG from paired-label experiments

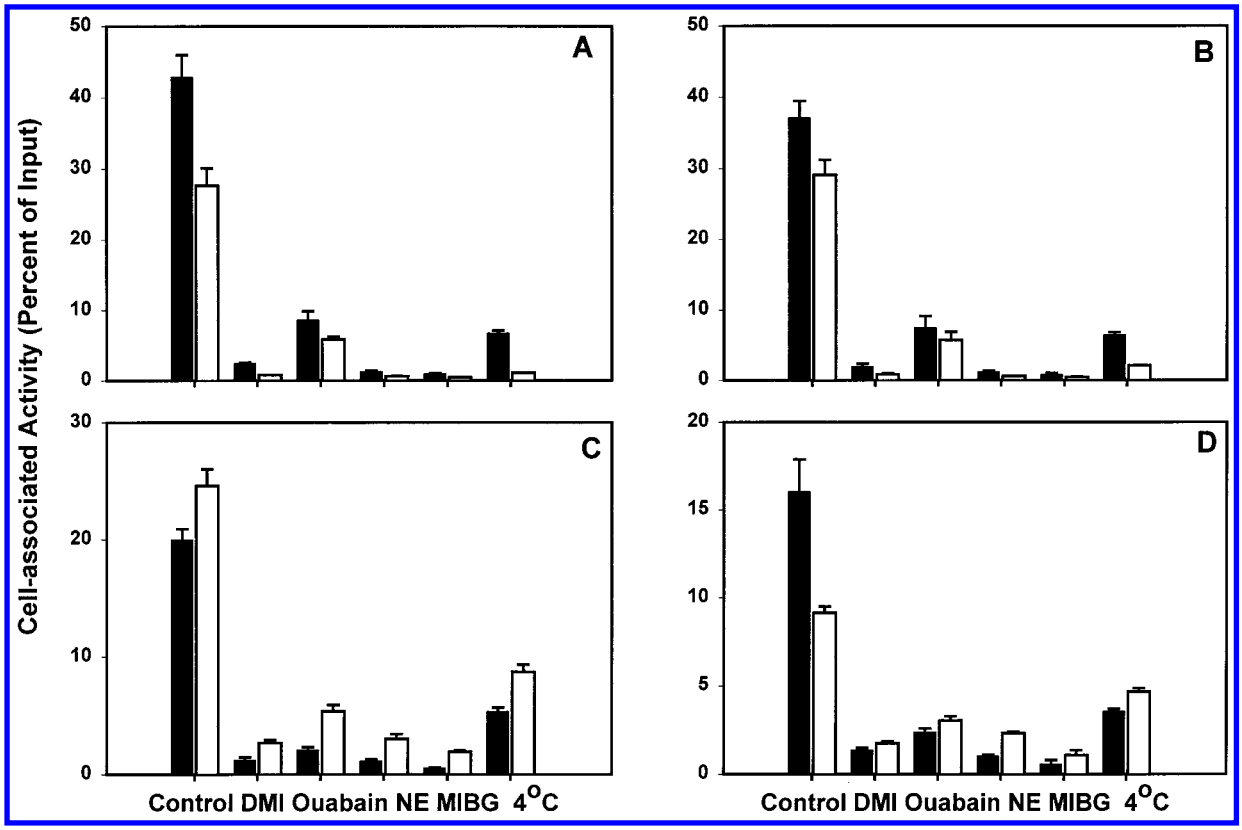

Figure 1. Uptake of [^{131}I]HIBG (A), *p*[^{131}I]AIBG (B), [^{131}I]CIBG (C), and ex-*p*[^{131}I]INBG (D) by SK-N-SH human neuroblastoma cells in vitro (open bars). Results obtained for [^{125}I]MIBG from respective paired-label assays are also shown (filled bars). The effect of various Uptake-1-blocking 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 activity was determined as a percent of input radioactivity.

remained fairly constant. For these compounds, values from the first equilibration are shown in Table 2. *p*[^{131}I]-AIBG was the most hydrophilic (0.32 ± 0.02) of all the compounds studied. Switching the amino group from the 4- to 5-position increased lipophilicity. While [^{131}I]HIBG had a partition coefficient of 0.60 ± 0.03, introduction of another hydroxyl group reduced the partition coefficient to 0.38 ± 0.02, and capping one of the hydroxyl groups in [^{131}I]DHIBG increased the lipophilicity back to 0.60 ± 0.04.

In Vitro Stability. In vitro stabilities of radioiodinated MIBG derivatives were determined by HPLC after incubating them in PBS at 37 °C for various periods of time. [^{131}I]FIBG, [^{131}I]CIBG, *p*[^{131}I]INBG, *m*[^{131}I]AIBG, and [^{131}I]HIMBG were stable up to 24 h with less than 5% of free iodide formed. While there was about 5−10% of free iodide in the *p*[^{131}I]AIBG and [^{131}I]HIBG preparations even at the beginning of incubation, no further deiodination was seen up to 4 h at 37 °C. [^{131}I]DHIBG deteriorated steadily with time with only about 30−35% of the total activity associated with the intact molecule at 4 h. While another third of the activity was free iodide, the remainder was an unidentified nonpolar compound. At 24 h, most of the activity corresponded to free iodide. Although little deiodination was seen in the case of *m*[^{131}I]INBG, a radioactive byproduct more polar than the parent compound accrued with time. For up to 4 h, the [^{131}I]NHIBG remained intact except for the formation of a small amount of a slightly more nonpolar byproduct. Three radioactive peaks were observed at 24 h with the majority of the activity associated with the above byproduct. A commensurate amount of the activity was associated with the intact molecule, and only 5−10% of the total activity corresponded to free iodide.

Table 3. Uptake of Various MIBG Analogues by SK-N-SH Cells in Vitro

MIBG analogue	% specific uptake[a]
MIBG	100.00 ± 0.00
4-hydroxy-3-iodobenzylguanidine	66.42 ± 3.92
4-amino-3-iodobenzylguanidine	80.48 ± 4.05
4-chloro-3-iodobenzylguanidine	116.73 ± 4.80
3-iodo-4-nitrobenzylguanidine	50.40 ± 3.77
3-iodo-5-nitrobenzylguanidine	1.24 ± 0.12
3,5-dihydroxy-5-iodobenzylguanidine	0.08 ± 0.03
4-hydroxy-3-iodo-5-methoxybenzylguanidine	0.69 ± 0.24
3-amino-5-iodobenzylguanidine	0.27 ± 0.10
N-hydroxy-3-iodobenzylguanidine	12.34 ± 1.14

[a] Specific uptake was obtained by subtracting uptake in the presence of DMI from that of control values. Each value is then normalized to the same value for MIBG obtained from paired-label assays; mean ± SD (n = 3−6).

Uptake of MIBG Analogues by SK-N-SH Cells in Vitro. The degree of uptake, and the influence of various interventional agents and inhibitory conditions, for four analogues is shown in Figure 1. The specific uptake, defined as the difference between control values and those obtained in the presence of DMI, for each new analogue as well as for *p*[^{131}I]AIBG and [^{131}I]HIBG is presented in Table 3. The 4-chloro derivative had a specific uptake of 117% that of [^{125}I]MIBG. The specific uptakes of *p*[^{131}I]AIBG and [^{131}I]HIBG were 80% and 60% that of [^{125}I]MIBG, suggesting that these derivatives retain the biological characteristics of MIBG not only with respect to the canine adrenomedullary uptake in vivo reported previously (Lee et al., 1986), but also with respect to the uptake in SK-N-SH human neuroblastoma cells in vitro. The switching of the amino group to the 5-position resulted in the complete loss of specificity (specific uptake of *m*[^{131}I]AIBG was 0.27% that of [^{125}I]-

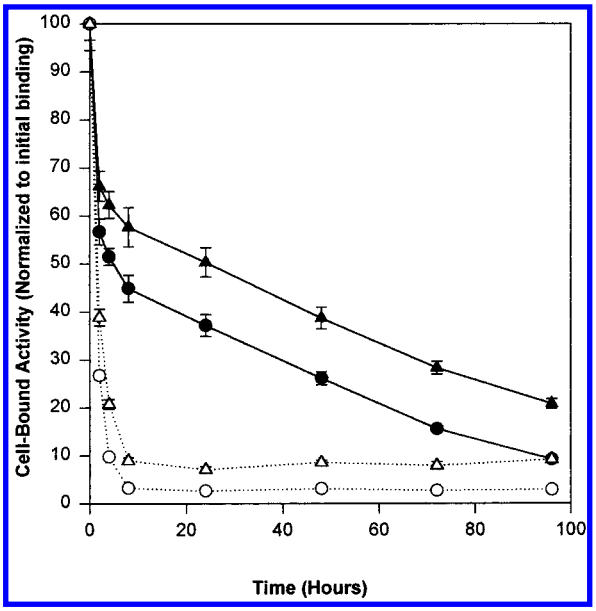

Figure 2. Paired-label exocytosis of [^{125}I]CIBG (▲, without DMI; △, with DMI) and [^{131}I]FIBG (●, without DMI; ○, with DMI) as a function of time. Cells were allowed to take up the tracers at 37 °C for 2 h. The medium containing the tracers was replaced with fresh medium without or with DMI, and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

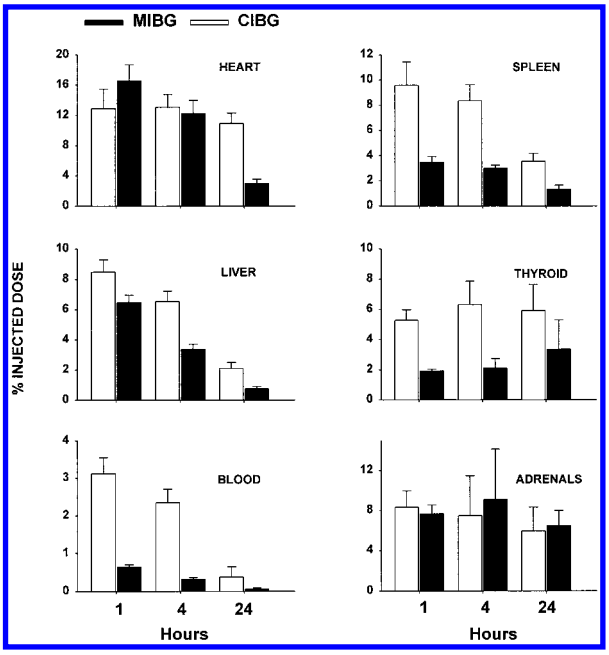

Figure 3. Paired-label uptake (%ID/g) of radioactivity in heart, spleen, liver, thyroid, blood, and adrenals at 1, 4, and 24 h after intravenous administration of [^{131}I]CIBG (□) and [^{125}I]MIBG (■).

MIBG). The 4-nitro derivative had a specific uptake about half that of [^{125}I]MIBG. As with the amino derivatives, switching the nitro group to the 5-position yielded a compound with a specific uptake of about 1% that of MIBG. Both dihydroxy- and hydroxy-methoxy-substituted derivatives did not have any significant uptake. While the *N*-hydroxy derivative showed some uptake, it was not substantial. The uptake of certain derivatives such as [^{131}I]CIBG and *p*[^{131}I]INBG was not reduced to the same level as [^{125}I]MIBG by some of the Uptake-1-blocking conditions, especially incubation at 4 °C.

Washout of CIBG from SK-N-SH Cells in Comparison to That of FIBG. Figure 2 shows the results obtained from a paired-label study of retention of [^{125}I]-CIBG and [^{131}I]FIBG by SK-N-SH cells. The amount of initially bound [^{125}I]CIBG activity retained was consistently higher than that of [^{131}I]FIBG at all time points with the difference increasing with time. Even at 96 h, the specifically retained [^{125}I]CIBG activity was about 2-fold higher than that for [^{131}I]FIBG.

Biodistribution. Detailed biodistribution data are presented for those novel MIBG derivatives that demonstrated a reasonable uptake in SK-N-SH human neuroblastoma cells in vitro. Figure 3 shows the %ID/g in selected normal mouse tissues obtained from a paired-label biodistribution of [^{131}I]CIBG and [^{125}I]MIBG (see Supporting Information for uptake in other tissues). The heart uptake of [^{131}I]CIBG (12.9 ± 2.6% ID/g) was less than that for [^{125}I]MIBG (16.6 ± 2.1% ID/g) at 1 h ($p < 0.05$); however, the ^{131}I/^{125}I ratio in the heart increased with time, reaching >3 at 24 h. The uptake of both [^{131}I]-CIBG and [^{125}I]MIBG was similar in the adrenals, the other Uptake-1 target tissue, and the difference in uptake was statistically significant only at 4 h (9.09 ± 5.03% ID/g vs 7.49 ± 3.98% ID/g; $p < 0.05$). The uptake of [^{131}I]CIBG in a number of other tissues was higher than that of [^{125}I]-MIBG, a finding similar to that observed for [^{131}I]FIBG (Vaidyanathan et al., 1997a). Compared to FIBG, one notable difference of CIBG was in its thyroid uptake. [^{131}I]-CIBG activity in thyroid was 2−3-fold higher ($p < 0.05$) than that seen for [^{125}I]MIBG, suggesting that the former was more susceptible to in vivo deiodination; in contrast, [^{131}I]FIBG was less susceptible to deiodination than [^{125}I]-MIBG. Also, unlike FIBG, the blood activity from [^{131}I]-CIBG was considerably higher than that of [^{125}I]MIBG. At 1 and 4 h, blood levels were 3.12 ± 0.43% ID/g and 2.35% ± 0.36% ID/g, respectively, for [^{131}I]CIBG compared to 0.64 ± 0.06% ID/g and 0.32 ± 0.05% ID/g, respectively, for [^{125}I]MIBG.

The heart uptake of *p*[^{131}I]INBG was consistently lower than that of [^{125}I]MIBG (14.6 ± 2.6, 11.7 ± 2.3, and 3.4 ± 0.6% ID/g versus 20.5 ± 2.4, 15.9 ± 2.6, and 3.8 ± 0.8% ID/g at 1, 4, and 24 h, respectively; $p < 0.05$ for 1 and 4 h), and a similar trend was seen in adrenals as well (Figure 4). The uptake of *p*[^{131}I]INBG in a number of other normal tissues was significantly higher than that for [^{125}I]MIBG. For example, the uptake of *p*[^{131}I]INBG in spleen, lungs, and blood was 3−40-fold higher over the 24 h period. In addition, the ^{131}I/^{125}I ratio in the thyroid reached a value of more than 6 at 24 h, suggesting that *p*[^{131}I]INBG is very susceptible to in vivo deiodination. The biodistribution of *m*[^{131}I]INBG was quite different from that of the para-isomer. Heart uptake of *m*[^{131}I]-INBG was only about one-tenth or less that of [^{125}I]MIBG at all time points. For example, at 1 h, the heart uptake of *m*[^{131}I]INBG was 1.89 ± 0.29% ID/g compared to 15.44 ± 1.84% ID/g for [^{125}I]MIBG ($p < 0.05$). Although the value of adrenal uptake for *m*[^{131}I]INBG at 1 h was roughly half that for [^{125}I]MIBG, no significant activity was left in the adrenals at 24 h (0.24 ± 0.02% ID/g vs 6.86 ± 0.5% ID/g for [^{125}I]MIBG). The uptake in a number of other tissues such as liver, spleen, and lung was significantly less than that of [^{125}I]MIBG. The thyroid uptake of *m*[^{131}I]INBG was 2−5-fold lower than that of [^{125}I]MIBG, suggesting that, unlike the para-isomer, this derivative was less likely to undergo in vivo deiodination.

Except in the heart, adrenals, thyroid, and the brain, the uptake of [^{131}I]NHIBG was similar to [^{125}I]MIBG in most tissues at all time points (Figure 5). The heart uptake of [^{131}I]NHIBG (12.2 ± 1.0 and 8.0 ± 1.1% ID/g) was about half and two-thirds ($p < 0.05$) that of [^{125}I]-MIBG (22.2 ± 2.6 and 12.0 ± 2.1% ID/g) at 1 and 4 h,

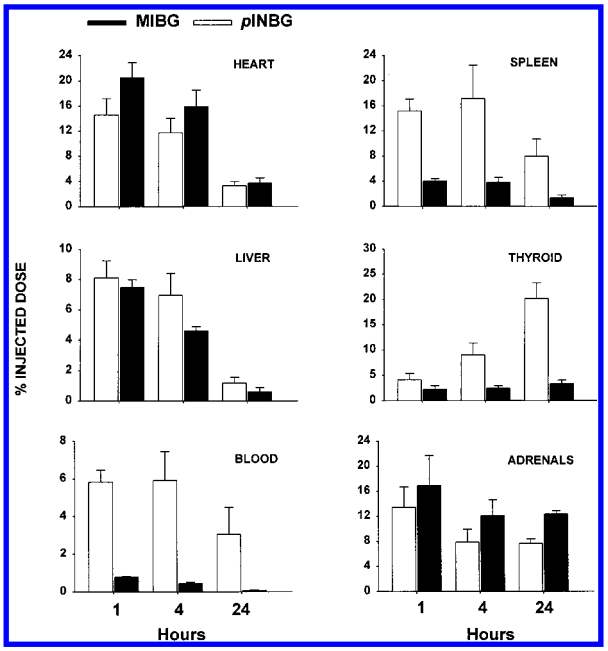

Figure 4. Paired-label uptake (%ID/g) of radioactivity in heart, spleen, liver, thyroid, blood, and adrenals at 1, 4, and 24 h after intravenous administration of *p*[^{131}I]INIBG (□) and [^{125}I]MIBG (■).

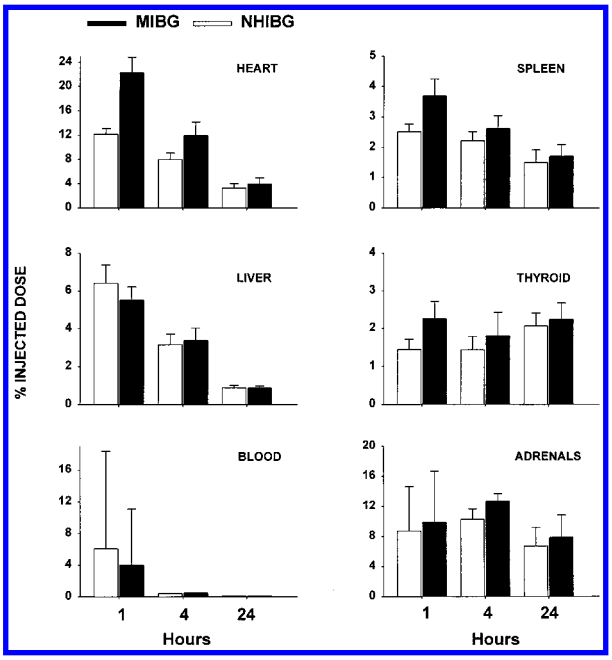

Figure 5. Paired-label uptake (%ID/g) of radioactivity in heart, spleen, liver, thyroid, blood, and adrenals at 1, 4, and 24 h after intravenous administration of [^{131}I]NHIBG (□) and [^{125}I]MIBG (■).

respectively, and at 24 h, the heart uptake of both tracers was similar. The adrenal uptake of [^{131}I]NHIBG was lower ($p < 0.05$ except at 1 h) than that of [^{125}I]MIBG, but the differences were not very substantial. Although not to a great degree, the thyroid uptake of [^{131}I]NHIBG was less than that of [^{125}I]MIBG at all time points ($p < 0.05$ only at 1 h). At 1 and 4 h, the brain uptake of [^{131}I]-NHIBG was about 4-fold higher than that of [^{125}I]MIBG, possibly due to its higher lipophilicity.

Neither heart nor adrenals had any significant uptake of [^{131}I]DHIBG at any time points. The heart uptake of [^{131}I]DHIBG at 1, 4, and 24 h was 1.12 ± 0.07% ID/g, 0.35 ± 0.06% ID/g, and 0.06 ± 0.01% ID/g, respectively, compared to 25.78 ± 5.08% ID/g, 14.76 ± 4.24% ID/g, and 4.24 ± 0.35% ID/g for [^{125}I]MIBG. Similarly, the adrenal uptake was also less than 10% that of [^{125}I]MIBG at all time points. A number of other tissues also sequestered [^{131}I]DHIBG to a considerably low degree. Thyroid uptake of [^{131}I]DHIBG was almost 3-fold higher than that of [^{125}I]-MIBG at 24 h, suggesting the in vivo instability of the former. While [^{131}I]HIMBG did not have any significant uptake in heart and adrenals, most of the [^{131}I]HIMBG-associated activity was eliminated via the urine within the first hour. The uptake of *m*[^{131}I]AIBG in major organs including the target organs, heart and adrenals, was considerably lower than that of [^{125}I]MIBG. The stomach and intestines exhibited a higher uptake of *m*[^{131}I]AIBG at the early time points. Thyroid uptake of *m*[^{131}I]AIBG was 2−3-fold lower than that of [^{125}I]MIBG, suggesting its inertness to deiodination in vivo.

The specificity of the uptake of the various MIBG analogues in heart and adrenals was determined by measuring the effect of DMI on the uptake in these tissues at 1 h postinjection. As shown in Table 4, DMI reduced the heart uptake of [^{131}I]CIBG to 58% of the control values; in comparison, [^{125}]MIBG uptake was reduced to 48% of its control values, and these reductions are statistically significant ($p < 0.05$). In adrenals, the uptake of [^{131}I]CIBG and co-injected [^{125}I]MIBG actually increased in animals pretreated with DMI. While the difference in [^{131}I]CIBG uptake was statistically significant ($p = 0.03$), that of [^{125}I]MIBG was not. The heart accumulation of *p*[^{131}I]INBG was reduced to 32% of the control values, and that of co-injected [^{125}I]MIBG was reduced to 45%. Adrenal uptake of *p*[^{131}I]INBG was also reduced by DMI pretreatment, but the difference was not statistically significant. While DMI did not have any effect on the [^{131}I]DHIBG uptake in heart, the uptake actually increased in the case of *m*[^{131}I]INBG. Considering the very low myocardial uptake of *m*[^{131}I]INBG, this increase may not have any significance. While the adrenal uptake of both *m*[^{131}I]INBG and [^{131}I]DHIBG was reduced in the DMI-treated animals, the differences were not statistically significant. The heart uptake of [^{131}I]-NHIBG was specific as shown by the fact that its uptake was reduced to about 48% of the control values when the mice were pretreated with DMI. As seen with [^{131}I]CIBG, DMI-treatment increased the adrenal uptake of [^{131}I]-NHIBG; however, the increase was not statistically significant. DMI did not have any effect on the heart uptake of [^{131}I]HIMBG. Although the absolute heart uptake of *m*[^{131}I]AIBG was considerably lower than that of [^{125}I]MIBG, it seems to be mediated by the Uptake-1 pathway as it was reduced to roughly half its value by DMI, and the difference was statistically significant.

DISCUSSION

The purpose of this study was to determine the effects of ring- and side-chain substitution in MIBG on the biological properties of the compound. A number of MIBG derivatives were prepared, and most of them, with the exception of CIBG and NHIBG, had polar substituents on the ring (Vaidyanathan et al., 2001). CIBG was developed to investigate whether the encouraging results obtained with FIBG could be extended by substituting fluorine with chlorine. The canine adrenomedullary concentration of FIBG was similar to that of MIBG at 0.5 h but increased with time up to 24 h; however, by 72 h, FIBG uptake was less than that of MIBG (Wieland, 1986). On the other hand, uptake of FIBG in SK-N-SH neuroblastoma cells in vitro and mouse heart in vivo at 72 h and beyond was significantly higher than that of

Table 4. Heart and Adrenal Uptake of Various MIBG Analogues in Control Mice and in Those Treated with DMI

	%ID/g[a]					
	heart			adrenals		
tracer	control	DMI	*p*	control	DMI	*p*
CIBG	21.1 ± 6.3	12.3 ± 2.8	0.02	8.5 ± 3.9	13.8 ± 2.4	0.03
	(24.7 ± 6.4)	(12.0 ± 2.3)	(0.00)	(8.1 ± 3.2)	(9.8 ± 0.8)	(0.29)
p-INBG	14.1 ± 1.6	4.5 ± 0.5	0.00	9.3 ± 2.0	7.24 ± 2.0	0.10
	(22.7 ± 2.0)	(10.3 ± 2.0)	(0.00)	(10.7 ± 2.2)	(6.9 ± 2.0)	(0.02)
m-INBG	1.9 ± 0.3	2.4 ± 0.3	0.04	6.8 ± 2.1	6.6 ± 1.2	0.87
	(15.4 ± 1.8)	(9.1 ± 1.1)	(0.00)	(11.3 ± 3.8)	(7.5 ± 1.0)	(0.17)
DHIBG	1.1 ± 0.1	1.1 ± 0.2	0.99	1.9 ± 1.3	1.7 ± 0.5	0.76
	(25.8 ± 5.1)	(11.6 ± 2.2)	(0.00)	(11.6 ± 4.0)	(9.8 ± 2.6)	(0.42)
HIMBG	1.0 ± 0.1	1.1 ± 0.2	0.36	1.7 ± 0.5	1.1 ± 0.2	0.05
	(19.5 ± 1.5)	(10.5 ± 1.3)	(0.00)	(15.4 ± 7.2)	(8.5 ± 0.8)	(0.07)
m-AIBG	2.8 ± 0.7	1.5 ± 0.3	0.00	6.0 ± 2.1	4.5 ± 1.1	0.19
	(20.3 ± 4.0)	(8.1 ± 1.7)	(0.00)	(14.1 ± 4.2)	(8.5 ± 1.1)	(0.02)
N-HIBG	12.1 ± 1.0	5.9 ± 0.5	0.00	8.7 ± 5.9	10.2 ±1.4	0.31
	(22.2 ± 2.6)	(8.2 ± 0.6)	(0.00)	(9.9 ± 6.8)	(11.3 ± 1.4)	(0.31)

[a] Mean ± SD ($n = 5$); values in parentheses are those for MIBG obtained from a paired-label study.

MIBG (Vaidyanathan et al., 1997a). Another of the analogues selected for study was NHIBG, an MIBG derivative with a hydroxyl group on the guanidine side chain. Although the hydroxyl group is polar, modifying the guanidine moiety with a hydroxyl substituent in fact reduces the polarity of guanidine derivatives (Baley and DeGrazio, 1973). Nevertheless, we were interested in studying the effect of an *N*-hydroxy substituent on the biological characteristics of MIBG.

The effect of various substituents on lipophilicity was generally as expected. While introduction of fluorine at the 4-position of MIBG did not alter the lipophilicity substantially, substituting a chlorine increased the lipophilicity of MIBG 4-fold. Introduction of polar substituents such as hydroxyl, nitro, and amino onto the ring indeed reduced the lipophilicity of MIBG. In the case of INBG and AIBG, switching the position of the amino or nitro group from the para to the ortho position resulted in an increased lipophilicity.

Based on studies of the in vitro stability of MIBG and its few analogues, it has been concluded that free iodide is the major decomposition product (Lee et al., 1986; Wafelman et al., 1994b). Very small amounts of *m*-iodobenzylamine and a compound more nonpolar than MIBG, among other impurities, have been detected in the case of [^{131}I]MIBG (Wafelman et al., 1994b). Most of the derivatives exhibited an in vitro stability comparable to that of MIBG. Although both *p*[^{131}I]AIBG and [^{131}I]-HIBG were purified by reversed-phase HPLC, analytical HPLC of a freshly prepared sample of these showed the presence of free iodide. As reported before for other radiotracers (Larsen et al., 1997), this may be due to radiolytic degradation during the Sep-pak concentration. The in vitro stability of the above benzylguanidine derivatives has been studied at 4 °C for extended periods of time (Lee et al., 1986). When *p*AIBG was labeled with ^{125}I, less than 1% of radioiodide was formed up to 5 days. On the other hand, 5% of free iodide was generated from *p*[^{131}I]AIBG at 30 h. [^{125}I]HIBG was less stable than *p*[^{125}I]AIBG and gave rise to about 2% radioiodide at 42 h. In the current study, the stability was studied for only a short duration; however, a higher, more biologically relevant temperature was used. No extensive deiodination was seen under these conditions.

[^{131}I]DHIBG, a [^{131}I]HIBG analogue with an additional hydroxyl group, degraded completely during the 24 h period. A considerable amount of an unidentified, nonpolar product was the major component at 4 h. Both [^{131}I]-DHIBG and this nonpolar product deiodinated completely by 24 h. A peak more nonpolar than the parent compound was also seen by HPLC in the case of [^{131}I]NHIBG. While *p*[^{131}I]INBG remained fairly intact, a considerable amount of the meta isomer decomposed to a product more polar than the parent benzylguanidine.

The specificity of the various MIBG analogues for NET was determined by paired-label uptake assays using SK-N-SH human neuroblastoma cells (Vaidyanathan and Zalutsky, 1993; Vaidyanathan et al., 1994, 1995, 1997a). Because the uptake is dependent on the passage number of the cells (Lashford et al., 1991; Vaidyanathan et al., 1995), the uptake of the [^{125}I]MIBG control varied from assay to assay. Only nca [^{131}I]CIBG showed specific uptake comparable to that of [^{125}I]MIBG; in fact, its specific uptake (116.7 ± 9.6%) was higher than that of [^{125}I]MIBG. This is similar to the result observed for both ^{18}F- and ^{131}I-labeled FIBG (Vaidyanathan et al., 1994, 1997a), and may be due in part to the increased lipophilicity as a result of the introduction of a halogen substituent.

The specific uptake of *p*[^{131}I]INBG prepared by exchange radioiodination (specific activity = ∼5 mCi/mg) was 50.4 ± 3.6% that of [^{125}I]MIBG. The lower uptake could not be due to the presence of the carrier, because no saturation in uptake was seen at this activity concentration when [^{131}I]MIBG of similar specific activity was used (Vaidyanathan and Zalutsky, 1993). Switching the $-NO_2$ group to the 5-position resulted in complete destruction of NET specificity. This lack of specificity indicates that NET may have some steric requirements for its substrates. [^{131}I]NHIBG had a specific uptake 12.3 ± 2.0% that of [^{125}I]MIBG, corroborating the earlier observation that alterations in the guanidine function are detrimental to NET-mediated uptake.

It was disappointing to note that [^{131}I]DHIBG, the MIBG analogue structurally similar to norepinephrine, did not have any significant uptake in SK-N-SH cells. Although norepinephrine is also very polar, the lack of specificity of [^{131}I]DHIBG for NET may be a result of its poor trans-membrane transport due to its very high polarity. The MIBG analogue with one hydroxyl group at the 4-position (HIBG) has been shown to be an excellent analogue of MIBG with respect to its adrenomedullary uptake in dogs (Lee et al., 1986). It was anticipated that capping of the 3-hydroxyl group in DHIBG with a group such as methyl (HIMBG) might enhance its specific uptake; however, this was not the case. It is unclear whether this is an intrinsic property

of HIMBG or whether it was rapidly converted to DHIBG within the cells.

Like HIBG, the 4-amino-substituted MIBG (*p*AIBG) has also been shown to be an excellent MIBG analogue (Lee et al., 1986). While it is easy to introduce iodine at the ortho and para positions of hydroxyl and amino groups of phenols and anilines, respectively, the resultant iodophenols and iodoanilines are more susceptible to deiodination. We developed a radiosynthesis of 3-amino-5-iodobenzylguanidine anticipating it to be more stable, and thus a better analogue than *p*[^{131}I]AIBG. However, it did not show any specificity for NET in the SK-N-SH cells. Based on the lack of specificity of various MIBG derivatives in which both 3- and 5-positions were occupied, it may be fair to conclude that the presence of subtituents at both meta positions in MIBG may be counterproductive. It has been reported that while 3,4-diiodobenzylguanidine exhibited high adrenomedullary uptake despite its in vivo instability, relocating the 4-iodo subtituent to the 5-position resulted in substantial reduction in the adrenomedulllary uptake (Wieland et al., 1984; Wieland, 1986).

Based on the above results, it is tempting to speculate that polar substituents in the ring of MIBG adversely affect its NET specificity. While HIBG and *p*AIBG exhibit heart and adrenomedullary uptake in dogs and monkeys, no studies have been done to demonstrate their specificity of uptake in tumor cells such as SK-N-SH. As described earlier, there are variabilities in MIBG uptake from tissue to tissue. To determine the specificity of these two compounds for potential oncologic applications, we investigated their uptake in SK-N-SH neuroblastoma cells. The results (Figure 1 and Table 3) clearly demonstrate that, although not to the same level as MIBG, these two compounds are taken up by SK-N-SH cells to a considerable degree, suggesting that polar groups such as hydroxyl and amino at the 4-positon do not destroy the specificity of uptake in this cell line. The lower uptake of [^{131}I]HIBG compared to that of *p*[^{131}I]AIBG is somewhat similar to their behavior in dog adrenal medulla. It may be possible that, once within the cells, [^{131}I]HIBG is deiodinated to a higher degree than *p*[^{131}I]AIBG.

One of the motivations for investigating CIBG is the enhanced retention of FIBG seen in SK-N-SH cells in vitro (Vaidyanathan et al., 1997a). From a paired-label study, it was clear that SK-N-SH cells retained [^{125}I]CIBG specifically to an even higher degree than [^{131}I]FIBG. Thus, potentially higher tumor doses might be delivered by [^{131}I]CIBG compared to [^{131}I]FIBG. At least part of the enhanced retention may be as a result of the higher lipophilicity of CIBG. It may also be possible that the halogen substituent alters the rate and the pathway of metabolism of MIBG. For example, MIBG is metabolized to HIBG, albeit to a small degree, within pheochromocytoma in humans (Mangner et al., 1986). It is not clear how the presence of a 4-substituent would alter this pathway. It is known that microsomal cytochrome P-450 hydroxylates *o*- and *m*-fluoroanilines at the para position. On the other hand, it defluorinates as well as *o*-hydroxylates *p*-fluoroaniline (Rietjens and Vervoort, 1989). Studies are in progress to determine in vitro metabolism of MIBG and its fluoro and chloro analogues.

A number of the novel analogues were further evaluated by determining their biodistribution in normal mice. Thyroid uptake of [^{131}I]CIBG was 2−3-fold higher than that for [^{125}I]MIBG, suggesting that, contrary to the in vitro stability results, chlorine at the 4-position makes MIBG more prone to deiodination in vivo. While fluorine substitution at the 4-position increased the stability of [^{131}I]MIBG (Vaidyanathan et al., 1997a), it had a destabilizing effect on the astatinated derivative (Vaidyanathan et al., 1997b). These results are somewhat enigmatic and cannot be easily rationalized. Another important difference in the biodistribution of [^{131}I]CIBG was its higher blood uptake. Because bone marrow is a critical organ with respect to the radiation dose it can receive, it is not desirable to have persistent activity in the circulation. Most of the MIBG in the circulation was found to be in the erythrocyte fraction, and its movement from erythrocyte to plasma is not clearly understood (Sisson and Wieland, 1986). Because more lipophilic compounds are sequestered into erythrocytes rapidly (Zimmerman et al., 1987; Zou et al., 1998), it is reasonable to suggest that CIBG is taken up and probably retained by erythrocytes to a greater extent than MIBG and FIBG.

The uptake of *p*[^{131}I]INBG in tissues such as spleen, blood, thyroid, and lungs was considerably higher than that of [^{125}I]MIBG. The uptake in these tissues was similar to that seen for free iodide (Garg et al., 1990). This suggests that, although it was found to be stable in vitro, *p*[^{131}I]INBG underwent rapid deiodination in vivo, and that the observed biodistribution may be that of a combination of free iodide and intact molecule. The isomeric compound *m*[^{131}I]INBG was developed to hopefully augment the inertness to deiodination. Paradoxically, while it was more unstable than *p*[^{131}I]INBG in vitro, in vivo results suggested it to be more stable. Nonetheless, results from both in vitro experiments using SK-N-SH cells and that from in vivo in mouse showed it to be a poor analogue of MIBG.

While the uptake of [^{131}I]NHIBG in adrenals and a number of other tissues was comparable to that of [^{125}I]-MIBG, it had a lower heart uptake at early time points. This corroborates the results obtained from in vitro experiments and suggests that introduction of a hydroxyl group on the guanidino function results in the diminution of NET specificity. Results shown for [^{131}I]DHIBG, [^{131}I]-HIMBG, and *m*[^{131}I]AIBG in Table 4 clearly demonstrate that, as seen in the in vitro system, these MIBG derivatives are not suitable substrates for NET. This suggests that this transporter possibly accepts aromatic substrates with one vacant meta position.

In conclusion, among a number of ring- and side-chain-substituted, radioiodinated MIBG analogues developed, only [^{131}I]CIBG showed higher uptake by SK-N-SH human neuroblastoma cells in vitro than [^{125}I]MIBG. However, its biodistribution data in normal mice suggest that it may not have any advantage over MIBG. While polar substituents are tolerated at the 4-position, it appears that the 5-position needs to be left unsubstituted. We are currently taking a prodrug approach to developing MIBG analogues with masked polar substituents. Hopefully with this strategy, tumor uptake may not be hindered, and at the same time faster normal tissue clearance of the deprotected metabolites may occur.

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Supporting Information Available: Tables (5, 6, and 7) containing numerical data of tissue uptake for CIBG, *p*INBG, and NHIBG for several mouse tissues (4 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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