quired to reduce the growth rate to 50% of the control.

The in vitro cytotoxicity against H. Ep. 2 human epidermoid carcinoma cells (ATCC CCL23) was evaluated in monolayer cultures. H. Ep. 2 cells were maintained in exponential growth in Basal Medium of Eagle with Hanks' salts (BME) (GIBCO Laboratories, Grand Island, NY) and 0.5 g of $NaHCO_3$ per L, supplemented with 15% heat inactivated (56 °C, 30 min) bovine calf serum (Hyclone Laboratories, Logan, UT). Cells were harvested from the surface of culture flasks, for subculturing or for counting, by using 0.005% trypsin (2 × crystalline, 3080 NF units/mg, GIBCO Laboratories, Grand Island, NY) and 0.1% EDTA, disodium salt, in BME without serum. For growth rate determinations, 2×10^4 cells were placed in replicate 25 cm^2 flasks, in control medium. After 1 day of incubation the medium was changed to compound-containing medium. Then growth was monitored by harvesting and counting the cells in two flasks from each treatment group, on days 1, 2, and 4 after adding the compounds. The data was plotted and analyzed as described previously[30b] and above for L1210 cells. The average control population doubling time was 19 h.

Nucleotide Extractions. L1210 cells were incubated as described in the Results and Discussion and the neutralized perchloric acid extracts were prepared as described previously.[2b] The nucleotides were separated by anion exchange chromatography (HPLC) as described previously[32] except that the pH of both elution buffers was 4.75 for studies on 7-aza-TCN and 4.65 for TCN. The UV absorbance of the eluate was monitored at 254 nm for detection of natural nucleotides, and at 312 nm (λ_{max} at pH 4.75) for detection of 7-aza-TCN nucleotides or 292 nm (λ_{max} at pH 4.65) for detection of TCN nucleotides.

Acknowledgment. This investigation was supported in part by research grants CH-299 and CH-312 from the American Cancer Society, in part by funds from the Department of Health and Human Services (Grant No. AI-25739), and in part by a grant to L.L.W. from the Office of the Vice President for Research, the University of Michigan. The authors (L.B.T.) would also like to thank Warner-Lambert/Parke-Davis for a grant for graduate student research support and Ms. Rae Miller for her expert preparation of the manuscript. The authors thank K. Z. Borysko, R. D. Newcomb, and T. J. Franks for their expert technical assistance.

Registry No. **2**, 125284-49-3; **3**, 129572-01-6; **4**, 129572-02-7; **5**, 109947-70-8; **6**, 129572-03-8; **7**, 129572-04-9; **10**, 129572-05-0; **11**, 129572-06-1; **13**, 129572-07-2; **14**, 129572-08-3.

(32) Wotring, L. L.; Townsend, L. B. *Cancer Res.* **1989**, *49*, 289.

Synthesis and Biochemical Evaluation of Tritium-Labeled 1-Methyl-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-1*H*-indazole-3-carboxamide, a Useful Radioligand for $5HT_3$ Receptors

David W. Robertson,* William Bloomquist, Marlene L. Cohen, Leroy R. Reid, Kathryn Schenck, and David T. Wong

Lilly Research Laboratories, Eli Lilly and Company, Lilly Corporate Center, Indianapolis, Indiana 46285.
Received April 17, 1990

The advent of potent, highly selective $5HT_3$ receptor antagonists has stimulated considerable interest in $5HT_3$ receptor mediated physiology and pharmacology. To permit detailed biochemical studies regarding interaction of the indazole class of serotonin (5HT) antagonists with $5HT_3$ receptors in multiple tissues, we synthesized 1-methyl-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-1*H*-indazole-3-carboxamide (LY278584, compound **9**) in high specific activity, tritium-labeled form. This radioligand was selected as a synthetic target because of its potency as a $5HT_3$-receptor antagonist, its selectivity for this receptor viz a viz other 5HT-receptor subtypes, and the ability to readily incorporate three tritia via the indazole *N*-CH_3 substituent. Alkylation of *N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-1*H*-indazole-3-carboxamide (**8**) with sodium hydride and tritium-labeled iodomethane, followed by HPLC purification, resulted in [^{3}H]-**9** with a radiochemical purity of 99% and a specific activity of 80.5 Ci/mmol. This radioligand bound with high affinity to a single class of saturable recognition sites in membranes isolated from cerebral cortex of rat brain. The K_d was 0.69 nM and the B_{max} was 16.9 fmol/mg of protein. The specific binding was excellent, and accounted for 83–93% of total binding at concentrations of 2 nM or less. The potencies of known $5HT_3$-receptor antagonists as inhibitors of [^{3}H]-**9** binding correlated well with their pharmacological receptor affinities as antagonists of 5HT-induced decreases in heart rate and contraction of guinea pig ileum, suggesting the central recognition site for this radioligand may be extremely similar to or identical with peripheral $5HT_3$ receptors.

Introduction

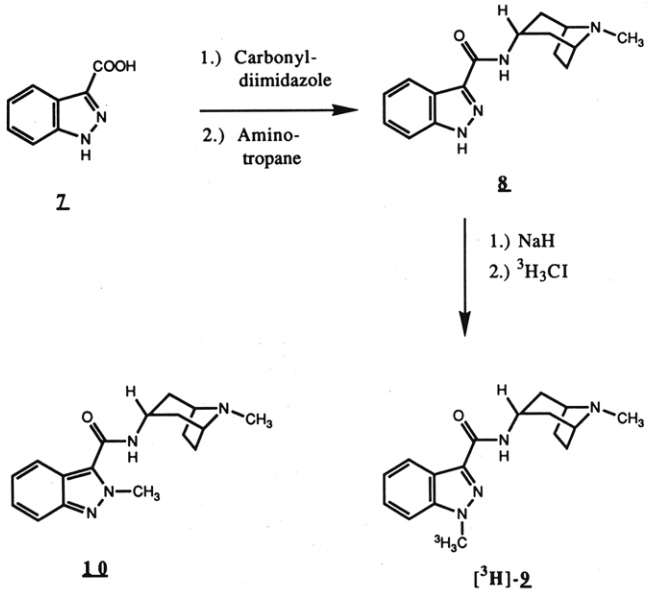
Although pharmacological data have long suggested that subtypes of serotonin (5-hydroxytryptamine, 5HT) receptors exist, the seminal work of Peroutka and Snyder provided biochemical evidence for the existence of $5HT_1$- and $5HT_2$-receptor subtypes.[1] During the past decade an explosion of information has emerged regarding the multiple molecular forms of 5HT receptors, and at least six subtypes are now accepted.[2]

The $5HT_3$-receptor subtype has attracted considerable attention recently, and our understanding of these receptors has increased dramatically over the past few years because of the discovery and widespread availability of potent and selective antagonists of these receptors.[3] The structures of some of the better characterized $5HT_3$-receptor antagonists are displayed in Chart I. These agents produce a variety of peripheral and central pharmacological effects in laboratory animals, and are being studied in man for the treatment of gastrointestinal motility disorders, migraine pain, chemotherapy-induced emesis, schizophrenia, anxiety, substance abuse, and other central nervous system (CNS) disorders.[3] Interest in the CNS

(1) Peroutka, S. J.; Snyder, S. H. *Mol. Pharmacol.* **1979**, *16*, 687.
(2) Robertson, D. W.; Fuller, R. W. *Annu. Rep. Med. Chem.* **1988**, *23*, 49.
(3) Fozard, J. R. In *The peripheral actions of 5-Hydroxytryptamine*; Fozard, J. R., Ed.; Oxford Medical Publications: Oxford, 1989; p 354.

0022-2623/90/1833-3176$02.50/0

Scheme I. Synthesis of [^{3}H]-9



indications for these compounds was heightened by the discovery that central binding sites were present for radiolabeled **5** (GR65630, an analogue of odansetron), **2** (ICS 205-930), **6** (quipazine), and related compounds.[4-8] Because some of these radioligands are limited by relatively high nonspecific binding, we prepared 1-methyl-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-1*H*-indazole-3-carboxamide (compound **9**, LY278584) in high specific activity, tritium-labeled form. In this paper we document the rationale for its selection, its synthesis, pharmacological data showing it is a potent peripheral 5HT$_3$-receptor antagonist, biochemical data demonstrating the labeled compound is a useful radioligand for central 5HT$_3$ receptors, and evidence suggesting similarities among central and peripheral 5HT$_3$ receptors.[9]

Results and Discussion

Compound Selection. Fludzinski and co-workers demonstrated that a series of tropanylamides of indazole-3-carboxylic acid were potent antagonists of peripheral 5HT$_3$ receptors.[10] Compound **8** (Scheme I) was essentially equipotent with **2** in antagonizing the von Bezold Jarisch reflex (vide infra, a 5HT$_3$ receptor mediated response) in rats. Furthermore, methylation at N-1 of the indazole (**9**, Scheme I) maintained receptor affinity while methylation at N-2 (**10**) led to a substantial decline in apparent 5HT$_3$-receptor affinity. Because of the potency of **9** as a 5HT$_3$-receptor antagonist, and the presence of a methyl substituent that could be used as a tritium anchor, we explored further this agent as an antagonist of 5HT$_3$ receptors.[11]

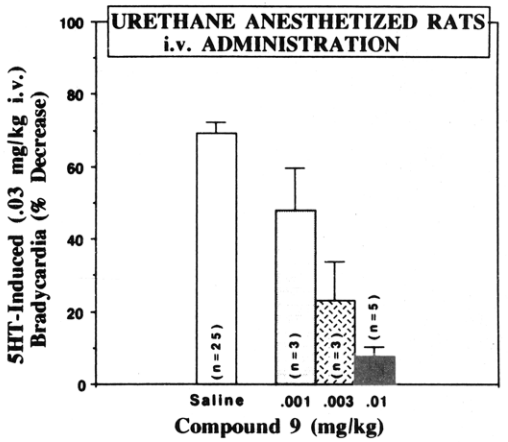


Figure 1. Dose-dependent inhibition of 5HT (30 μg/kg, iv) induced bradycardia by **9** in urethane-ansthestized rats. The drug was given iv 15 min before 5HT challenge. Data represent means ± SEM, and the number of animals for each group is given in parentheses as an inset.

Table I. Affinity of **9** for Subtypes of Central 5HT Receptors[a]

radioligand	5HT receptor	K_i, nM
[^{3}H]-8OHDPAT	5HT$_{1A}$	>1000
[^{3}H]-5HT	5HT$_{1B}$	>1000
[^{3}H]-mesulergine	5HT$_{1C}$	>1000
[^{3}H]-5HT	5HT$_{1D}$	>1000
[^{3}H]-ketanserin	5HT$_2$	>1000
[^{3}H]-**9**	5HT$_3$	1.62 ± 0.23

[a] Binding studies were conducted as described in the Experimental Section.

Table II. Inhibition of [^{3}H]-**9** Binding to Rat Cortical Membranes by 5HT$_3$ Receptor Ligands

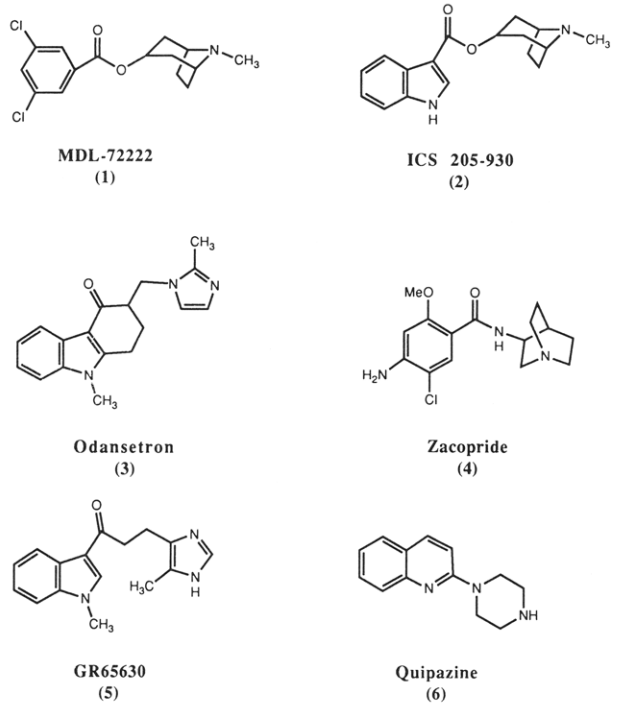
compound	K_i, nM	pK_i
2	1.28 ± 0.27 (8)	8.89
metoclopramide	502 ± 133 (7)	6.30
odansetron	2.46 ± 0.57 (8)	8.61
zacopride	0.42 ± 0.10 (8)	9.38
1	26.05 ± 9.18 (9)	7.59
quipazine	3.04 ± 0.65 (5)	8.52
8	4.26 ± 1.07 (4)	8.37
9	1.62 ± 0.17 (4)	8.79
10	1048 ± 173 (4)	5.98
11[b]	7.52 ± 4.26 (4)	8.12

[a] K_i values were derived from IC_{50} values according to the method of Cheng and Prusoff.[22] The data represent means ± SEM, and the number of replicates is provided in parentheses. [b] The ester analogue of **8**, prepared according to published procedures.[10]

In rats, intravenously administered 5HT activates the von Bezold–Jarisch reflex, resulting in a transient, dose-dependent bradycardia. This reflex response to 5HT results from activation of right ventricular 5HT$_3$ receptors, followed by a centrally mediated increase in vagal outflow.[12] In the present studies, iv administration of 5HT (30 μg/kg) to urethane-anesthetized rats resulted in a 72.0 ± 2.5% decrease in heart rate, consistent with previous studies.[13] Intravenous administration of unlabeled **9**, 15 min prior to 5HT, led to a dose-dependent inhibition of 5HT-mediated bradycardia (Figure 1). These effects were evident at doses of **9** as low as 1 μg/kg, and the ED_{50} was

(4) Kilpatrick, G. J.; Jones, B. J.; Tyers, M. B. *Nature* **1987**, *330*, 746.
(5) Barnes, N. M.; Costall, B.; Naylor, R. J. *J. Pharm. Pharmacol.* **1988**, *40*, 548.
(6) Peroutka, S. J.; Hamik, A. *Eur. J. Pharmacol.* **1988**, *148*, 297.
(7) Watling, K. J.; Aspley, S.; Swain, C. J.; Saunder, J. *Eur. J. Pharmacol.* **1988**, *149*, 397.
(8) Nelson, D. R.; Thomas, D. R. *Biochem. Pharmacol.* **1989**, *38*, 1693.
(9) A portion of these data have appeared in communication form: Wong, D. T.; Robertson, D. W.; Reid, L. R. *Eur. J. Pharmacol.* **1989**, *166*, 107.
(10) Fludzinski, P.; Evrard, D. A.; Bloomquist, W. E.; Lacefield, W. B.; Pfeifer, W.; Jones, N. D.; Deeter, J. B.; Cohen, M. L. *J. Med. Chem.* **1987**, *30*, 1535.
(11) Beecham scientists independently discovered the potent 5HT$_3$ receptor antagonist properties of this series of molecules and granisetron, a homologue of LY278584, has reached advanced clinical trials; see: Addelman, M.; Erlichman, C.; Fine, S.; Warr, D.; Murray, C. *J. Clin. Oncol.* **1990**, *2*, 337.
(12) Fozard, J. R.; Host, M. *Br. J. Pharmacol.* **1982**, *77*, 520P.
(13) Cohen, M. L.; Bloomquist, W.; Gidda, J. S.; Lacefield, W. B. *J. Pharmacol. Exp. Ther.* **1989**, *248*, 197.

Chart I. Structures of Some $5HT_3$-Receptor Antagonists



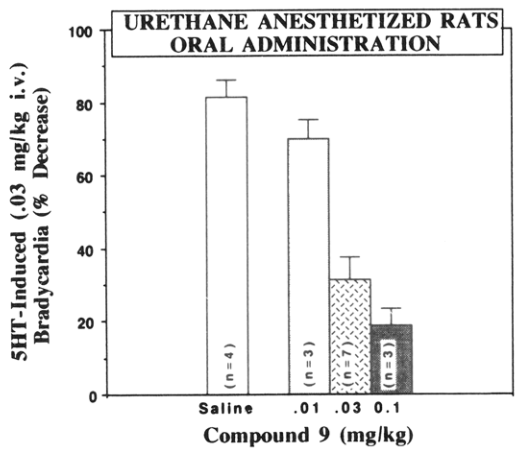


Figure 2. Dose-dependent antagonism of 5HT-mediated bradycardia by **9** after oral administration to urethane-anesthetized rats. Serotonin (30 μg/kg, iv) was given to the rats 1 h after administration of the indicated doses of **9**. Data represent means ● SEM, and the number of animals for each group is given in parentheses as an inset.

less than 3 μg/kg iv, demonstrating that **9** is a potent $5HT_3$ receptor antagonist. In previous studies with **2** under identical conditions, iv administration of 3 μg/kg led to a 66% decrease in 5HT-induced bradycardia, suggesting that this drug and **9** have comparable potencies.[13] After oral administration, **9** produced a dose-dependent reduction in the von Bezold Jarisch reflex response to 5HT, with an ED_{50} between 10 and 30 μg/kg (Figure 2). Thus, the oral potencies of **9** and zacopride were comparable, whereas both drugs were more potent orally than either **2** or odansetron.[13] Finally, the duration of action of **9** after oral administration was investigated, and the results are displayed in Figure 3. Oral dosing of 100 μg/kg of **9** resulted in an 80% reduction in the von Bezold–Jarisch reflex 1 h postadministration. Antagonism of $5HT_3$ receptors was manifest 3 h later, but had vanished 6 h postadministration. These data suggested that **9** had a duration of action intermediate to that of odansetron and zacopride, and comparable to that of **2**.[13] Thus, **9** is a potent and relatively

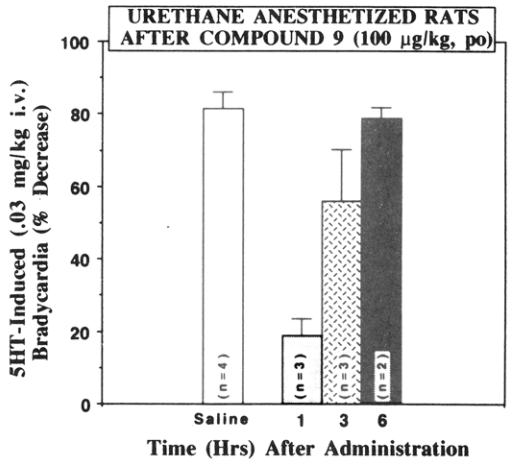


Figure 3. Time course for inhibition of 5HT-induced bradycardia by **9**. Serotonin (30 μg/kg, iv) was given to the rats at the indicated times after administration of **9** (100 μg/kg, po). Data represent means ± SEM, and the number of animals for each group is given in parentheses as an inset.

long-acting antagonist of $5HT_3$ receptors after both iv and oral administration.

The suitability of **9** as a potential radioligand for $5HT_3$ receptors was probed by determining its affinity for all known 5HT receptor subtypes (Table I). The compound was essentially devoid of affinity for $5HT_{1A}$, $5HT_{1B}$, $5HT_{1C}$, $5HT_{1D}$, or $5HT_2$ receptors, indicating substantial selectivity as a $5HT_3$-receptor ligand. Thus, interaction with other 5HT-receptor subtypes would be unlikely to complicate binding experiments using the radiolabeled drug.

Synthesis of 9. Experiments using [^{3}H]-**5**, [^{3}H]quipazine, and [^{3}H]zacopride suggested that the density of $5HT_3$ receptors is relatively low in brain membranes.[4-6] Therefore, we anticipated that biochemical studies with labeled **9** would require material of high specific activity. Tritium-labeled iodomethane was selected as the reagent of choice because it can be obtained in high specific activity, and can be used to introduce three tritia simultaneously. Compound **9** had been previously prepared by coupling of 1-methylindazole-3-carboxylic acid with 3α-tropanylamine.[10] However, a synthesis that enabled introduction of the 1-methyl substituent in the final step was required, and the route shown in Scheme I proved to be successful. Indazole-3-carboxylic acid was prepared as previously described[10] and then coupled with 3α-tropanylamine with use of carbonyldiimidazole in DMF, yielding **8** in 54% yield after chromatographic purification. Reaction of this material with sodium hydride in DMF, followed by addition of 1 equiv of iodomethane, produced essentially homogeneous **9**. HPLC analysis under conditions where starting material, **9**, and its 2-methylated regioisomer, **10**, were well-separated, suggested that little, if any, of the regioisomer was produced in this alkylation. The precise orientation of this alkylation was confirmed by nuclear Overhauser effect (NOE) experiments. When the new methyl resonance at δ 4.16 was irradiated, one NOE was observed at δ 7.76, the resonance of the aromatic hydrogen at position 7 of the indazole. These data require the methyl substituent arising from the alkylation to be located on N-1 of the indazole. Moreover, this material was identical chromatographically and spectroscopically with material prepared from authentic 1-methylindazole-3-carboxylic acid, thus establishing the virtually complete regioselectivity of this base-induced indazole methylation.

Alkylation of **8** with sodium hydride and tritium-labeled iodomethane (85 Ci/mmol) proceeded as expected to af-

ford [^{3}H]-**9** with approximately 40% radiochemical purity, and HPLC purification increased the radiochemical purity to 99%. The UV spectrum of [^{3}H]-**9** was indistinguishable from that of unlabeled material, and the specific activity was 80.5 Ci/mmol (see the Experimental Section). The compound appears to be relatively stable in radiolabeled form, and storage for 1.5 months in an ethanol solution resulted in a 3% decrease in radiochemical purity.

[^{3}H]-9 Biochemical Studies. [^{3}]-**9** bound in a saturable fashion to rat cortical membranes, and the binding reached saturation at approximately 2 nM. Scatchard replot of the binding isotherms suggested the recognition sites for [^{3}H]-**9** were homogeneous; the B_{max} was 16.86 fmol/mg of protein and the K_d was 0.69 nM. Whereas some 5HT$_3$ radioligands have high nonspecific binding,[4-7] the specific binding of [^{3}H]-**9** was approximately 80% of total binding at concentrations close to the K_d. Further details regarding the biochemical characterization of [^{3}H]-**9** have been communicated previously.[9]

Whereas **9** exerted virtually no effect on the binding of radioligands to 5HT$_1$ receptor subtypes and 5HT$_2$ receptors (IC_{50} values exceeded 1000 nM; Table I), antagonists of 5HT$_3$ receptors, from diverse structural classes, inhibited the specific binding of [^{3}H]-**9** to brain cortical membranes at low nanomolar concentrations. For example, the K_i values for inhibition of [^{3}H]-**9** binding (2 nM) were 1.28, 2.46, 0.42, and 26.05 nM for **2**, odansetron, zacopride, and **1**, respectively. The rank order of binding of these 5HT$_3$-receptor antagonists using [^{3}H]-**9** was similar to data obtained using other 5HT$_3$-receptor radioligands.[4-8] Compound **8**, the indazole precursor to [^{3}H]-**9**, was slightly less potent than **9** itself (K_i values were 4.26 and 1.62 nM, respectively). The ester analogue of **8**, compound **11** (1*H*-indazole-3-carboxylic acid, 8-methyl-8-azabicyclo[3.2.1]-oct-3-yl ester), was slightly less potent than the amide analogues, but the 2-methylindazole analogue **10** had only modest affinity (K_i = 1048 nM) for 5HT$_3$ receptors labeled by [^{3}H]-**9**.

While the relative affinities of these compounds for [^{3}H]-**9** binding sites in the CNS matched their apparent pharmacological affinities for 5HT$_3$ receptors in the rat heart, we quantified this comparison further. Using [^{3}H]-**9**, we were particularly interested in determining whether evidence for subtypes of 5HT$_3$ receptors could be obtained with this ligand; Richardson and co-workers have proposed the existence of three subtypes of 5HT$_3$ receptors based upon assay-dependent differences in potency among 5HT$_3$ receptor antagonists.[14,15] For example, **1** was considerably less potent than **2** in antagonizing 5HT-induced contractions in the guinea pig ileum,[14] suggesting this drug does not interact effectively with enteric neurons.[16] The potency differences were less when the compounds are examined in rat brain[6] and neuroblastoma cells.[17] In contrast, cocaine displayed roughly equivalent potency as a 5HT$_3$-receptor antagonist, regardless of the assay system.[15] We approached this problem by examining the affinity of several structurally diverse 5HT$_3$-receptor antagonists in heart and ileum, and comparing affinity constants to those determined in brain via displacement of [^{3}H]-**9**.

To probe possible differences between the cardiac and central 5HT$_3$ receptors, -log K_i values for displacement of [^{3}H]-**9** from rat cortical membranes were compared to -log ED_{50} values for blocking 5HT-induced bradycardia in the rat, and the data are plotted in Figure 4. The correlation between these two activities was excellent (0.90) and highly significant ($p < 0.01$), despite the fact that one measurement was in vivo and the other was in vitro. The -log K_i values of these compounds for central 5HT$_3$ receptors was also compared to their -log K_B values for functional blockade of 5HT$_3$ receptors in the guinea pig ileum, and the data are displayed in Figure 5. Although this comparison involves different species, again the correlation was highly significant (r = 0.93; $p < 0.01$).

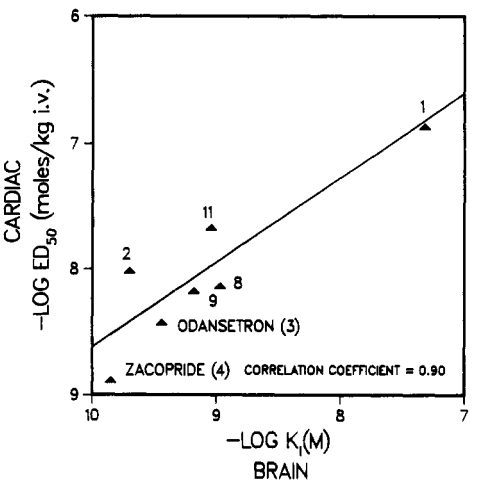


Figure 4. Correlation of the abilities of structurally diverse compounds to block 5HT$_3$ receptors in the rat heart and to bind to 5HT$_3$ receptors in the rat cortex. The -log K_i values for displacement of [^{3}H]-**9** were derived from data in Table II. The -log ED_{50} values for inhibition of 5HT-induced bradycardia were obtained as described in the Experimental Section.

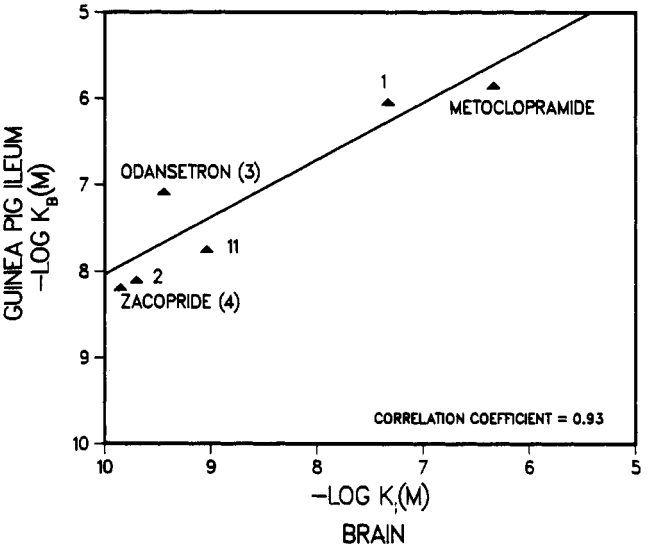


Figure 5. Correlation of the abilities of 5HT$_3$-receptor antagonists to block 5HT$_3$ receptors in the guinea pig ileum and to bind to 5HT$_3$ receptors in the rat cortex. The -log K_i values for displacement of [^{3}H]-**9** from cortical 5HT$_3$ receptors were derived from data in Table II. The -log K_B values for inhibition of 2-methylserotonin-induced contractions of the guinea pig ileum were obtained as described in the Experimental Section.

The data presented in Figures 4 and 5 do not support the notion of major differences among 5HT$_3$ receptors in rat heart, rat brain, and guinea pig ileum; in fact, these correlative studies suggest these receptors are similar. However, it should be noted that a limited number of compounds were used in these correlative studies and that the data points are distributed heterogeneously. Moreover, it is evident from Figures 4 and 5 that the slopes of the

(14) Richardson, B. P.; Engel, G.; Donatsch, P.; Stadler, P. A. *Nature* **1985**, *316*, 126.
(15) Richardson, B. P.; Engel, G. *Trends Neurosci.* **1986**, *9*, 424.
(16) Fozard, J. R. *Trends Pharmacol. Sci.* **1987**, *8*, 501.
(17) Hoyer, D.; Neijt, H. C. *Mol. Pharmacol.* **1988**, *33*, 303.

regression lines were less than unity, which could be taken as evidence for subtle differences in $5HT_3$ receptors among these three tissues. In addition to differences among the actual receptors, it is also conceivable that differences exist among the $5HT_3$ receptor transduction mechanisms. Because the $5HT_3$ receptor may be a potassium channel or is at least closely coupled to a potassium channel,[18] these possible differences could involve potassium channel gating mechanisms and/or differences among these tissues in transmembrane potentials.[19] In any event, detailed molecular studies of the $5HT_3$ receptors in these tissues will be needed to define precisely differences and similarities.

Summary and Conclusions

In this paper we presented pharmacological evidence showing that **9** is a potent, orally effective and long-acting $5HT_3$-receptor antagonist. We described an efficient preparation of **9** in high specific activity, tritium-labeled form, and the biochemical characterization of its interactions with central 5HT receptors. Using several structurally diverse $5HT_3$-receptor antagonists, we demonstrated that the affinities of these compounds for central [^{3}H]-**9** recognition sites correlated with their potencies as antagonists of rat ventricular and guinea pig ileal $5HT_3$ receptors. These data did not reveal evidence for multiple $5HT_3$ receptors as has been previously postulated. The excellent correlation of the binding of several $5HT_3$ receptor ligands and their functional antagonism of $5HT_3$ receptors suggest that [^{3}H]-**9** is labeling central $5HT_3$ receptors. Although several $5HT_3$ receptor antagonists have been radiolabeled and used in receptor binding studies, [^{3}H]-**9** appears to be particularly useful because of its low nonspecific binding and high specific activity. Because of its favorable pharmacodynamic and biochemical properties, [^{3}H]-**9** may be a useful probe of $5HT_3$ receptors to aid in elucidating the precise role these receptors play in mediating the physiological and pathophysiological actions of 5HT.

Experimental Section

Methods. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and were not corrected. Proton magnetic resonance (^{1}H NMR) spectra were obtained by use of a Bruker WM-270 spectrometer. Difference NOE (nuclear Overhauser effect) spectra were obtained by subtracting free induction decays accumulated with the decoupler off-resonance from similar accumulations with particular resonances irradiated, followed by Fourier transformation of the difference signals. The procedure was not optimized for maximum NOE measurement; the usual irradiation period was 2.0 s, followed by a preaccumulation delay of 0.03 s. Mass spectra were recorded from a Varian MAT CH-5 spectrometer. HPLC studies were performed on a Waters 6000A chromatograph, and the detector was a Waters operated at 315 nM. For the HPLC specific activity determinations, equal fractions from the column were collected in vials containing PCS scintillation fluid (Amersham), and the radioactivity was measured in a Packard Model 3375 liquid scintillation spectrometer. Microanalytical data were provided by the Physical Chemistry Department of the Lilly Research Laboratories; only symbols of elements analyzed are given, and they were within 0.4% of theoretical values unless indicated otherwise.

Except where noted, a standard procedure was used for product isolation. This involved quenching by addition to water, filtration or exhaustive extraction with a solvent (washing of extract with aqueous solutions, on occasion), drying over an anhydrous salt, and evaporation of solvent under reduced pressure. Particular solvents, aqueous washes (if needed), and drying agents are mentioned in parentheses after the phrase "product isolation".

***N*-(8-Methyl-8-azabicyclo[3.2.1]oct-3-yl)-1*H*-indazole-3-carboxamide (8).** Carbonyldiimidazole (5.5 g, 34 mmol) was added in one portion to a solution of 1*H*-indazole-3-carboxylic acid (5 g, 30 mmol) in 50 mL of DMF, and the reaction was stirred for 30 min at room temperature. A solution of 3α-tropanylamine (4.75 g, 34 mmol) in 25 mL of DMF was added dropwise and then the reaction was stirred at room temperature for 16 h. Solvent was removed under reduced pressure and the product was purified by preparative silica gel HPLC eluted isocratically with ammonium hydroxide/methanol/methylene chloride (1:20:79, v/v). This yielded 5.0 g (54%) of product with a melting point of 223–225 °C: ^{1}H NMR ($CDCl_3$) δ 1.80–2.43 (m, 8 H, tropane H's), 2.35 (s, 3 H, NCH_3), 3.24 (m, 2 H, bridgehead H), 4.37 (q, 1 H, methine H), 7.28 (t, 1 H, indazole H5), 7.43 (t, 1 H, indazole H6), 7.51 (d, 1 H, indazole H4), 7.59 (d, 1 H, amide H), 8.41 (d, 1 H, indazole H7), 10.93 (bs, 1 H, indazole NH); mass spectrum (70 eV), *m/e* (relative intensity) 284 (45, M^+), 83 (100). Anal. ($C_{16}H_{20}N_4O$) C, H, N.

1-Methyl-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-1*H*-indazole-3-carboxamide (Unlabeled 9). Sodium hydride (27 mg of a 60% dispersion in mineral oil, 0.66 mmol) was added in one portion to a solution of *N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-1*H*-indazole-3-carboxamide (200 mg, 0.66 mmol) in 25 mL of DMF, and the reaction was stirred at room temperature for 15 min. Iodomethane (99 mg, 0.70 mmol) was then added dropwise. After 2 h, solvent was removed under reduced pressure to provide essentially homogeneous product as an oil. HPLC analysis was performed with a reverse-phase Waters C18 (4.5 mm × 25 cm) column eluted with acetonitrile/ammonium dihydrogen phosphate/water (20:0.5:79.5) at 2300 psi and at a flow rate of 2 mL/min. Under these conditions, retention times for starting material, product, and 2-methylated byproduct were 4.55, 7.53, and 3.95 min, respectively. Formation of the maleate salt and recrystallization from ethyl acetate/ethanol provided 162 mg (59%) of product as white crystals with mp 193–195 °C. Anal. ($C_{21}H_{27}N_4O_5$) C, H, N.

1-[^{3}H$_3$]Methyl-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-1*H*-indazole-3-carboxamide ([^{3}H]-9). Sodium hydride (4.4 mg of an 80% dispersion in oil, 0.147 mmol) was added to a solution of *N*-(8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-1*H*-indazole-3-carboxamide (39.9 mg, 0.14 mmol) in 2 mL of dry DMF, and the reaction was stirred for 15 min at ambient temperature. [^{3}H]-Iodomethane (10–15 Ci at 85 Ci/mmol) was distilled into the reaction, and stirring was continued at ambient temperature for 2 h. Volatile materials were removed in vacuo, and the labile tritium was removed by repeated evaporations to dryness from methanol (3 × 5 mL). The product (13.9 Ci) was dissolved in methanol, and analysis by reverse-phase TLC in methanol/triethylamine (100:1) indicated approximately 40% radiochemical purity.

An 800 mCi portion of this impure material was purified by HPLC on a μBondapak C18 (25 cm × 10 mm) column eluted with acetonitrile/water/triethylamine (500:500:1) at 3 mL/min. Solvents were removed in vacuo, and the residue was dissolved in ethanol, resulting in 418 mCi of [^{3}H]-**9**. The chemical identity was ascertained by identical chromatographic mobility with an authentic sample of **9**, by UV spectroscopy, and by DCI mass spectrometry using ammonia as reagent gas. The radiochemical purity was 99% as determined by reverse-phase HPLC on a μBondapak C18 (30 cm × 46 mm) column eluted isocratically with acetonitrile/0.5% ammonium dihydrogen phosphate in water (20:80).

Determination of Specific Activity. Compound **9** has an ultraviolet absorbance pattern with a maximum at 302 nm (ϵ = 4203). The UV spectrum of [^{3}H]-**9** was indistinguishable from that of unlabeled compound. The concentration of a sample of [^{3}H]-**9** in ethanol was measured with use of the absorbance at 302 nm, and the radioactivity present was then determined. These measurements were repeated on samples containing several concentrations of labeled compound, and the specific activity was calculated to be 80.5 Ci/mmol.

Pharmacological Methods. Serotonin-Induced Activation of von Bezold–Jarisch Reflex in Urethane-Anesthetized Rats. Male Sprague-Dawley rats (200–300 g, Harlan Industries) were anesthetized with urethane (1.25 g/kg, ip), a tracheotomy

(18) Derkach, V.; Surprenant, A.; North, R. A. *Nature* **1989**, *339*, 706.
(19) Robertson, D. W.; Steinberg, M. I. *J. Med. Chem.* **1990**, *33*, 1529.

was performed, and an endotracheal tube was inserted (PE240). The carotid artery was cannulated and connected to a Gould Statham P2310 pressure transducer via a 23-guage needle. A femoral vein was exposed, cannulated with polyethylene tubing (PE50), and used for iv drug administration. Heart rate and blood pressure were monitored with use of the pressure transducer signal and a cardiotachometer coupler (Beckman 9857B).

For iv evaluation of 5HT$_3$-receptor antagonists, an initial response to 5HT was generated. When 5HT-induced bradycardia returned to control levels (within 5 min), either antagonist or saline was administered, and 5HT-induced bradycardia was elicited again 15 min after antagonist or saline administration. For oral studies, fasted, conscious rats were dosed (5 mL/kg) by gavage with either antagonist or vehicle 1 h prior to 5HT challenge; 15–20 min before 5HT challenge, rats were anesthetized with urethane and surgically prepared as indicated above. For studies examining the duration of drug action, the 5HT$_3$-receptor antagonist or vehicle was administered orally to conscious rats. Rats were then anesthetized, surgically prepared, and challenged with 5HT at the time indicated after oral administration of the 5HT$_3$-receptor antagonist. Drugs were prepared daily in either distilled water or saline, with subsequent dilutions in physiological saline.

Determination of Apparent Antagonist Dissociation Constants at 5HT$_3$ Receptors in Guinea Pig Ileum. Noncumulative contractile concentration–response curves for 2-methylserotonin, a selective 5-HT$_3$ receptor agonist,[14] were obtained by a stepwise increase in concentration. EC_{50} values were taken as the concentration of 2-methylserotonin that produced half-maximal contraction. After control responses to 2-methylserotonin were obtained, ilea were incubated with appropriate concentrations of antagonist for 1 h. Noncumulative responses to 2-methylserotonin (tissues were washed between 2-methylserotonin additions) were then repeated in the presence of the antagonist that was added in the wash buffer between 2-methylserotonin additions. In each tissue, only one antagonist concentration was examined, and in each experiment one tissue was not treated with the antagonist and served as a control to correct for time-related changes in sensitivity.

Apparent antagonist dissociation constants (K_B) were determined for each concentration of antagonist according to the following equation:[20]

$$K_B = \frac{[B]}{(\text{dose ratio} - 1)}$$

where [B] is concentration of antagonist and dose ratio is EC_{50} of the agonist in the presence of the antagonist divided by control EC_{50}. These results were then expressed as the negative logarithm of K_B (i.e., $-\log K_B$). Calculations were performed according to the procedures of Bemis et al.[21]

Preparation of Membranes. Male Sprague–Dawley rats weighing 125–150 g (Harlan Industries, Cumberland, IN) were killed by decapitation. The cerebral cortex was dissected from the brain over ice and homogenized in 9 volumes of 0.32 M sucrose. The homogenate was centrifuged at 1000g for 10 min. The supernatant was centrifuged at 17000g for 20 min. The pellet was suspended in 100 volumes of 50 mM Tris-HCl (pH 7.4), incubated at 37 °C for 10 min, and centrifuged at 50000g for 10 min. The process was repeated three times and the final pellet was suspended in the medium for radioligand binding.

Binding Assay. Radioligand binding assays were modified from methods described previously.[4] Cortical membranes in aliquots of 600–900 μg of protein were incubated at 25 °C for 30 min in 2 mL of medium containing 50 mM Tris-HCl (pH 7.4), 10 μM pargyline, 0.6 mM ascorbic acid, 5 mM $CaCl_2$, 2 nM [^{3}H]-**9** (80.5 Ci/mmol), and reference drugs at five or more concentrations in triplicate samples. The filters were washed twice with 5 mL of chilled medium and placed in scintillation vials with 10 mL of PCS (Amersham/Searle) scintillation fluid. Specific binding was defined as the radioactivity of samples in the absence and the presence of 10 μM 5HT and accounted for 93% and 83% of total binding at 0.2 and 2 nM [^{3}H]-**9**, respectively.

5HT Receptor Subtype Binding Assays. Cortical membranes of rat brain were used in binding of [^{3}H]-8OHDPAT (8-hydroxy-2-(dipropylamino)tetralin, 142.9 Ci/mmol) to 5HT$_{1A}$ receptors, [^{3}H]-5HT (30 Ci/mmol) to 5HT$_{1B}$ receptors in the presence of 100 nM 8OHDPAT and Sch23390, and [^{3}H]ketanserin (90 Ci/mmol) to 5HT$_2$ receptors. Membranes isolated from bovine choroid plexus were used for [^{3}H]mesulergine (85 Ci/mmol) binding to 5HT$_{1C}$ receptors, while bovine striatal membranes were used for binding of [^{3}H]-5HT to 5HT$_{1D}$ receptors in the presence of 100 nM Sch23390. Spiperone at 10 μM was used to establish nonspecific [^{3}H]ketanserin binding. Other conditions were similar to those used in [^{3}H]-**9** binding to 5HT$_3$ receptors.

Acknowledgment. We thank Dale Duckworth for his assistance with the analytical HPLC data, Douglas Erickson, Joseph Krushinski, and Tony Lavagnino for their chemical assistance, Kathy Cloutier for literature work, and Pamela Edmonds and Denise Hunter for preparation of the manuscript. Finally, we thank Mr. Tom Thompson and his colleagues of the Amersham Corp. for their collaboration in the synthesis of [^{3}H]-**9**.

(20) Furchgott, R. F. In *Handbook of Experimental Pharmacology*; Baschko, H., Muscholl, E., Eds.; Springer-Verlag: Berlin, 1972; Vol. 33, pp 283–335.

(21) Bemis, K. G. *Am. Stat. Assoc. Biopharm. Proc.* **1985**, *1985*, 72.

(22) Cheng, Y. C.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.