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Ligand-Directed Functional Heterogeneity of Histamine H_1 Receptors: Novel Dual-Function Ligands Selectively Activate and Block H_1-Mediated Phospholipase C and Adenylyl Cyclase Signaling[#]

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Ligand-directed signaling of H_1 receptors

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1-phenyl-3-dimethylamino-1,2,3,4-tetrahydronaphthalene (PAT)

5-phenyl-7-(dimethylamino)-5,6,7,8-tetrahydro-9*H*-benzocycloheptane (PAB)

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ABSTRACT

The autacoid and neurotransmitter histamine activates the H_1 G protein-coupled receptor (GPCR) to stimulate predominantly phospholipase (PL) C/inositol phosphates (IP) signaling, and, to a lesser extent, adenylyl cyclase (AC)/cAMP signaling, in a variety of mammalian cells and tissues, as well as, H_1-transfected clonal cell lines. This study reports that two novel H_1 receptor ligands developed in our laboratory, (–)-*trans*-1-phenyl-3-dimethylamino-1,2,3,4-tetrahydronaphthalene (*trans*-PAT) and (±)-c*is*-5-phenyl-7-(dimethylamino)-5,6,7,8-tetrahydro-9*H*-benzocycloheptane (*cis*-PAB), activate H_1 receptors to selectively stimulate AC/cAMP formation and PLC/IP formation, respectively, in CHO cells transfected with guinea pig H_1 receptor cDNA. *Trans*-PAT and *cis*-PAB also are shown to be functionally selective antagonists of H_1-linked PLC/IP and AC/cAMP signaling, respectively. While *cis*-PAB H_1 receptor activity is shown to be typically competitive, *trans*-PAT displays a complex interaction with the H_1 receptor that is not competitive regarding antagonism of saturation binding by the standard H_1 antagonist radioligand [^{3}H]-mepyramine or H_1/PLC/IP functional activation by histamine. *Trans*-PAT, however, does competitively block H_1/PLC/IP functional activation by *cis*-PAB. Molecular determinants for *trans*-PAT vs. *cis*-PAB differential binding to H_1 receptors, that presumably leads to differential activation of AC/cAMP vs. PLC/IP signaling, likely involves stereochemical factors as well as more subtle steric influences. Results suggest the *trans*-PAT and *cis*-PAB probes will be useful to study molecular mechanisms of ligand-directed GPCR multifunctional signaling. Moreover, since most untoward cardiovascular, respiratory, and gastrointestinal H_1 receptor-mediated effects proceed via the PLC/IP pathway, PAT-type agonists that selectively enhance H_1-mediated AC/cAMP signaling provide a mechanistic basis for exploiting H_1 receptor activation for drug design purposes.

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The histamine H_1 protein is a G protein-coupled receptor (GPCR) first cloned and characterized from bovine adrenal gland in 1991 (Yamashita, 1991). The cDNA genes encoding the H_1 receptor from other species were cloned soon after, including for human (De Backer, 1993). Southern blot analysis with H_1 receptor probes indicates that there are no related genes in various species and there is no compelling evidence for H_1 receptor subtypes (Smit et al., 1999), though, inter-species heterogeneity regarding H_1 pharmacology is known (Seifert et al., 2003). In most types of mammalian smooth muscle, endothelial, and brain tissue, histamine activation of H_1 receptors triggers $G\alpha_q$ protein activation with subsequent stimulation of phospholipase (PL) C and increased intracellular formation of inositol phosphates (IP) and diacylglycerol (Hill et al., 1997).

In mammalian brain and adrenal gland, activation of H_1 receptors also stimulates adenylyl cyclase (AC) and intracellular formation of adenosine 3',5'-cyclic monophosphate (cAMP). In rat brain, H_1-mediated stimulation of cAMP formation is enhanced by protein kinase (PK) C activation and is dependent on intra- and extracellular calcium (Garbarg and Schwartz, 1988). In bovine adrenal cells, H_1-mediated stimulation of cAMP formation also is dependent on extracellular calcium (Marley et al., 1991). Meanwhile, in H_1-transfected CHO (CHO-H_1) cells, histamine H_1 activation augments forskolin-stimulated cAMP formation by a mechanism not sensitive to extracellular calcium, nor PKC activation, and, is pertussis toxin insensitive (Leurs et al., 1994). Thus, while details like G protein, kinase, and calcium involvement are not clear, H_1 receptors also can modulate AC/cAMP signaling in addition to PLC/IP signaling. This dual signaling mechanism now is thought to be common among GPCRs.

The phenomenon of multiple signaling pathways associated with a single GPCR can be described within the framework of the three-state model of GPCR activation (Leff et al., 1997),

wherein, GPCRs isomerize between inactive and constitutively active states (Kenakin, 2001). GPCR activation causes dissociation of heterotrimeric (α,β,γ) G protein subunits – the Gα subunit can then activate transducer protein (e.g., PLC, AC) to alter second messenger concentration. In addition to a role for G-$\beta\gamma$ subunits in signal transduction (Clapham and Neer, 1997), it also is now realized the same GPCR can couple to different Gα proteins to result in "multifunctional signaling" (Milligan, 1993). A critical assumption of the GPCR multifunctional signaling theory is that a heterogeneity of active receptor conformations exists and that agonist ligands differ in their ability to induce, stabilize, or select among receptor conformations, as described in the "stimulus trafficking" hypothesis (Kenakin, 2001). It follows that, upon binding, agonist ligand chemical structural parameters are among the most important determinants of GPCR conformation that influences type of Gα protein and signaling pathway activated.

Previously, it was reported that the novel selective histamine H_1 ligand, (–)-*trans*-1-phenyl-3-dimethylamino-1,2,3,4-tetrahydronaphthalene (*trans*-PAT, Fig. 1), stimulates tyrosine hydroxylase activity and dopamine synthesis in rat and guinea pig forebrain in vitro and in vivo, by activating presynaptic H_1 receptors (Booth et al., 1999; Choksi et al., 2000). This effect of *trans*-PAT is similar to that observed for the endogenous agonist histamine (Fleckenstein et al., 1993) and effects of both ligands can be blocked specifically by typical H_1 antagonists such as triprolidine (Fig. 1). Based on these results, *trans*-PAT was proposed as a putative agonist at H_1 receptors linked to modulation of tyrosine hydroxylase, the rate-limiting enzyme in catecholamine neurotransmitter synthesis (Choksi et al., 2000). Subsequently, it was reported that *trans*-PAT also behaves as a potent antagonist (pA_2 = 9.2) regarding H_1-mediated

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contraction of guinea pig ileum, and, *trans*-PAT fully blocks H_1 receptor activation of PLC/IP formation in CHO-H_1 cells (Booth et al., 2002). Meanwhile, radioreceptor competition binding results, using the standard H_1 antagonist radioligand [^{3}H]-mepyramine (Fig. 1), show the Hill coefficient (n_H) for the slope of the displacement curve by *trans*-PAT consistently is ~ 0.9 using membranes prepared from rodent tissues and H_1-transfected clonal cell lines (Booth et al., 1999; Choksi et al., 2000; Booth et al., 2002), characteristic of agonist ligand binding at a GPCR, according to the ternary complex model with limiting availability of G protein (De Lean et al., 1980). The complex H_1 receptor activity shown by *trans*-PAT also has been observed in pilot studies for another PAT-type derivative, (±)-c*is*-5-phenyl-7-(dimethylamino)-5,6,7,8-tetrahydro-9*H*-benzocycloheptane (*cis*-PAB, Fig. 1), that behaves as an H_1 agonist or antagonist, depending on the functional assay (Moniri and Booth, 2004). The preliminary functional results reported for *trans*-PAT and *cis*-PAB are reminiscent of activities shown by certain dopamine D_2 receptor ligands that are agonists at postsynaptic D_{2L} receptors but antagonists at presynaptic D_{2L} receptors (Kilts et al., 2002; Mottola et al., 2002). The term "functional selectivity" was used to describe this phenomenon wherein a ligand that acts as an agonist at a GPCR linked to one particular signaling pathway may act as an antagonist at the same receptor linked to another signaling pathway. Functional selectivity can be exploited for drug design purposes as a practical application of the multifunctional signaling and stimulus trafficking hypotheses.

In this paper, we report the unique H_1 binding and selective functional activity (PLC/IP vs. AC/cAMP signaling) of *cis*-PAB and *trans*-PAT, two novel H_1 ligands that are from the same chemical class but differ primarily with regard to stereochemistry (Fig. 1). Results of these studies suggest delineation of ligand molecular structural parameters that determine functionally

selective binding should be an important consideration involved in designing GPCR-active drugs with predictable and selective pharmacotherapeutic effects vs. untoward side effects.

Materials and Methods

Chemicals

(1*R*,3*S*)-(–)-*Trans*-1-phenyl-3-*N*,*N*-dimethylamino-1,2,3,4-tetrahydronaphthalene (*trans*-PAT, Fig. 1) was synthesized as previously described (Wyrick et al., 1993). Briefly, the benzylstyrylketone was cyclized to the tetralone intermediate and reduced to the (±)-*cis*- and (±)-*trans*-tetralols. This diastereomeric tetralol mixture was converted to the free amine, followed by dimethylation and fractional recrystallization, to isolate (±)-*trans*-PAT. The (±)-*trans*-PAT enantiomeric mixture was converted to the (–)-camphorsulfonic acid diastereomeric salt and resolved by fractional recrystallization to yield the pure (1*R*,3*S*)-(–)-*trans*-PAT isomer (Wyrick et al., 1993; Bucholtz et al., 1998, 1999). To synthesize (5*R*,7*S*/5*S*,7*R*)-(±)-*cis*-5-phenyl-7-(dimethylamino)-5,6,7,8-tetrahydro-9*H*-benzocycloheptane (*cis*-PAB, Fig. 1) (Wyrick et al., 1995), the corresponding diphenylpentenone was cyclized to the tetrahydrobenzocycloheptanone and reduced to the (±)-*cis*- and (±)-*trans*-tetrahydrobenzocycloheptanol diastereomers, which could be separated using silica gel column chromatography. Conversion of the (±)-*cis*-alcohols to the free amine, followed by dimethylation, gave (±)-*cis*-PAB as a solid that could be purified as the HCl salt; (±)-*trans*-PAB resisted crystallization and remained a gum. Resolution of the (5*R*,7*S*/5*S*,7*R*)-(±)-*cis*-PAB enantiomers currently is underway.

[^{3}H]-Mepyramine (specific activity = 20 Ci/mmol), L-[1-^{14}C]-tyrosine (54 Ci/mmol), and [2-^{3}H(N)]-myoinositol (24 Ci/mmol) were purchased from Perkin-Elmer Life Science (Boston, MA). Other compounds were obtained in highest purity from Sigma-Aldrich (St. Louis, MO).

CHO cell transfection and culture

Chinese hamster ovary cells deficient in dihydrofolate reductase (CHO-K1) were stably transfected with the guinea pig histamine H_1 receptor cDNA (Traiffort et al., 1994). Clonal transfects expressing the H_1 receptor (CHO-H_1) were selected for in α-minimal essential media without ribonucleosides and supplemented with 10% fetal bovine serum and 2 mM L-glutamine. For binding and functional studies, CHO-H_1 cells were grown to 90% confluence in 75 cm^2 tissue culture flasks containing α-minimum essential medium, supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 0.1% penicillin-streptomycin (100 units/100 μg/ml), in a humidified atmosphere of air:CO_2 (95:5%) at 37° C.

Null-transfected CHO-K1 cells were cultured as above and used to verify that effects observed were H_1 receptor-dependent (selective H_1 antagonists also were used). Essentially, no H_1 radioligand specific binding was detected in membranes prepared from CHO-K1 cells as [^{3}H]-mepyramine total and nonspecific saturation binding was 480 ± 49 and 460 ± 27 fmol/mg protein (mean ± S.E.M.), respectively. Also, essentially no histamine receptor-mediated IP or cAMP second messenger formation was detected in lysates of CHO-K1 cells as second messenger levels were 99 ± 1.3% basal control values (mean ± S.E.M.) after exposure to 10 μM histamine for 15-45 min.

Radioreceptor assays

Radioligand competition and saturation binding assays were performed using membrane homogenate prepared from CHO-H_1 cells, as previously reported (Booth et al., 2002). For competition binding assays, membranes were incubated with ~K_D concentration of the standard H_1 antagonist radioligand [^{3}H]-mepyramine (1.0 nM), plus, test ligand (0.01 – 10,000 nM). For

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saturation isotherms, membranes were incubated 0.01 to 10.0 nM [^{3}H]-mepyramine, some conditions included the H_1 antagonist triprolidine, *trans*-PAT, or *cis*-PAB, where possible, at ~$K_{0.5}$, 10-times $K_{0.5}$, or 100-times $K_{0.5}$. concentration. Both assays used 50mM Na^+-K^+ phosphate buffer (total assay volume was 0.4 ml), incubation was for 30 min at 25 °C, and non-specific binding was defined by triprolidine (10 μM). Inhibition data were analyzed by nonlinear regression using the sigmoidal curve-fitting algorithms in Prism 3.0 (Graphpad, San Diego, CA) to determine IC_{50} and Hill coefficient (n_H). In light of the incompletely characterized nature of the interaction between the H_1 receptor and the novel ligands used here, ligand affinity is expressed as an approximation of K_i values by converting IC_{50} data to $K_{0.5}$ values using the equation $K_{0.5} = IC_{50}/ 1 + L/K_D$, where L is the concentration of radioligand having affinity K_D (Cheng and Prusoff, 1973). Each experimental condition was run in triplicate and each experiment was performed a minimum of three times to determine S.E.M..

Measurement of [^{3}H]-inositol phosphates formation in CHO-H_1 cells

Formation of [^{3}H]-inositol phosphates ([^{3}H]-IP) was measured in CHO-H_1 cells, as described previously (Booth et al., 2002). Briefly, CHO-H_1 cells were incubated overnight in 12-well culture plates (ca. 7.0 x 10^4 cells/well) with [^{3}H]-myo-inositol (0.4 μCi), a precursor of the PLC-β substrate phosphatidylinositol. Aliquots of drug stocks were added in triplicate in the presence of 50 mM LiCl (total well volume = 0.5 ml) and incubation continued at 37°C for 45 min. After aspiration of media, wells were placed on ice and lysed by incubation with 50 mM formic acid (15 min). Formic acid was neutralized with ammonium hydroxide and well contents were added to individual AG1-X8 200-400 formate resin anion exchange columns. Ammonium formate/formic acid (1.2 M/0.1 M) was used to elute [^{3}H]-IP directly into scintillation vials for

counting of tritium. Resulting data were analyzed using the nonlinear regression algorithm in Prism 3.0, and are expressed as mean percent control [^{3}H]-IP formation, and potencies are expressed as concentrations required to produce 50% maximal [^{3}H]-IP formation (EC_{50}) ± S.E.M ($n \geq 3$).

Measurement of cAMP formation in CHO-H_1 cells

Formation of cAMP was measured in CHO-H_1 cells grown in 24-well plates, pre-incubated for 5 min in serum-free media in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (1.0 mM), followed by addition of drug stocks in serum-free media. After incubation for 15 min at 37° C, plates were placed on ice and the cells lysed by addition of 0.1 M HCl followed by sonication. Well contents were centrifuged (700 x *g*, 10 min) individually and aliquots of the supernatants were used in the direct-cAMP immunoassay kit (Assay Designs Inc, Ann Arbor, MI). Data were expressed as mean percent control cAMP formation as obtained by linear standard curve extrapolation, and potencies are expressed as concentrations required to produce 50% maximal cAMP formation (EC_{50}) ± S.E.M ($n \geq 3$).

Results

H_1 radioreceptor binding assays using CHO-H_1 cell membranes

Competition binding analysis: Representative H_1 radioligand (the antagonist [^{3}H]-mepyramine) displacement curves for *trans*-PAT and *cis*-PAB, in comparison to the endogenous agonist histamine and the standard competitive H_1 antagonist triprolidine (Hill et al., 1997), are shown in Fig. 2. Curves are sigmoidal in shape and span 3-4 log ligand concentration units to achieve complete radioligand displacement, characteristic of competitive displacement of ~K_D radioligand concentration from a single population of GPCRs. Values for $K_{0.5}$ ± S.E.M. (nM) for triprolidine, *trans*-PAT, *cis*-PAB, and histamine are 0.93 ± 0.16, 1.15 ± 0.36, 175.0 ± 16.0, and 18,300 ± 1,200, respectively. The Hill coefficient (n_H) for the slope of the competitive displacement curve is ~1.0 (0.98 ± 0.08) for the H_1 antagonist triprolidine. Meanwhile, n_H <1.0 for *trans*-PAT, *cis*-PAB, and histamine (n_H ± S.E.M. = 0.87 ± 0.14, 0.77 ± 0.03, and 0.72 ± 0.09, respectively), characteristic of agonist ligand binding at a GPCR, according to the ternary complex model with limiting availability of G protein (De Lean et al., 1980).

Saturation binding analysis: Fig. 3A-C shows representative saturation binding curves for [^{3}H]-mepyramine alone and in the presence of triprolidine, *cis*-PAB, and *trans*-PAT, at increasing concentration (~$K_{0.5}$, ~10-times $K_{0.5}$, and ~100-times $K_{0.5}$, except for *cis*-PAB where maximum solubility was 30 μM). As shown in Fig. 3A, the B_{max} value for [^{3}H]-mepyramine binding in absence of triprolidine is 320 ± 11 fmol/mg protein and this value is not significantly different in presence of 1.0, 10, and 100 nM triprolidine (ANOVA p=0.1). Meanwhile, the K_D value for [^{3}H]-mepyramine binding increases from 0.51 ± 0.01 nM in the absence of triprolidine, to, 1.24 ± 0.05, 3.64 ± 0.01, and 16.3 ± 2.50 nM, in the presence of 1.0, 10, and

100 nM triprolidine, respectively (ANOVA $p<0.003$). These results (no change in B_{max} but decreased affinity of radioligand in presence of increasing concentration of competitor), suggest that triprolidine binds to H_1 receptors in accordance with typical Michaelis-Menton type competitive kinetics. Fig. 3B shows that analogous results are obtained using *cis*-PAB as displacing ligand, i.e., there is no significant change in [^{3}H]-mepyramine B_{max}, but, K_D value increases from 0.43 ± 0.02 nM in absence of *cis*-PAB, to, 0.52 ± 0.01 nM, 2.50 ± 0.03 nM, and 7.31 ± 0.31 nM, in the presence of 0.1, 0.5, and 1.0 μM *cis*-PAB, respectively (ANOVA $p<0.0001$). Thus, it appears *cis*-PAB binding to H_1 receptors also is according to typical competitive kinetics. In contrast, Fig. 3C shows that when the displacing ligand is *trans*-PAT, the [^{3}H]-mepyramine B_{max} value decreases from 260 ± 4.9 fmol/mg protein in absence of *trans*-PAT, to, 230 ± 1.1, 160 ± 4.4 and 52 ± 0.6 fmol/mg protein, in presence of 1.0, 10, and 100 nM *trans*-PAT, respectively (ANOVA $p<0.0001$). Also, Fig. 3C shows that K_D for [^{3}H]-mepyramine increases from 0.34 ± 0.05 nM in absence of *trans*-PAT, to, 1.21 ± 0.32, 1.64 ± 0.31, and 2.61 ± 0.14 nM, in presence of 1.0, 10, and 100 nM *trans*-PAT, respectively (ANOVA $p<0.002$). These results (decreased B_{max} value and decreased affinity of radioligand in presence of increasing concentrations of displacing ligand), suggest *trans*-PAT binding to H_1 receptors is complex and not according to typical competitive kinetics. This unique *trans*-PAT binding interaction with H_1 receptors is not obvious from the results of Fig. 2, where K_D amount of [^{3}H]-mepyramine is used to label H_1 receptors, i.e., only half the total H_1 receptor population is labeled. Likewise, it appears that when concentration of *trans*-PAT begins to exceed $K_{0.5}$, it binds to H_1 receptors in a manner that is not competitive, decreasing the total number of H_1 receptors that can be labeled by [^{3}H]-mepyramine (B_{max}) and also decreasing apparent H_1 affinity of [^{3}H]-mepyramine (K_D).

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Stimulation of PLC/IP formation in CHO-H_1 cells

Fig. 4 shows that histamine stimulates PLC/[^{3}H]-IP formation in a concentration-dependent manner in CHO-H_1 cells, with E_{max} of ~ 100 μM (~ 900% basal control activity) and EC_{50} of ~ 3 μM; the effect at EC_{50} is fully blocked by the competitive H_1 antagonist triprolidine (Fig. 4 inset). Also shown in Fig. 4, *cis*-PAB stimulates formation of [^{3}H]-IP in CHO-H_1 cells in a concentration-dependent manner. Although solubility problems limited maximal *cis*-PAB concentrations to ~ 30 μM, it could be determined that E_{max} is ~ 10 μM (~ 200 % basal control activity; ~ 20% histamine E_{max}) and EC_{50} is ~ 140 nM; the effect at EC_{50} is fully blocked by triprolidine (Fig. 4 inset). Even at concentrations up to 30 μM, *trans*-PAT had no effect to stimulate [^{3}H]-IP formation in CHO-H_1 cells (Fig. 4).

Although *cis*-PAB is ~ 20-fold more potent than histamine at stimulating [^{3}H]-IP formation (EC_{50} about 140 nM vs. 3.0 μM), the maximal stimulation produced by *cis*-PAB is about 20% of the histamine maximal response. These results suggest that in comparison to the endogenous agonist histamine, *cis*-PAB is a partial agonist regarding H_1-mediated stimulation of PLC/IP formation – accordingly, *cis*-PAB should appear as an antagonist in the presence of histamine in this H_1 functional assay. Fig. 5 shows that, in fact, histamine H_1-mediated stimulation of PLC/[^{3}H]-IP formation is competitively antagonized by *cis*-PAB, as indicated by a histamine EC_{50} value that increases as concentration of *cis*-PAB increases (shift to the right in concentration–response curve), i.e., histamine EC_{50} is ~ 3 μM in absence of *cis*-PAB, but, histamine EC_{50} increases to ~ 8 and ~ 25 μM in presence of 0.1 and 1.0 μM *cis*-PAB, respectively (ANOVA $p<0.0001$). Meanwhile, histamine E_{max} for stimulation of PLC/IP

formation is achieved, regardless of the concentration of *cis*-PAB present. As described by Kenakin (1993), competitive antagonism by a weak partial agonist (i.e., *cis*-PAB) can be treated as competitive agonism by the antagonist, as the error in the apparent K_B value introduced by the weak partial agonist is negligible. Assuming competitive antagonism by *cis*-PAB, the data in Fig. 5 yields an apparent K_B value of 180 nM.

The Fig 5 inset shows that, as expected, the H_1 antagonist triprolidine also competitively antagonizes histamine stimulation of [^{3}H]-IP formation. This result is indicated by histamine EC_{50} values that increase concomitant with increasing concentration of triprolidine, i.e., the histamine EC_{50} is ~ 4 μM in absence of triprolidine, but, increases to ~ 40 and ~ 300 μM in presence of 0.01 and 0.01 μM triprolidine, respectively (ANOVA $p<0.0001$). For triprolidine, the data in Fig. 5 yield an apparent K_B value of 2.0 nM (nearly 100-times more potent than *cis*-PAB, consistent with its higher H_1 affinity). Overall, the results in Figs. 4 and 5 confirm the H_1 competitive antagonist activity of triprolidine and identify *cis*-PAB as a novel potent partial agonist (in comparison to histamine) at H_1 receptors that activate PLC/IP formation.

Results summarized in Fig. 6 show that, in contrast to the competitive antagonism observed for *cis*-PAB and triprolidine (Fig. 5), *trans*-PAT antagonism of histamine H_1-mediated stimulation of PLC/[^{3}H]-IP formation apparently is not competitive. For instance, the histamine EC_{50} is ~ 3 μM in the absence of *trans*-PAT, but, the EC_{50} value increases to ~ 10, 17, and 27 μM in presence of 0.01, 0.1, and 1.0 μM *trans*-PAT, respectively (ANOVA $p<0.0001$). Also, the histamine E_{max} decreases from 100% response in the absence of *trans*-PAT, to, ~ 95%, 60%, and 50% response in presence of increasing concentrations of *trans*-PAT (ANOVA $p<0.0001$). Meanwhile, as shown in the Fig. 6 inset, *trans*-PAT antagonism of *cis*-PAB H_1-mediated stimulation of PLC/[^{3}H]-IP formation appears to be competitive. For instance, while the *cis*-

PAB EC_{50} is ~ 85 nM in the absence of *trans*-PAT, the EC_{50} value increases to ~ 850 and 2,600 nM in the presence of 0.01 and 0.1 μM *trans*-PAT, respectively (ANOVA $p<0.0001$). Also, consistent with competitive antagonism, the *cis*-PAB E_{max}, is unchanged in the absence or presence of *trans*-PAT (ANOVA $p=0.2$).

Stimulation of AC/cAMP formation in CHO-H_1 cells

Results in Fig. 7 show that histamine stimulates AC/cAMP formation over basal control levels in a concentration-dependent manner in CHO-H_1 cells, with E_{max} ~ 100 μM (~ 170% basal control activity) and EC_{50} ~ 2.1 μM. Moreover, the novel H_1 ligand *trans*-PAT also stimulates AC/cAMP in this assay system with efficacy and potency similar to histamine. Although solubility problems limited maximal *trans*-PAT concentrations to ~ 1.0 mM, it could be determined that E_{max} is ~ 160% basal level at 100 μM and EC_{50} is ~ 2.0 μM. Maximal response for both histamine and *trans*-PAT is comparable to the AC activator forskolin (~ 173% basal at 1.0 μM) here and the histamine response also is comparable to that observed in bovine adrenal cells (~ 200%) (Marley et al., 1991). At EC_{50}, both histamine and *trans*-PAT H_1-mediated increases in cAMP are significantly different than basal ($p<0.01$), and, effects of both ligands are fully blocked by the H_1 antagonist triprolidine (data not shown). At concentrations 0.01-100 μM, *cis*-PAB alone had no effect on cAMP formation, but, it could competitively antagonize H_1-mediated stimulation of AC/cAMP formation produced by *trans*-PAT (Fig. 7, inset) and histamine (data not shown)

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Discussion

Results of these studies indicate that the novel H_1 ligands *trans*-PAT and *cis*-PAB can display mutually opposing activity and selectively activate and block different H_1-linked intracellular signaling pathways, i.e., the AC/cAMP and PLC/IP signaling cascades. The endogenous agonist histamine, on the other hand, non-selectively activates both H_1 pathways. The literature now documents many observations of the same GPCR activating different intracellular signaling pathways. For example, adrenergic α_{1B} receptors, which predominantly couple to $G\alpha_q$/PLC to stimulate IP formation, also can couple to $G\alpha_S$/AC to stimulate cAMP formation in CHO cells expressing α_{1B} cDNA (Horie et al., 1995). The reverse also is observed, e.g., histamine H_2 receptors, which predominantly couple to $G\alpha_S$/AC/cAMP, also can couple to $G\alpha_q$/PLC/IP in COS cells expressing H_2 cDNA (Kuhn et al., 1996). The 5-HT_{2C} receptor, that is phylogenetically closely related to H_1 receptors (Smit et al., 1999), predominantly couples to $G\alpha_q$ to activate PLC but also can modulate AC/cAMP formation through $G\alpha_i$ (Cussac et al., 2002). Recent evidence also suggests that H_1 receptors may mediate antinociception via $G_{i/o}$ signaling in vivo (Galeotti et al., 2002). Meanwhile, GPCRs are not limited to just two different G protein partners – many of the 30 or so different 5-HT receptors couple with several different G proteins and can modulate a half-dozen different intracellular signaling pathways (Raymond et al., 2001).

Examples of GPCR signaling promiscuity have been demonstrated using native intact cells, primary cultures, and in vivo animal models (Allgeier et al., 1994; Arey et al., 1997; Galeotti et

al., 2002), suggesting that multifunctional signaling among GPCRs is not necessarily an artifact of receptor over-expression in clonal cell lines. On the other hand, receptor and/or G protein over expression might reveal both qualitative and quantitative ligand-GPCR responses that might not be detected in native cell and tissue preparations. Nevertheless, while the H_1-linked AC/cAMP signaling revealed here for *trans*-PAT may be due, in part, to receptor and/or G protein over expression, the lack of effect for *cis*-PAB to activate H_1/AC/cAMP signaling in comparison to *trans*-PAT and histamine in the same assay system indicates that the effect is ligand-specific. Clearly, too, the activity of *trans*-PAT as a functionally selective full agonist regarding H_1/AC/cAMP signaling is a concentration-dependent phenomenon. Thus, while *trans*-PAT binds to the H_1 receptor (Fig. 2) and blocks H_1/PLC/IP signaling (Fig. 3C) at concentrations as low as ~1.0 nM, significant activation of H_1/AC/cAMP signaling does not occur until concentration of *trans*-PAT approaches ~1.0 μM (Fig. 7). While the physiological relevance of concentration-dependent functional selectivity is presently unclear, preliminary results presented in this paper suggest that specific ligands can be designed to selectively activate or block specific intracellular signaling pathways for multifunctional signaling GPCRs.

Molecular mechanisms to account for GPCR multifunctional signaling involve the concept of “GPCR permissiveness” that assumes a high degree of flexibility in the interactions between a ligand, receptor, and G protein (Raymond, 1995). These interactions occur mainly between the G proteins and the second and third intracellular loops and carboxy-terminal tail of the receptor. Some factors that influence this interaction include receptor–G protein ratios and amounts, alternative GPCR splicing, and conformational changes in the G protein and/or receptor. In the present study, amount and ratio of H_1 receptors and G protein types expressed in the CHO-H_1

cells probably is a factor in signaling. This is suggested by the robust potency and efficacy of histamine to stimulate H_1-mediated PLC/IP formation (Fig. 3) such that EC_{50} (~ 2.6 μM) is well below $K_{0.5}$ (~ 18 μM) and the maximal effect is nearly a 10-fold increase over basal IP formation. In contrast, a 3-fold increase in basal IP formation by histamine is the maximum we found reported using mammalian brain, adrenal, retina, and ileum tissue preparations (Hill et al., 1997). Meanwhile, receptor splice variants are not likely to play a role in the ligand-directed signaling results observed here because the CHO-H_1 cells were transfected with H_1 cDNA, and, in any event, the authors could find no evidence in the literature for the existence of H_1 splice variants. Receptor conformational changes, however, likely are a factor for the current results, given that ligand chemical structure is among the most important molecular determinants for GPCR conformation that leads to activation of signaling (Kenakin, 2001). Furthermore, there is the phenomenon of spontaneous "pre-coupling of receptor–Gα protein complexes" (Leff et al., 1997) that explains observed GPCR constitutive activity, now well documented, including for H_1 receptors (Bakker et al., 2000). Such spontaneous receptor–Gα protein coupling suggests that ligand binding influences stabilization of fluctuating GPCR–Gα conformations and that ligand chemical structural parameters determine which conformation will be stabilized, induced, or selected (Kenakin, 2001).

The molecular determinants for *trans*-PAT vs. *cis*-PAB differential binding to the H_1 active site that leads to differential activation of the AC/cAMP vs. PLC/IP signaling pathways likely are due, in large part, to stereochemical factors. The H_1 receptor is known to be highly sensitive to the stereochemistry of PAT/PAB-type ligands. For example, *cis*-PAT is an H_1 antagonist that blocks H_1-medited stimulation of tyrosine hydroxylase and dopamine synthesis by *trans*-PAT in

rat forebrain (Choksi et al., 2000). Likewise, rank order of PAT isomer H_1 affinity depends on stereochemistry ([1*R*,3*S*]-[–]-*trans* > [1*S*,3*S*]-[–]-*cis* > [1*S*,3*R*]-[+]-*trans* > [1*R*,3*R*]-[+]-*cis*) and varies ~ 50-fold ($K_{0.5}$ ~ 1 - 50 nM), with *S*-chirality at the C3 amine position (Fig. 1) being the most important structural determinate for binding (Buchlotz et al., 1998; 1999). Interestingly, only the (–)-*trans*-PAT isomer has H_1 agonist activity (AC activation) – the other isomers are H_1 antagonists regarding PLC and AC activation (Booth et al., 2002; Moniri and Booth, 2004). Previous molecular modeling studies (Bucholtz et al., 1999) suggest that the protonated amine moiety of PAT/PAB-type compounds forms an ionic bond with the H_1 Asp^{116} residue (guinea pig numbering) in transmembrane helix (TMH) 3. The equivalent TMH3 Asp^{116} residue is highly conserved among biogenic amine neurotransmitter GPCRs and mutagenesis studies suggest this residue interacts with a positively charged amine moiety of endogenous agonists and other ligands (Savarese and Fraser, 1992), including for the H_1 receptor (Ohta et al., 1994) and the serotonin $5HT_2$ receptor family (Wang et al., 1993; Kristiansen et al., 1996) that is phylogenetically closely related to H_1 (Smit et al., 1999). While the main structural difference between *trans*-PAT and *cis*-PAB is stereochemical, there also is added conformational flexibility in the hexane (PAT) vs. heptane (PAB) heterocyclic ring systems, and molecular modeling studies indicate both structural parameters influence orientation of the dimethylamino and appended phenyl moieties (Bucholtz et al., 1999). Thus, in addition to differential interactions of the *trans*-PAT and *cis*-PAB amine moieties with H_1 TMH 3 residue(s), the phenyl moieties of these ligands may form differential π–π electron binding interactions with aromatic amino acids of the H_1 receptor binding pocket. In this regard, mutational analysis and molecular dynamics simulations of the H_1 receptor (Elz et al., 2000) and the related $5\text{-}HT_{2A}$ serotonin receptor (Kroeze et al., 2002) suggests that π–π stacking interactions occur between bound aromatic-

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containing ligands and aromatic amino acid residues in TMH 5 and 6. Mutagenesis studies to probe the molecular interactions of *trans*-PAT vs. *cis*-PAB with H_1 active site amino acid residues currently are underway in our laboratory.

Presently, we have not succeeded in separating the (5*R*,7*S*) and (5*S*,7*R*) enantiomers of (±)-*cis*-PAB. We note that the nearly 5-fold lower efficacy of *cis*-PAB in comparison to histamine with regard to H_1-mediated activation of PLC/IP formation (Fig. 5) might involve antagonism of one *cis*-PAB enantiomer by the other, analogous to (+)-*trans*-PAT antagonism of (–)-*trans*-PAT regarding H_1-mediated activation of tyrosine hydroxylase and dopamine synthesis (Booth et al., 1999; Choksi et al., 2000). Based on studies with PAT, we predict that (5*S*,7*R*)-*cis*-PAB will be the more active enantiomer at H_1 receptors since this isomer has the critical dimethylamino moiety in the same configuration as (1*R*,3*S*)-(–)-*trans*-PAT (stereochemical nomenclature rules result in differential *R,S* designation for the amine moieties of the two molecules even though they share the same 3-D configuration – see Fig. 1).

The binding interaction between *trans*-PAT and the H_1 receptor appears to be complex given that it is not competitive with respect to antagonism of H_1 saturation binding by [^{3}H]-mepyramine (Fig. 3C) and H_1/PLC/IP functional activation by histamine (Fig. 6). Meanwhile, the H_1/PLC/IP functional interaction between *trans*-PAT and *cis*-PAB is typically competitive (Fig. 6 inset), suggesting that there is overlap in the H_1 binding pharmacophores of *trans*-PAT and *cis*-PAB. Here again, mutagenesis studies will be helpful to sort H_1 receptor molecular recognition determinants for *trans*-PAT vs. *cis*-PAB functionally selective binding that presumably leads to differential activation of AC/cAMP vs. PLC/IP signaling.

To the best of our knowledge, the present results are the first to show that different but structurally related ligands can be developed to selectively activate or block two different intracellular signaling pathways for the same GPCR, i.e., H_1 receptors linked to AC/cAMP and PLC/IP signaling. Previously, it has been shown that a novel ligand ([Gly^1,Arg^{19}]hPTH-[1-28]) could be designed to selectively activate one of the two signaling pathways (AC/cAMP vs. PLC/IP) associated with the type 1 parathyroid GPCR (Takasu et al., 1999). The PAT/PAB-type probes, however, will be uniquely useful to study molecular determinants of switching mechanisms involved in GPCR multifunctional signaling. Such functionally selective ligands targeted to GPCRs also hold promising pharmacotherapeutic utility. For example, activation of H_1-linked PLC/IP signaling in peripheral smooth muscle and endothelial tissues can present clinically as respiratory distress (bronchial constriction), diarrhea (gastrointestinal contractions), and, edema and hypotension (increased vascular permeability), especially associated with an allergic response. *Trans*-PAT, however, is a lipophilic molecule that can penetrate brain tissue to selectively activate H_1-linked AC/cAMP signaling, presumably leading to modulation of tyrosine hydroxylase activity and catecholamine neurotransmitter synthesis (Choksi et al., 2000; Moniri and Booth, 2004). As most untoward cardiovascular, respiratory, and gastrointestinal H_1-mediated effects proceed via the PLC/IP pathway (Hill et al., 1997), PAT-type drugs that selectively enhance H_1-mediated AC/cAMP signaling in brain provide a mechanistic basis for exploiting H_1 receptors as pharmacotherapeutic targets in neuropsychiatric and neurodegenerative disorders involving alterations in catecholamine neurotransmission.

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FOOTNOTES

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Figure Legends

Fig. 1. Structures of histamine, (–)-*trans*-1-phenyl-3-dimethylamino-1,2,3,4-tetrahydronaphthalene (*trans*-PAT; 1*R*,3*S* enantiomer), (±)-c*is*-5-phenyl-7-(dimethylamino)-5,6,7,8-tetrahydro-9*H*-benzocycloheptane (*cis*-PAB; 5*S*,7*R* enantiomer shown), triprolidine, and [^{3}H]-mepyramine.

Fig. 2. Representative H_1 radioligand ([^{3}H]-mepyramine) displacement curves for triprolidine, *trans*-PAT, *cis*-PAB, and histamine (HA) using CHO-H_1 membranes

Fig. 3A-C. Representative [^{3}H]-mepyramine saturation binding curves in absence and presence of competing H_1 ligands.

3A: In absence of triprolidine, B_{max} = 320 ± 11 fmol/mg protein, K_D = 0.51 ± 0.01 nM; in presence of 1.0, 10, and 100 nM triprolidine, respectively, B_{max} = 310 ± 11, 300 ± 10, and 300 ± 12 fmol/mg protein (ANOVA $p>0.1$), K_D = 1.24 ± 0.05, 3.64 ± 0.01, and 16.3 ± 2.50 nM (ANOVA $p<0.003$).

3B: In absence of *cis*-PAB, B_{max} = 220 ± 7.4 fmol/mg protein, K_D = 0.43 ± 0.02 nM; in presence of 0.1, 0.5, and 1.0 μM *cis*-PAB, respectively, B_{max} = 230 ± 11, 220 ± 8.9, and 200 ± 9.0 fmol/mg protein (ANOVA $p>0.1$), K_D = 0.52 ± 0.01 nM, 2.50 ± 0.03 nM, and 7.31 ± 0.31 nM (ANOVA $p<0.0001$).

3C: In absence of *trans*-PAT, B_{max} = 260 ± 4.9 fmol/mg protein, K_D = 0.34 ± 0.05 nM; in presence of 1.0, 10, and 100 nM *trans*-PAT, respectively, B_{max} = 230 ± 1.1, 160 ± 4.4, and 52 ± 0.6 fmol/mg protein (ANOVA $p<0.0001$), K_D = 1.21 ± 0.32, 1.64 ± 0.31, and 2.61 ± 0.14 nM (ANOVA $p<0.002$).

Fig. 4. H_1 receptor-mediated stimulation of PLC/[^{3}H]-IP formation in CHO-H_1 cells

Histamine (HA): E_{max} = 940 ± 40% basal control activity at 100 μM, EC_{50} = 2.6 ± 0.2 μM; stimulation observed at ~ EC_{50} is significantly different (***, *t*-test $p<0.001$) from basal control activity and is fully blocked by 1.0 μM triprolidine (inset).

Cis-PAB: E_{max} = 21 ± 2.9% histamine value (200 ± 8% basal control activity) at 10 μM, EC_{50} = 140 ± 2.2 nM; stimulation observed at ~ EC_{50} is significantly different (**, *t*-test $p<0.01$) from basal control activity and is fully blocked by 1.0 μM triprolidine (inset).

Fig. 5. Antagonism of histamine H_1 receptor-mediated stimulation of PLC/[^{3}H]-IP formation in CHO-H_1 cells by *cis*-PAB and triprolidine

Histamine EC_{50} = 2.93 ± 0.82 μM in absence of *cis*-PAB; EC_{50} = 7.53 ± 0.86 and 25.0 ± 0.94 μM in presence of 0.1 and 1.0 μM *cis*-PAB, respectively (ANOVA $p<0.001$). Histamine E_{max} = 97.5 ± 4.0% (1170 ± 33.6% basal control activity) and this value does not differ from that obtained in presence of 0.1 or 1.0 μM *cis*-PAB (ANOVA $p>0.8$).

Inset: Histamine EC_{50} = 3.82 ± 0.83 μM in absence of triprolidine, EC_{50} = 40.4 ± 0.9 and 310 ± 9.4 μM in presence of 0.01 and 0.1 μM triprolidine, respectively (ANOVA $p<0.0001$). Histamine E_{max} = 99 ± 3.0% (890 ± 1.2% basal) and this value does not differ from that obtained in presence of 0.01 and 0.1 μM triprolidine (ANOVA $p>0.7$).

Fig. 6. Antagonism of histamine and *cis*-PAB H_1 receptor-mediated stimulation of PLC/[^{3}H]-IP formation in CHO-H_1 cells by *trans*-PAT

Histamine EC_{50} = 2.87 ± 0.79 μM in absence of *trans*-PAT; EC_{50} = 9.88 ± 0.82, 17.11 ± 0.80, and 27.03 ± 0.67 μM in presence of 0.01, 0.1 and 1.0 μM *trans*-PAT, respectively (ANOVA $p<0.001$). Histamine E_{max} = 107.1 ± 5.3% (940 ± 40% basal control activity) in absence of *trans*-PAT; E_{max} = 94.5 ± 4.1% (720 ± 7% basal), 61.5 ± 2.7% (540 ± 24% basal), and 47.8 ± 4.3% (410 ± 12% basal), in presence of 0.01, 0.1 and 1.0 μM *trans*-PAT, respectively (ANOVA $p<0.001$).

Inset: *Cis*-PAB EC_{50} = 85.6 ± 0.4 nM in absence of *trans*-PAT; EC_{50} = 851.1 ± 0.2 and 2,580 ± 0.2 nM in presence of 0.01 and 0.1 μM *trans*-PAT, respectively (ANOVA $p<0.0001$). *Cis*-PAB E_{max} = 103 ± 14.0% (230 ± 3.2% basal control activity) and this value does not differ from that obtained in presence of 0.01 and 0.1 μM *trans*-PAT (ANOVA $p>0.2$).

Fig. 7. H_1 receptor-mediated stimulation of AC/cAMP formation in CHO-H_1 cells

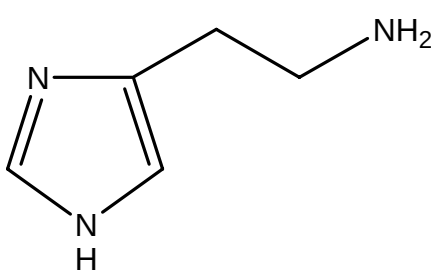
Histamine (HA): E_{max} = 170 ± 25% basal control activity at 100 μM, EC_{50} = 2.1 ± 0.4 μM; stimulation observed at ~ EC_{50} is significantly different (*t*-test $p<0.001$) from basal control activity and is fully blocked by 1.0 μM triprolidine (not shown).

Trans-PAT: E_{max} = 85 ± 8.5% histamine value (160 ± 9.0% basal control activity) at 100 μM, EC_{50} = 2.0 ± 0.5 μM; stimulation observed at ~ EC_{50} is significantly different (*t*-test $p<0.001$) from basal control activity and is fully blocked by 1.0 μM triprolidine (not shown).

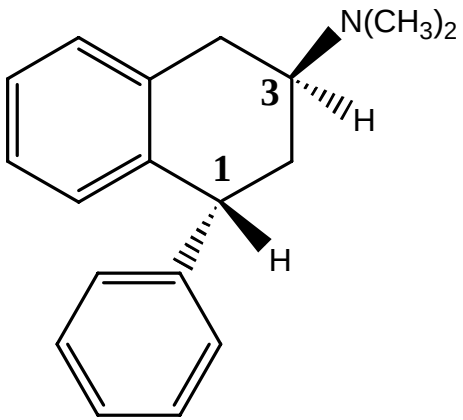
The AC activator forskolin (FSK) was used as positive control (112 ± 12.9 % histamine E_{max} or 173 ± 5% basal activity at 1.0 μM).

Inset: Antagonism of *trans*-PAT H_1 receptor-mediated stimulation of AC/cAMP formation in CHO-H_1 cells by *cis*-PAB.

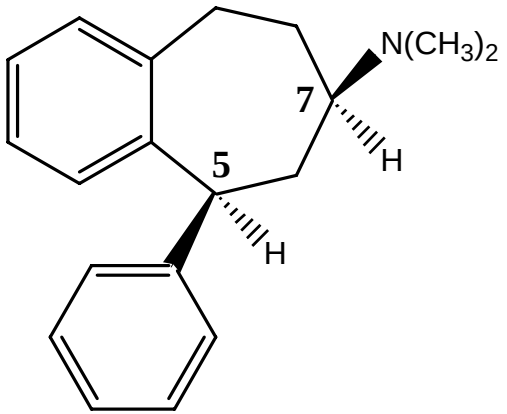
Trans-PAT EC_{50} = 4.2 ± 0.9 μM in absence of *cis*-PAB; EC_{50} = 9.2 ± 0.3 μM, 74 ± 0.6 and 160 ± 0.7 μM in presence of 0.05, 0.1 and 1.0 μM *cis*-PAB, respectively (ANOVA $p<0.0001$). *Trans*-PAT E_{max} = 100 ± 3% (150 ± 4.3% basal control activity) and this value does not differ from that obtained in presence of 0.05, 0.1 and 1.0 μM *cis*-PAB (ANOVA $p>0.9$).



Histamine



***trans*-PAT**



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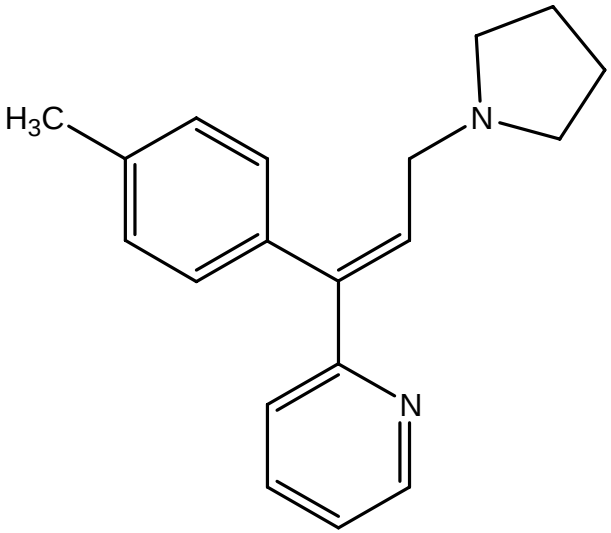
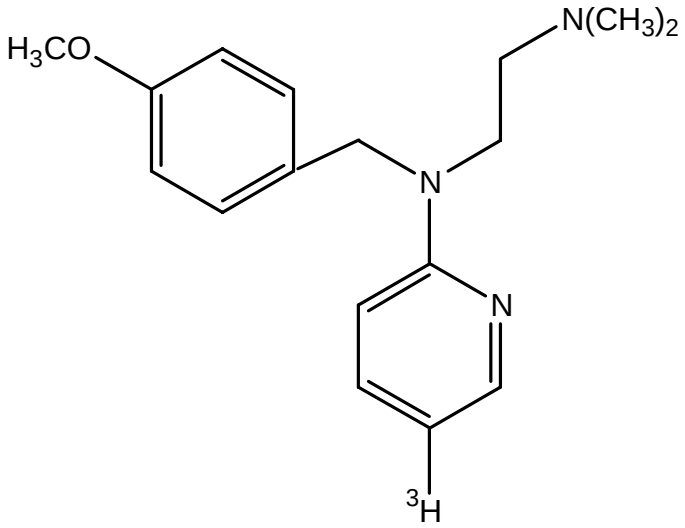


Triprolidine



[^{3}H]-Mepyramine

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