Risperidone Irreversibly Binds to and Inactivates the h5-HT7 Serotonin Receptor

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ABSTRACT

Risperidone displays a novel mechanism of antagonism of the h5-HT7 receptor. Pretreatment of the cells with 5 or 20 nM risperidone, followed by removal of the drug from the media, renders the 5-HT7 receptors unresponsive to 10 nM 5-HT for at least 24 h. Thus, risperidone seems to be producing a rapid, long-lasting inactivation of the h5-HT7 receptor. Whole-cell radioligand binding studies indicate that risperidone interacts in an irreversible or pseudo-irreversible manner with the h5-HT7 receptor, thus producing the inactivation. Internalization of the h5-HT7 receptor was not detected by monitoring green fluorescent protein-labeled fluorescent forms of the h5-HT7 receptor exposed to 20 nM risperidone. Ten other antagonists were tested for h5-HT7-inactivating properties, and only 9-OH-risperidone and methiothepin were found to demonstrate the same anomalous properties as risperidone. These results indicate that the h5-HT7 receptor may possess unique structural features that allow certain drugs to induce a conformation resulting in an irreversible interaction in the intact membrane environment. This may indicate that the h5-HT7 receptor is part of a subfamily of G-protein-coupled receptors (GPCRs) possessing this property or that many GPCRs have the potential to be irreversibly blocked, but only select drugs can induce this effect. At the very least, the possibility that highly prescribed drugs, such as risperidone, are irreversibly antagonizing GPCR function in vivo is noteworthy.

G-protein-coupled receptors (GPCRs) are the largest single family of receptors expressed in the mammalian genome (Leffkowitz, 2004). Approximately 600 genes express GPCRs, which serve to detect the levels of a multitude of extracellular and intracellular substances and induce intracellular changes designed to adapt to the changes in the extracellular environment. Synthetic agonists mimic endogenous substances in stimulating GPCRs, and competitive antagonists act by binding to the receptor but causing no effect, thereby preventing agonist stimulation of the receptor. Competitive antagonists display two hallmark characteristics: 1) antagonist inhibition can be overcome by increasing agonist levels while maintaining constant competitive antagonist levels and 2) agonist concentration-response curves are shifted to the right in the presence of a constant level of competitive antagonist (Egan et al., 2000). Additions and exceptions to these rules have been observed, including receptor reserve, which predicts maximal receptor functionality at agonist fractional occupancies less than 100%, and constitutive activity, which is the degree of receptor stimulation observed in the absence of any agonist (Teitler et al., 2002; Kenakin, 2004; Purohit et al., 2005).

Risperidone was one of the first of a new generation of antipsychotic drugs developed based on potency as a 5-HT2A and D2 dopamine receptor antagonist (Leysen et al., 1988; Meltzer et al., 1989). These “atypical” antipsychotic drugs have been found to be superior to the original “typical” antipsychotic drugs in that they produce fewer extrapyramidal side-effects and are beneficial in treating the negative symptoms of psychosis (Meltzer et al., 1989). As part of an ongoing investigation into the neurochemical effects of atypical antipsychotic drugs, we produced a cell line expressing a constitutively active mutant form of the h5-HT7 receptor (Purohit et al., 2004, 2005). Additions and exceptions to these rules have been observed, including receptor reserve, which predicts maximal receptor functionality at agonist fractional occupancies less than 100%, and constitutive activity, which is the degree of receptor stimulation observed in the absence of any agonist (Teitler et al., 2002; Kenakin, 2004; Purohit et al., 2005).

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ABBREVIATIONS: GPCR, G-protein-coupled receptor; PCR, polymerase chain reaction; GFP, green fluorescent protein; ANOVA, analysis of variance; 5-HT, 5-hydroxytryptamine; HEK, human embryonic kidney; Ro-20-1724, 4-[3-butoxy-4-methoxyphenyl]-methyl]-2-imidazolidinone.
h5-HT7 receptor. Therefore, the present study was performed to investigate, in greater detail, the mechanism of action of risperidone at the h5-HT7 receptor expressed in HEK-293 cells. Risperidone was a potent inhibitor of cAMP production induced by 10 μM 5-HT, with an IC50 approximately equal to its Kd value of 2 nM. Competitive antagonism predicts that risperidone’s potency should have been dramatically reduced in the presence of 5-HT at a concentration ~200-fold over its EC50 at the h5-HT7 receptor. Therefore, we conducted a series of experiments to determine the paradoxical high potency of risperidone in inhibiting 5-HT stimulation of the h5-HT7 receptor. Our results indicate that risperidone interacts in an irreversible or pseudo-irreversible manner, producing an inhibition of h5-HT7 receptor activity that seems to be rapid, potent, and essentially complete. This mechanism of action seems to be observed only with the h5-HT7 receptor, in that risperidone has been demonstrated to be a classic competitive antagonist at other serotonin and dopamine receptors.

GPCR desensitization has been studied extensively in vitro (Lefkowitz and Williams, 1978; Sibley and Lefkowitz, 1985; Freedman and Lefkowitz, 1996; Gaintinidov et al., 2004; Lefkowitz et al., 1983; Lefkowitz, 2004). As a rule, receptor desensitization occurs because of a prolonged interaction of a high concentration of an agonist with the receptor. The time course of the desensitization involves hours and often results in a partial loss of activity (usually between 30 and 50%).

This process has been shown to be due to the phosphorylation of agonist-occupied GPCRs followed by the binding of arrestin proteins that uncouple the receptor from GTP-binding proteins. A GPCR inactivation process occurring through the interaction of an antagonist with the receptor, at low concentrations, complete within 30 min (see Results), is a novel observation. It is noteworthy that methiothepin and risperidone were reported to produce an insurmountable antagonism of 5-HT7-mediated contraction of dog basilar arteries, whereas six other antagonists produced a competitive antagonism (Terron and Falcon-Neri, 1999). Thus, the results and interpretations described herein involving recombinant cell function in vitro are likely to be occurring in vivo.

Materials and Methods

Radioligand Binding. Radioligand binding studies in membrane homogenates were performed as described previously (Purohit et al., 2005). HEK cells stably expressing h5-HT7 receptors (100 mm dish; ~100% confluent) were scraped and collected in 50 mM Tris-HCl, 0.5 mM EDTA, and 10 mM MgSO4, pH 7.6 (at 23°C); centrifuged at 10,000g for 30 min; homogenized using a Polytron homogenizer (Kinematica, Basel, Switzerland); and centrifuged again at 10,000g for 30 min. The membranes were resuspended in 50 mM Tris-HCl, 0.5 mM EDTA, 10 mM MgSO4, and 0.1% ascorbic acid, pH 7.6 (at 23°C). Assays were performed in triplicate 1.0-ml volumes containing 10 μg of membrane protein (which was added last). Assays containing 2 nM [3H]H15-HT (24 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA), 1 nM [3H]mesulergine (82 Ci/mmol; GE Healthcare, Little Chalfont, Buckinghamshire, UK), or 1 nM [3H]risperidone (43 Ci/mmol; Janssen Pharmaceutica, New Brunswick, NJ) (Gommeren et al., 1997) were performed in the absence and presence of 10 μM clozapine to detect the level of available h5-HT7 receptors. Specific binding was generally ~85–95% of total binding. Tubes were incubated for 30 min at 37°C and filtered, and the filters were washed with 10 ml of Tris buffer. Radioactivity was measured using a liquid scintillation counter at an efficiency of 40%. Experimental results were analyzed using Prism Software (GraphPad Software, San Diego, CA). Protein content of the samples was determined with the use of a BCA protein assay kit (Pierce Chemical, Rockford, IL).

Whole-cell radioligand binding studies were performed as described previously with minor modifications (Shenoy et al., 2006). Radioligands were incubated with the cells for 30 min in the presence and/or absence of nonradioactive drugs, the media was removed, cells were rinsed with PBS, and cells were lysed with 3% trichloroacetic acid. The dissociation rate experiments involved a 30-min incubation with radioligand, addition of 10 μM mesulergine, and addition of 3% TCA at the appropriate time points. The released radioactivity was added to scintillation vials and counted on a Beckman scintillation counter (efficiency 40%).

CAMP Assay. A modification of the procedure of Salomon et al. (1974) was used. Cells were plated in six-well plates (2 × 106 cells/well) for 24 h and then incubated with [3H]adenine (4 μCi/well) for 24 h. Ro-20-1724 (0.25 mM), a phosphodiesterase inhibitor, was included in all subsequent media treatments. Typical drug treatment experiments involved a 30-min pretreatment with media or drug followed by exposure to 5-HT for 15 min. Where applicable, media was aspirated and replaced three times to remove drugs, followed by exposure to 5-HT for 15 min. Control experiments demonstrated that this procedure produced no effect on the cells’ responsiveness to 5-HT (see Results), and [3H]risperidone, [3H]H15-HT, and [3H]mesulergine tracer experiments demonstrated that three washes eliminated >99% of the drug from the original media. Cells were then lysed, and total cAMP was isolated and eluted using Dowex column chromatography. [3H]cAMP levels were assessed using liquid scintillation counting in Ultima Gold XR. For radioligand binding studies, subsequent to the final aspiration, radioligand binding buffer was added, and cells were treated as described above (see Radioligand Binding).

Creation of GFP Fusion Proteins. h5-HT7-GFP fusion proteins were created using the pEGFP-N1 vector from Clontech (Mountain View, CA). This vector contains multiple cloning sites upstream and in frame with the GFP, enabling the creation of a fusion protein with the GFP attached to the C terminus of the receptor. PCR was performed to amplify the entire open reading frame of the h5-HT7 with primers designed to remove the stop codon, while adding SstI and BamHI restriction sites at the 5’ and 3’ ends, respectively. SstI/BamHI digests of the PCR products were ligated into the pEGFP vector in frame with the GFP DNA sequence. DNA sequencing was performed (Center for Comparative Functional Genomics facility) to confirm that no additional mutations were created during the PCR step. DH5α Escherichia coli were transformed with the vector/receptor cDNA and grown overnight in Luria-Bertani broth with selection antibiotic, and DNA were extracted using a miniprep kit from QIAAGEN (Valencia, CA).

Confocal Microscopy in Living Cells. HEK-293 cells were transiently transfected with h5-HT7-GFP fusion protein in 100-mm dishes. Twenty-four hours after transfection, cells were seeded in serum-free medium on polyllysine-coated glass coverslips and incubated in serum-free medium overnight before microscopy. The cells were imaged live in PBS on a confocal imaging system (LSM-510 META; Zeiss, Thornwood, NY) using a 63 × 1.4 numerical aperture oil immersion objective at room temperature. The GFP was excited with the 488 nm line from an argon laser and emission collected using a band pass filter at 500 to 550 nm. The pinhole was set at 1.32 Airy units that results in a z-resolution of 2 μm. Several images of GFP expressing cells were collected to establish a baseline and then either 300 nM clozapine or 20 nM risperidone was added to the cells, and incubation was continued for an additional 30 min. Images were collected at a rate of 1 image/min for 30 min. Cells expressing the GFP fusion protein were also imaged in phosphate-buffered saline for only 30 min to establish that receptors did not internalize in the absence of added drug (data not shown). Images were collected using the Aim software (Zeiss) and the figures were assembled using Adobe Photoshop.
Results

Contrary to classic competitive antagonism, risperidone was found to inhibit 50 nM and 10 μM 5-HT-stimulated cAMP with identical IC_{50} values (K_{i} = 2.6 nM) (data not shown). To further investigate the mechanism producing this unexpected effect of risperidone, the effects of risperidone and clozapine on increasing concentrations of 5-HT was investigated (Fig. 1). The inhibition by 20 nM risperidone of 5-HT-mediated stimulation of cAMP accumulation in HEK-293 cells stably expressing h5-HT_{7} receptors was clearly insurmountable at 5-HT concentrations as high as 10 μM. The EC_{50} of 5-HT at the h5-HT_{7} receptor was 64 nM (Fig. 1), indicating that 10 μM 5-HT was 156-fold in excess of its EC_{50}. Risperidone at 20 nM is 10-fold in excess of its K_{i} value for the h5-HT_{7} receptor (Purohit et al., 2005). Likewise, clozapine was included at a concentration of 300 nM, which is ~10-fold over its K_{i} for the h5-HT_{7} receptor (Purohit et al., 2005). Unlike risperidone, clozapine's inhibition of 10μM 5-HT was surmountable, consistent with a classic competitive antagonism mechanism of action. Figure 1B demonstrates classic competitive antagonism by risperidone of 5-HT-stimulated inositol phosphate accumulation at r5-HT_{2A} receptors. The rightward shift of the 5-HT concentration-response curve and no significant decrease in maximal stimulation are hallmarks of classic competitive antagonism.

The results in Fig. 1B, compared with the effect of risperidone demonstrated in Fig. 1A (insurmountable antagonism), clearly demonstrate the unusual nature of risperidone's actions at the h5-HT_{7} receptor, while demonstrating that risperidone did act as a classic competitive antagonist at the r5-HT_{2A} receptor. The data in Fig. 1 indicate that the mechanism of action of risperidone in inhibiting 5-HT-mediated stimulation of cAMP accumulation was different from that of clozapine and was not due to classic competitive antagonism.

Figure 2 demonstrates the unusual effect of 20 nM risperidone, 100 nM 9-OH-risperidone, and 30 nM methiothepin in producing a dramatic inactivation of the h5-HT_{7} receptor after removing the drug (washout). Cells were treated with no drug (A), 20 nM risperidone (B), 100 nM 9-OH-risperidone (C), 30 nM methiothepin (D), or 300 nM clozapine (E) for 30 min. The media was aspirated and replaced with fresh media three times, and 10 μM 5-HT was then added to the wells. After 15 min, the level of cAMP accumulation was measured. Although clozapine was present at saturating concentrations, there was no inhibition of 10 μM 5-HT, which was 588-fold over its EC_{50} (Fig. 1). However, risperidone, 9-OH-risperidone, and methiothepin produced a profound loss of 10 μM 5-HT-induced cAMP stimulation. These results are consistent with the unusual effects observed in Fig. 1 for risperidone. Mesulergine, penfluridol, amperozide, and cinanserin were found to behave in a classic competitive manner (Fig. 2). Zotepine, fluperlapine, ziprasidone, fluphenazine, chlorpromazine, perphenazine, and thioridazine were also found to produce no inhibition of 10 μM 5-HT stimulation (data not shown).

A possible explanation for the effects of risperidone observed in Figs. 1 and 2 is an irreversible or pseudo-irreversible interaction of risperidone with the h5-HT_{7} receptor. To determine whether this was occurring, radioligand binding experiments were performed on intact cells stably expressing h5-HT_{7} receptors. Figure 3 displays the effect of a 30-min
preincubation with risperdone, 9-OH-risperdone, methiothepin, or clozapine on \([^3H]\)-5-HT binding. The drugs were removed by repeated aspiration and addition of fresh media, and \[^3H\]-5-HT radiolabeling studies were performed on the intact cells (see Materials and Methods). In parallel with the cAMP production studies (Fig. 2), risperdone, 9-OH-risperdone, and methiothepin pretreatment significantly inhibited subsequent radiolabeling of the h5-HT7 receptors, whereas pretreatment with clozapine produced no effect on the level of \[^3H\]-5-HT specific binding observed. These results indicate that risperdone, 9-OH-risperdone, and methiothepin seem to be interacting irreversibly with the h5-HT7 receptor binding site, which would explain the results observed in Fig. 2.

To more directly demonstrate this irreversible interaction, \[^3H\]risperdone specific binding to intact cells was investigated (Fig. 4A). In this experiment, h5-HT7 receptors were labeled with 1 nM \[^3H\]risperdone or 1 nM \[^3H\]mesulergine for 30 min. The media was removed and cells washed three times, and the amount of radioligand bound to the receptor was determined. The presence of \[^3H\]risperdone-specific binding after repeated washing of the cells was a direct demonstration of the irreversible interaction of risperdone with the h5-HT7 receptor. Figure 4B displays the lack of dissociation of specific \[^3H\]risperdone binding after equilibration with 5-HT, receptor–expressing cells and the addition of 10 \(\mu M\) mesulergine. It is noteworthy that this same pretreatment with risperdone did not produce an irreversible effect when the cells were homogenized and membrane homogenates were pretreated with risperdone and then radiolabeled (data not shown). This might indicate that a soluble factor or a factor loosely associated with the plasma membrane was involved in the irreversible interaction observed in the whole-cell preparation. Figure 5 displays the time course of the rapid loss of h5-HT7 receptors exposed to 5 nM risperdone. These results demonstrate a far more rapid inactivation process than the classical desensitization phenomena associated with GPCRs (see Discussion).

To determine the time course of the recovery of function after removal of drugs, cAMP accumulation assays and whole-cell radioligand binding assays were performed 24 h subsequent to application and removal of the antagonists (Fig. 6). At the 24-h time point, there was only a slight recovery of both function and radioligand binding. These results indicate that the inactivation was an extremely long-lasting effect, and the functional effect and the radioligand binding effect were further evidence that risperdone, 9-OH-risperdone, and methiothepin were binding irreversibly to the h5-HT7 receptor.

To determine the concentration-dependent effect of risperdone on the 5-HT concentration-dependent stimulation of h5-HT7 receptors, cells were exposed to 1, 3, and 5 nM risperdone, washed, and exposed to varying concentrations of 5-HT (Fig. 7). The results demonstrate a concentration-dependent effect on the maximal 5-HT stimulation with no significant effect on EC50 values (69, 87, and 44 nM for control, 1 and 3 nM risperdone pretreatments, respectively).
These results are consistent with a concentration-dependent inactivation of h5-HT7 receptors as a result of the increasing levels of risperidone interacting with the receptors during the 30-min pretreatment.

GPCR internalization has received a great deal of attention as a factor in desensitization mechanisms (Shenoy et al., 2006). To determine whether internalization is a key factor in the rapid inactivation of the h5-HT7 receptor, GFP-tagged h5-HT7 receptors were expressed in HEK cells, and the effects of 5-HT, risperidone, and clozapine on internalization of the receptors was monitored with confocal microscopy (Fig. 8). No obvious internalization was produced by risperidone or clozapine under conditions that produce inactivation of the GFP-tagged h5-HT7 receptors. The h5-HT7-GFP fusion protein was found to demonstrate the same inactivating response to risperidone and 9-OH-risperidone as the native h5-HT7 receptor (data not shown). These observations indicate that a robust, rapid internalization mechanism is apparently not involved in the inactivation of the h5-HT7 receptor observed in our studies.

### Fig. 5
Time course of risperidone-induced inactivation of h5-HT7 receptors. Cells were preincubated with 5 nM risperidone for the indicated times, followed by treatment with 10 μM 5-HT for 15 min. The $t_{1/2}$ is the mean ± S.E.M. of three independent experiments.

### Fig. 7
Concentration-dependent inactivation of h5-HT7 receptor function by risperidone. Cells were pre-exposed to 0, 1, 3, and 5 nM risperidone for 30 min, washed, and stimulated with 10 μM 5-HT. Maximal 5-HT stimulation ranged from 60,000 to 80,000 dpm. Results are the means ± S.E.M. of three independent experiments performed in triplicate. There were no significant differences in the EC$_{50}$ values for 5-HT (63, 69, 87, and 44 nM for control, 1, 3, and 5 nM risperidone, respectively). There was a significant difference in the maximal (10$^{-8}$ M 5-HT) stimulation for control, 1, 3, and 5 nM risperidone treatments ($p < 0.01$; ANOVA).

### Fig. 8
Confocal imaging of GFP-tagged h5-HT7 receptors: lack of acute effects of risperidone and clozapine on receptor internalization. HEK-293 cells expressing GFP-tagged h5-HT7 receptors were imaged live in the absence (zero time, A and C) and 30 min after the addition of 20 nM risperidone (B and D). Likewise, h5-HT7-GFP–expressing cells were imaged in the absence (zero time, E and G) and 30 min after the addition of 300 nM clozapine (F and H). Scale bar, 10 μm.
**Discussion**

The data presented strongly indicate that risperidone, 9-OH-risperidone, and methiothepin produce an irreversible or pseudo-irreversible interaction with the h5-HT7 receptor. This irreversible interaction results in a rapid inactivation of the h5-HT7 receptor after exposure to any of these three drugs. The irreversible interaction of risperidone, 9-OH-risperidone, and methiothepin seems to be a somewhat unusual effect, because the majority of antagonists produce classic competitive antagonism.

The biological and pharmacological implications of this work are not trivial. A study of dog cerebral artery 5HT7-mediated relaxation produced results corresponding to our results (Terron and Falcon-Neri, 1999). In that study, methiothepin and risperidone were found to produce an surmountable antagonism of 5HT7-mediated relaxation of an in vivo prostaglandin-induced cerebral arterial constriction. Clozapine, mesulergine, and spiperone were found to produce surmountable antagonism in that study, corresponding to the surmountable antagonism of the h5-HT7 receptor produced by the drugs in our studies. The inactivation of cerebral arterial 5-HT7 receptors by risperidone would be predicted to produce an elevated, long-lasting basal constriction of the cerebral artery, because the 5-HT7 receptor seems to contribute to a vasodilatory response. This effect, in turn, might be expected to produce a propensity to cerebrovasculature constriction and stroke in situations in which the cerebral artery is stimulated to contract. It is noteworthy that risperidone has been reported to produce an elevated likelihood of stroke in patients treated with risperidone (Wooltorton, 2002; Schneider et al., 2006). The higher incidence of stroke due to dementia and/or Alzheimer's disease (Wooltorton, 2002; Schneider et al., 2006). The higher incidence of stroke in the patients treated with risperidone may have nothing to do with the h5-HT7 receptor inactivation noted in our studies. However, the inactivating effect noted herein does provide a possible pharmacological mechanism for the higher incidence of stroke in the patients treated with risperidone. Given the enormous population of patients that have been and are being prescribed risperidone, any unusual interaction or effect of this drug on a significant pharmacological target, such as brain and/or cardiovascular h5-HT7 receptors, is worthy of note and further investigation.

The unusual effect of risperidone, 9-OH-risperidone, and methiothepin on the h5-HT7 receptor raises the possibility that this is not just an idiotyncratic effect of these drugs on this particular GPCR. An irreversible antagonist interaction with the m1 muscarinic receptor has been reported (Grant and El Fakahany, 2005). It is possible that some of the drugs commonly used and categorized as competitive antagonists may be producing irreversible interactions not observed with the currently accepted methods of receptor antagonism testing. Concentration-dependent inhibition of agonist stimulation provides an IC50 value for an antagonist but does not provide a mechanism. Although most studies of antagonists do include experiments that reveal mechanisms (i.e., Schid analyses) many published studies do not. For instance, the classic paper on the potent antagonist properties of risperidone at the 5-HT2 receptor includes an inhibition curve but not a Schid analysis (Leysen et al., 1988). Our own studies using recombinant cells demonstrate the classic competitive antagonistic properties of risperidone at the r5-HT2A receptor (Fig. 1B). It is clear that a majority of drugs being developed as therapeutics undergo detailed mechanistic studies that would reveal irreversible or pseudo-irreversible effects. However, the studies described herein emphasize the importance of not assuming a competitive mechanism for a drug at one receptor based on a competitive mechanism demonstrated at another receptor.

Finally, the lack of irreversibility observed in the interaction of risperidone with the h5-HT7 receptor on membrane preparations that have been subjected to repeated homogenization and resuspension (data not shown) may be a key observation. This result implies that a factor present in the intact cells (and in the isolated cerebral dog arterial preparation) may be key to the irreversible interaction and is lost during the membrane preparation. This factor may be common to GPCRs or may be associated with a subset of GPCRs. Given the importance of GPCRs as cellular components and as drug targets, any novel factor that modulates drug-receptor interaction with GPCRs is a notable contribution. Studies to determine the reason for the difference in the risperidone-h5-HT7 receptor interaction between the intact cell preparations and the homogenate preparations should reveal important novel mechanistic insights into GPCR function.

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**References**


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