

# In vitro pharmacological characterization of SPN-810M (molindone)

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**Background:** Impulsive aggression (IA) is considered a maladaptive form of aggression that is reactive and overt and occurs outside of the acceptable social context. Many children and adolescents with attention-deficit/hyperactivity disorder (ADHD) display clinically significant aggression, with the predominant subtype being IA. However, there is currently no Food and Drug Administration-approved medication specifically to treat IA. The pathophysiology of IA is not fully understood, although it has been suggested to include the dopamine, norepinephrine, and serotonin systems.

**Methods:** SPN-810 (extended-release molindone) is being developed for the novel indication of IA and is currently being studied in patients treated for ADHD. Molindone is an indole derivative and a dopamine D<sub>2</sub> receptor antagonist.

**Results:** The in vitro pharmacological studies described in the current manuscript demonstrate that the active substance molindone (SPN-810M) is a potent antagonist for the dopamine receptors, D<sub>2S</sub> and D<sub>2L</sub>, and the serotonin receptor, 5-HT<sub>2B</sub>, at therapeutic concentrations. The in vitro studies further demonstrate that the antagonist effect of SPN-810M is due to the parent drug and not the metabolites, and that the antagonism is not affected by the presence of norepinephrine or dopamine neurotransmitters. In addition, studies investigating the potential differential effects of the enantiomers of SPN-810M have demonstrated that the R(-) enantiomer is more potent than S(+), showing greater regulatory effect on D<sub>2S</sub> and D<sub>2L</sub> receptors.

**Conclusion:** Overall, the results of the in vitro SPN-810M pharmacological studies provide some insight into how SPN-810M modulates the serotonin and dopamine pathways that play a role in IA.

**Keywords:** dopamine, serotonin, SPN-810M, impulsive aggression

## Introduction

Impulsive aggression (IA) is considered a maladaptive form of aggression that is reactive, overt, and occurring outside the acceptable social context.<sup>1,2</sup> The presence of IA features in patients diagnosed with neuropsychiatric disorders such as attention-deficit/hyperactivity disorder (ADHD) amplifies the risk of poor outcomes even when treatment of the primary disorder is optimized.<sup>3</sup> Many children and adolescents with ADHD display clinically significant aggression, with the predominant subtype being IA.<sup>3</sup> IA becomes clinically distinguishable once ADHD-targeted therapy is maintained. This has been demonstrated in the Multimodal Treatment Study of Children with ADHD, in which 54% of the children with ADHD presented with clinically significant aggression prior to 14 months of treatment (medication with or without behavioral therapy); however, despite treatment, 44% of these children with aggres-

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sion at baseline exhibited persistent IA.<sup>1</sup> Although there are guideline recommendations for adjunctive targeted therapy when IA persists with ADHD treatment,<sup>1,4-6</sup> the agents being used have not proven to be adequately effective and have tolerability issues; furthermore, there are limited data for clinicians due to the paucity of randomized clinical trials.<sup>3</sup> Currently, there is no US Food and Drug Administration (FDA)-approved medication specifically indicated to treat IA that may be observed in children with ADHD, and therefore there is a need for an effective medication with an improved tolerability profile.<sup>3,7</sup>

Although alterations in the central nervous system (CNS) biology have been implicated, the pathophysiology of IA is not fully understood.<sup>1,3</sup> It has been hypothesized that IA is associated with the recruitment of the acute threat response system structures — the amygdala, hypothalamus, and periaqueductal gray.<sup>8</sup> The recruitment of these structures in order to initiate IA may reflect the functioning of the ventromedial prefrontal cortex, the dorsomedial prefrontal cortex, and the anterior insular cortex.<sup>8</sup> Abnormalities in central serotonergic, noradrenergic, and dopaminergic function may also be involved in IA.<sup>9,10</sup> In addition, IA has been associated with dopamine hyperactivity as a result of deficient serotonergic functioning and regulation of the dopamine system in brain regions such as the nucleus accumbens and prefrontal cortex, which are linked to reward-related motivation.<sup>11</sup>

Dopamine binds to five subtypes of G-protein-coupled receptors, categorized as D<sub>1</sub>-like (D<sub>1</sub> and D<sub>5</sub>) or D<sub>2</sub>-like (D<sub>2</sub>, D<sub>3</sub>, and D<sub>4</sub>) receptors.<sup>12,13</sup> Through alternative splicing, two isoforms of the D<sub>2</sub> receptors are generated, D<sub>2S</sub> (short) and D<sub>2L</sub> (long), which include 29 additional amino acids in the third intracellular loop.<sup>12,13</sup> The D<sub>2</sub> receptors are known targets of antipsychotic drugs.<sup>12,13</sup> Serotonergic neurons originate from the raphe nuclei and relay information using various neural pathways and serotonin receptors.<sup>14</sup> Seven serotonin receptors have been identified to date: 5-HT<sub>1</sub> through to 5-HT<sub>7</sub>.<sup>14</sup> The role of serotonin in IA is not completely understood, although low levels of serotonin have been associated with IA.<sup>11</sup> The activation or antagonism of some of the serotonin receptors has been shown to inhibit aggressive behavior, while that of others increases this behavior.<sup>14-16</sup>

Although it is not yet completely known how SPN-810 (extended-release molindone) reduces IA, previous receptor binding studies have shown that molindone does indeed block the D<sub>2</sub> receptors.<sup>17</sup> This current manuscript will discuss in vitro pharmacological studies and elucidate the effect of molindone (SPN-810M) on the dopamine and serotonin receptors that have been implicated in IA.

## Materials and methods

### Compounds tested

Molindone Hydrochloride (SPN-810M) (USP reference standard) (Supernus Pharmaceuticals, Inc., Rockville, MD, USA); Molindone Hydrochloride SPN-810M P1-2 metabolite and Molindone Hydrochloride (SPN-810M) P3-2 metabolite (Advinus Therapeutics Ltd., Bangalore, India); Molindone (SPN-810M) R(-) enantiomer and S(+) enantiomer (Supelco, Bellefonte, PA, USA).

### Radioligand binding

For D<sub>2S</sub> and D<sub>2L</sub> radioligand binding, the methods described by Hayes et al<sup>18</sup> were followed. Briefly, D<sub>2S</sub> and D<sub>2L</sub> receptors in human recombinant Chinese hamster ovary (CHO) cells were labeled with 0.16 nM [<sup>3</sup>H]-spiperone. All cell lines described in this manuscript were purchased commercially and authorization for the ethical use of these cell lines was obtained prior to usage (additional information on all cell lines and their source is provided in Table S1). Assays (in duplicate) were conducted in incubation buffer containing 50 mM Tris-HCl, pH 7.4; 1.4 mM ascorbic acid; 0.001% BSA; and 150 mM NaCl. Non-specific binding was defined with 10 μM haloperidol. Following 120-minute incubation at 25°C, radioligand binding was quantified. Using the methods described in Bonhaus et al<sup>19</sup> with slight modifications, 5-HT<sub>2B</sub> receptors were labeled with 1.2 nM [<sup>3</sup>H]-lysergic acid diethylamide in human recombinant CHO-K1 cells. Assays were conducted in incubation buffer containing 50 mM Tris-HCl, pH 7.4; 4 mM CaCl<sub>2</sub>; and 0.1% ascorbic acid. Non-specific binding was defined with 10 μM 5-HT. Following 60-minute incubation at 37°C, radioligand binding was quantified.

### [<sup>35</sup>S]GTPγS binding

A GTPγS binding assay was used to evaluate the antagonist activity of SPN-810M at the human D<sub>2S</sub> and D<sub>2L</sub> receptors expressed in transfected CHO cells. Total [<sup>35</sup>S]GTPγS (guanine 5'-O-[γ-thio]triphosphate) binding was performed in duplicate based on the method used by Senogles et al, with minor modifications.<sup>20</sup> Briefly, test compounds were preincubated with the membranes (D<sub>2S</sub>: 0.05 mg/mL; D<sub>2L</sub>: 0.1 mg/mL) and GDP (guanosine 5'-diphosphate) (D<sub>2S</sub>: 3 μM; D<sub>2L</sub>: 10 μM) in modified HEPES buffer (20 mM HEPES, pH 7.4; 100 mM NaCl; 10 mM MgCl<sub>2</sub>; 1 mM EDTA; 1 mM DTT) for 20 minutes and scintillation proximity assay beads were then added for another 60 minutes at 30°C. The reaction was initiated by 0.3 nM [<sup>35</sup>S]GTPγS for an additional 30 minutes at 30°C for D<sub>2S</sub> and 15 minutes at 30°C for D<sub>2L</sub> incubation period. The bound [<sup>35</sup>S]GTPγS was detected using a scintillation counter (PerkinElmer Microbeta).

## Impedance measurement and cellular dielectric spectroscopy

To evaluate the antagonist activity of SPN-810M at the human  $D_{2S}$  receptor expressed in transfected HEK-293 cells, the CellKey (cellular dielectric spectroscopy) detection method was used to measure effects of the compound on impedance modulation. Cells were seeded onto 96-well plates coated with fibronectin at  $2 \times 10^5$  cells/well in HBSS buffer + 20 mM HEPES (Thermo Fisher Scientific, Waltham, MA, USA) with 0.1% BSA and equilibrated for 45 minutes at 28°C. Plates were placed onto the system and measurements were made at 28°C. HBSS (basal and stimulated controls), reference antagonist ( $IC_{50}$  determination), or the test compounds were preincubated for 15 minutes before the addition of HBSS (basal control) or the reference agonist dopamine at 30 nM ( $EC_{80}$ ). Impedance measurements were monitored for 10 minutes. The standard reference antagonist butaclamol was tested in each experiment at several concentrations to generate a concentration-response curve from which its  $IC_{50}$  value was calculated. Assays were run in duplicate. The analysis of other receptors (eg, 5-HT<sub>1A</sub>) used similar assay methods.

## Phosphoinositide hydrolysis assays and homogeneous time-resolved fluorescence (HTRF)

HEK-293 cells and CHO cells expressing 5-HT<sub>2A</sub> and 5-HT<sub>2B</sub>, respectively, were used in the IP-one HTRF<sup>®</sup> assay kit. For each assay, the test compound was incubated with the cells ( $5 \times 10^5$ /mL) in stimulation buffer of IP<sub>1</sub> Tb kit and 5-HT (5-HT<sub>2A</sub>: 100 nM; 5-HT<sub>2B</sub>: 30 nM) for 30 minutes at 37°C. The assays were done in duplicate. The analysis of other receptors (eg, D<sub>1</sub>, D<sub>3</sub>, D<sub>5</sub>, 5-HT<sub>2C</sub>, 5-HT<sub>6</sub>, and 5-HT<sub>7</sub>) used similar assay methods. Measurements were performed at the excitation wavelength of 337 nm, and the emission wavelength of 620 nm (donor) and 665 nm (acceptor). The HTRF ratio was calculated by dividing the acceptor signal (665 nm) by the donor signal (620 nm), which normalizes the signal and corrects the results for effects due to the optical characteristics of the media and assay components.

## Results

### Radioligand binding and functional activity of SPN-810M on the dopamine and serotonin receptors

SPN-810M was tested for potential agonist or antagonist effect on neurotransmitter receptors, specifically toward the  $D_{2S}$ ,  $D_{2L}$ , and 5-HT<sub>2B</sub> receptors. The radioligand binding

assays demonstrated that SPN-810M inhibited binding in response to an agonist for the  $D_{2S}$ ,  $D_{2L}$ , and 5-HT<sub>2B</sub> receptors (Figure 1).

Using a concentration range of 3 nM–10  $\mu$ M, SPN-810M demonstrated an antagonist effect on  $D_{2S}$  in the impedance-based cellular functional assay performed on human recombinant HEK-293 cells. The concentration of SPN-810M at which percent inhibition of control agonist response is reduced by half ( $IC_{50}$ ) was identified as 0.14  $\mu$ M. Furthermore, in the [<sup>35</sup>S]GTP $\gamma$ S binding assay, SPN-810M at concentrations ranging from 1 nM to 10  $\mu$ M showed a similar  $IC_{50}$  of 0.0844  $\mu$ M in response to control agonist for  $D_{2S}$  (Figure 2A). Similarly, SPN-810M (1 nM–10  $\mu$ M) was found to act as an antagonist for the  $D_{2L}$  receptor with an  $IC_{50}$  of 0.11  $\mu$ M in the [<sup>35</sup>S]GTP $\gamma$ S binding assay (Figure 2B). At the SPN-810M concentrations tested (3 nM–10  $\mu$ M), the inhibitory effect on D<sub>1</sub>, D<sub>3</sub>, and D<sub>5</sub> receptors was not as potent as that noted for  $D_{2S}$  and  $D_{2L}$ , with  $IC_{50}$  values ranging from 3.2  $\mu$ M to 8.3  $\mu$ M. In addition, there was no significant inhibition of the alpha-adrenergic receptors.

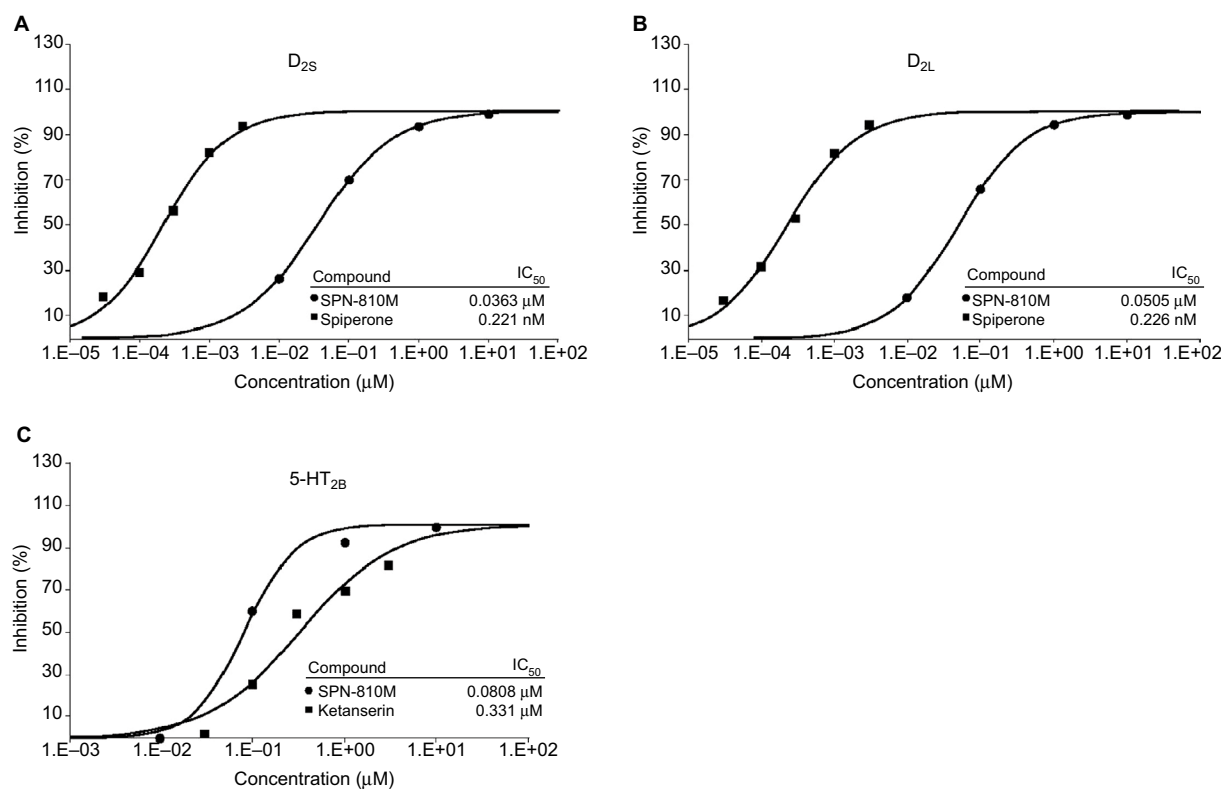
The phosphoinositide hydrolysis functional assay using concentrations of SPN-810M ranging from 3 nM to 10  $\mu$ M in human recombinant CHO cells identified SPN-810M as an antagonist for serotonin 5-HT<sub>2B</sub> with the  $IC_{50}$  as 0.41  $\mu$ M (Figure 2C). The functional assay also identified the  $IC_{50}$  of 5-HT<sub>2A</sub>, which was 14  $\mu$ M, demonstrating that SPN-810M was more potent in acting as an antagonist for 5-HT<sub>2B</sub> vs 5-HT<sub>2A</sub>.

### Human metabolites of SPN-810M

The major human metabolites of SPN-810M, P1-2 and P3-2, were evaluated for antagonistic effects on the dopamine and serotonin receptors. The binding assays identified no binding affinity for P1-2 on the tested receptors. Although the metabolite P3-2 had a slight antagonist effect on 5-HT<sub>2B</sub> at concentrations greater than 10  $\mu$ M, the phosphoinositide hydrolysis functional assay demonstrated an antagonist effect only at 100  $\mu$ M. Additional binding assays revealed that the SPN-810M metabolites P1-2 and P3-2 did not bind to other neurotransmitter receptors, such as norepinephrine, GABA, glutamate, histamine, or acetylcholine receptors.

### Potential effect of dopamine and norepinephrine on the in vitro pharmacology profile of SPN-810M

Additional assays were carried out to determine the effect of 0.1  $\mu$ M, 1  $\mu$ M, and 10  $\mu$ M dopamine or norepinephrine on SPN-810M antagonism of  $D_{2S}$ ,  $D_{2L}$ , and 5-HT<sub>2B</sub>.  $D_{2S}$



**Figure 1** Radioligand binding assays of SPN-810M for (A)  $D_{2S}$ , (B)  $D_{2L}$ , and (C)  $5\text{-HT}_{2B}$ . The  $IC_{50}$  for  $D_{2S}$ ,  $D_{2L}$ , and  $5\text{-HT}_{2B}$  was 0.0363  $\mu\text{M}$ , 0.0505  $\mu\text{M}$ , and 0.0808  $\mu\text{M}$ , respectively.

**Abbreviation:**  $IC_{50}$ , the concentration of the inhibitor at which the response (or binding) is reduced by half.

antagonistic activity of SPN-810M was unaffected by the presence of 0.1  $\mu\text{M}$  dopamine or norepinephrine (Table 1). The antagonist effect of SPN-810M on the  $D_{2L}$  receptor was also unaffected by the presence of norepinephrine at concentrations tested (0.1  $\mu\text{M}$ , 1  $\mu\text{M}$ , and 10  $\mu\text{M}$ ), with an  $IC_{50}$  ranging from 0.05  $\mu\text{M}$  to 0.11  $\mu\text{M}$  (Table 1). Likewise, the antagonist effect of SPN-810M on  $5\text{-HT}_{2B}$  was not affected in the presence of dopamine or norepinephrine ( $IC_{50}$  ranging from 0.19  $\mu\text{M}$  to 0.45  $\mu\text{M}$ ; Table 1). No agonist effect of SPN-810M on  $5\text{-HT}_{2A}$ ,  $5\text{-HT}_{2B}$ ,  $5\text{-HT}_{2C}$ ,  $5\text{-HT}_6$ ,  $5\text{-HT}_7$ ,  $D_1$ ,  $D_{2S}$ ,  $D_{2L}$ ,  $D_3$ , or  $D_5$  was observed in the presence of dopamine or norepinephrine.

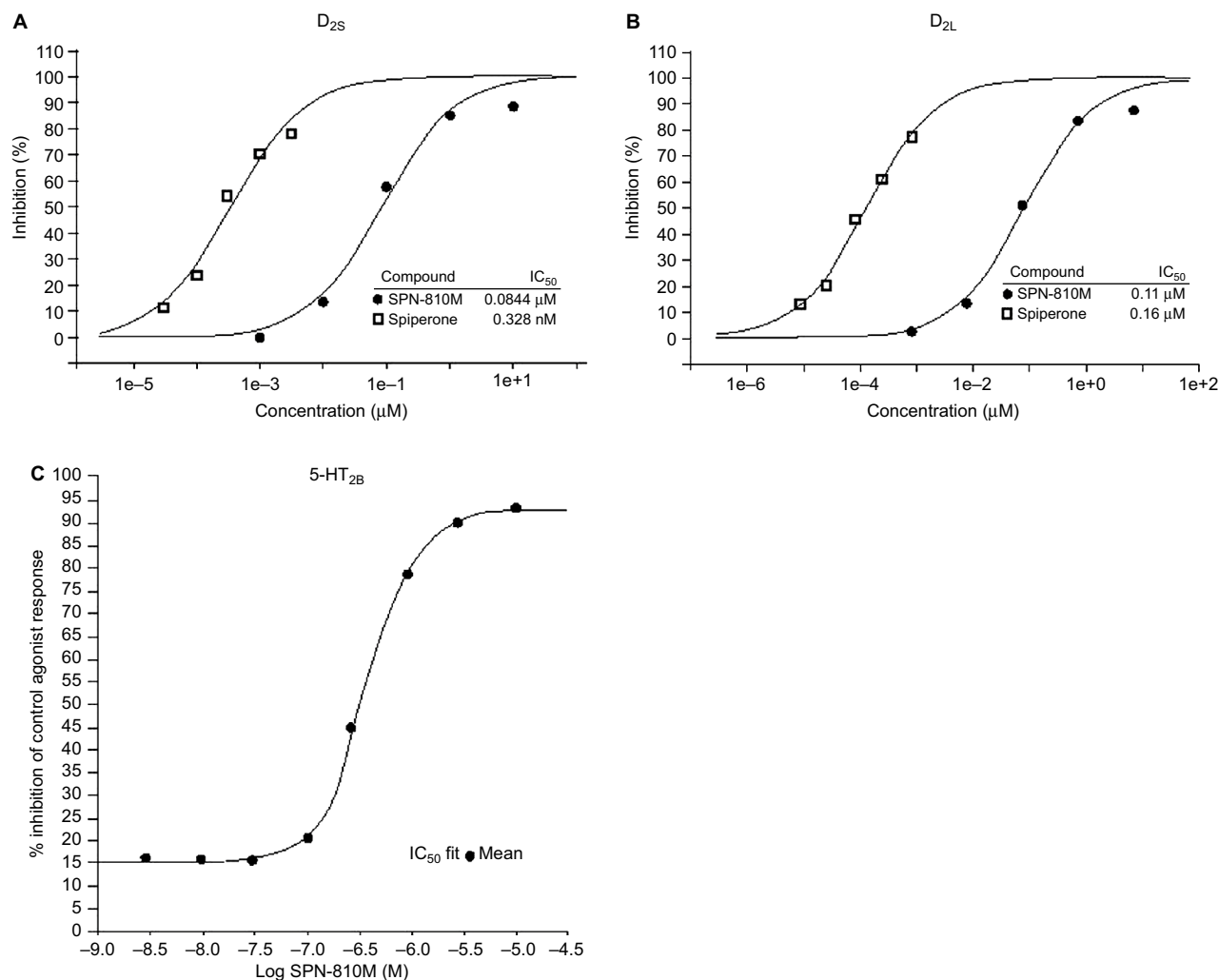
### SPN-810M enantiomers: R(−) enantiomer vs S(+) enantiomer

Results from the [ $^{35}\text{S}$ ]GTP $\gamma\text{S}$  binding assays demonstrate that antagonist potency of the R(−) enantiomer of SPN-810M is ~100-fold and 300-fold higher for  $D_{2S}$  and  $D_{2L}$ , respectively, than the S(+) enantiomer, based on the  $IC_{50}$  (Table 2). Further experiments demonstrate that the R(−) enantiomer also has a greater inhibitory effect than the S(+) enantiomer for the  $5\text{-HT}_{2B}$  receptor ( $IC_{50}$ =0.2  $\mu\text{M}$  vs 1.8  $\mu\text{M}$ , respectively; Table 2).

## Discussion

The results of the in vitro pharmacology studies demonstrated that at concentrations within the clinically relevant range for the intended therapeutic dose (36 mg/day) of SPN-810 (extended-release molindone), SPN-810M is a potent antagonist for the  $D_{2S}$  and  $D_{2L}$  dopamine receptors and the serotonin receptor  $5\text{-HT}_{2B}$ ; it is not as potent for the  $5\text{-HT}_{2A}$  receptor. The findings also indicate that R(−) is a more potent enantiomer of SPN-810M than S(+), showing greater effect on  $D_{2S}$  and  $D_{2L}$  receptors, and that the parent drug, SPN-810M, and not the major human metabolites (P1-2 and P3-2), has the antagonist effect on the target dopamine or serotonin receptors. In addition, the P1-2 and P3-2 metabolites did not demonstrate any inhibitory effect on several other neurotransmitter receptors. Finally, the data show that the presence of dopamine and norepinephrine neurotransmitters does not interfere with the antagonistic effect of SPN-810M on  $D_{2S}$ ,  $D_{2L}$ , and  $5\text{-HT}_{2B}$ , suggesting that known concomitant psychostimulants used to treat ADHD would not affect the pharmacological activity of SPN-810M in clinical settings.

SPN-810 (extended-release molindone) has been shown to improve IA as investigated in patients treated for ADHD.<sup>7,21,22</sup> The results of these in vitro pharmacology studies provided



**Figure 2** Effect of SPN-810M on (A) D<sub>2S</sub>, (B) D<sub>2L</sub>, and (C) 5-HT<sub>2B</sub>. IC<sub>50</sub> was determined using GTPγS assay for D<sub>2S</sub> and D<sub>2L</sub>, and HTRF for 5-HT<sub>2B</sub>. The IC<sub>50</sub> for D<sub>2S</sub>, D<sub>2L</sub>, and 5-HT<sub>2B</sub> was 0.0844 μM, 0.11 μM, and 0.41 μM, respectively.

**Abbreviations:** HTRF, homogeneous time-resolved fluorescence; IC<sub>50</sub>, the concentration of the inhibitor at which the response (or binding) is reduced by half.

**Table 1** IC<sub>50</sub> of SPN-810M on neurotransmitter receptors D<sub>2S</sub>, D<sub>2L</sub>, and 5-HT<sub>2B</sub> in the presence of neurotransmitters dopamine and norepinephrine<sup>a</sup>

| Receptor           | Norepinephrine |         |         | Dopamine |         |         |
|--------------------|----------------|---------|---------|----------|---------|---------|
|                    | 0.1 μM         | 1 μM    | 10 μM   | 0.1 μM   | 1 μM    | 10 μM   |
| D <sub>2S</sub>    | 0.034 μM       | ND      | ND      | ND       | ND      | ND      |
| D <sub>2L</sub>    | 0.11 μM        | 0.05 μM | 0.06 μM | ND       | ND      | ND      |
| 5-HT <sub>2B</sub> | 0.28 μM        | 0.26 μM | 0.19 μM | 0.45 μM  | 0.37 μM | 0.44 μM |

**Notes:** <sup>a</sup>IC<sub>50</sub> determined by impedance-based assay, [<sup>35</sup>S]GTPγS assay, and phosphoinositide hydrolysis assay for D<sub>2S</sub>, D<sub>2L</sub>, and 5-HT<sub>2B</sub>, respectively.

**Abbreviations:** IC<sub>50</sub>, the concentration of the inhibitor at which the response (or binding) is reduced by half; ND, not determined.

**Table 2** IC<sub>50</sub> of SPN-810M enantiomers for neurotransmitter receptors D<sub>2S</sub>, D<sub>2L</sub>, and 5-HT<sub>2B</sub>

| Receptor           | S(+) enantiomer | R(-) enantiomer |
|--------------------|-----------------|-----------------|
| D <sub>2S</sub>    | 4.85 μM         | 0.048 μM        |
| D <sub>2L</sub>    | 6.64 μM         | 0.021 μM        |
| 5-HT <sub>2B</sub> | 1.8 μM          | 0.2 μM          |

**Abbreviation:** IC<sub>50</sub>, the concentration of the inhibitor at which the response (or binding) is reduced by half.

insight regarding the potential mechanism, suggesting it does so mainly by modulating dopamine and also serotonin.

## SPN-810M: a potent antagonist for D<sub>2S</sub>, D<sub>2L</sub>, and 5-HT<sub>2B</sub>

Molindone has been found to be a potent antagonist for D<sub>2</sub> receptors.<sup>17</sup> Gene expression profiling has demonstrated that

molindone's effect on lipid metabolism is markedly weaker than that of the antipsychotics clozapine and risperidone.<sup>17,23</sup> Molindone does not act like antipsychotics such as haloperidol, as it has been shown to elicit little to no parkinsonism or extrapyramidal symptoms and has a low propensity for weight gain.<sup>24,25</sup> The results of the current radioligand binding assays and functional activity studies extend these findings and identify SPN-810M as an antagonist for  $D_{2S}$ ,  $D_{2L}$ , and  $5-HT_{2B}$ . SPN-810M also demonstrated an inhibitory effect on the  $D_1$ ,  $D_3$ ,  $D_5$ , and  $5-HT_{2A}$  receptors, but to a much lesser degree. The majority of atypical antipsychotics have a high affinity for  $5-HT_{2A}$ ,<sup>26</sup> although amisulpride is an example of a highly effective atypical antipsychotic that does not occupy any  $5-HT_{2A}$  receptors.<sup>27</sup> In any case, the lack of  $5-HT_{2A}$  potency suggests that SPN-810M does not act like known atypical antipsychotic drugs (with a high affinity for  $5-HT_{2A}$ ).<sup>26</sup> Overall, these in vitro results suggest that SPN-810M at clinically relevant doses is pharmacologically distinct from both typical and atypical antipsychotic drugs, with the exception of amisulpride.

Previous studies have shown that single enantiomers and their racemates can be equipotent in in vitro and in vivo pharmacological tests<sup>28</sup> or that each enantiomer can have a different potency, as seen with the dopamine  $D_1$ -like receptor partial agonist SKF 83959 enantiomer R(+) and the S(-) enantiomers, MCL 202 and MCL 201, respectively.<sup>29</sup> Results from the present study investigating the differential effects of the enantiomers of SPN-810M show that the racemic molecule and the R(-) enantiomer did not demonstrate significant differences in their in vitro action. However, the R(-) form showed ~100, 300, and eight times more potency as an antagonist for  $D_{2S}$ ,  $D_{2L}$ , and  $5-HT_{2B}$  receptors, respectively, than the S(+) enantiomer. The fact that the potency was similar for the R(-) enantiomer on  $D_{2S}$  and  $D_{2L}$  at 0.048  $\mu$ M and 0.021  $\mu$ M, respectively, suggests that the SPN-810M R(-) enantiomer could play a role in the  $D_2$  autoreceptor of the dopaminergic system. In particular, SPN-810M may affect both the presynaptic  $D_{2S}$  autoreceptors and the postsynaptic  $D_{2L}$  receptors.<sup>12,13</sup>

Active metabolites of certain drugs, such as triptans, can induce CNS side effects by binding to 5-HT or other receptors.<sup>30</sup> The major human metabolites (P1-2 and P3-2) of SPN-810M did not display antagonistic effects on the dopamine or serotonin receptors in vitro. Furthermore, the metabolites did not demonstrate an inhibitory effect on several other neurotransmitter receptors tested, such as norepinephrine, GABA, glutamate, histamine, or acetylcholine receptors. This finding is in agreement with a study conducted by Balsara et al, which concluded that a molindone metabolite does not

exert neuroleptic activity.<sup>31</sup> The lack of an effect due to the SPN-810M metabolites suggests that SPN-810M is not likely to have metabolite-related CNS side effects, improving the tolerability in a clinical setting.

## The effect of psychostimulants on SPN-810M antagonism of $D_{2S}$ , $D_{2L}$ , and $5-HT_{2B}$

Psychostimulants are considered first-line pharmacotherapy for the treatment of ADHD and refractory aggression, and they usually improve ADHD symptoms as well as aggressive behaviors by increasing synaptic dopamine levels in the brain.<sup>32-35</sup> Other pharmacotherapies for ADHD include norepinephrine reuptake inhibitors.<sup>4</sup> However, IA symptoms that are present prior to treatment persist in ~44% of children with ADHD, despite ADHD therapy.<sup>1,36</sup> Studies suggest that IA may respond to adjunctive treatments administered with optimized first-line pharmacotherapy for ADHD.<sup>1,37</sup> As such, the additional assays described above were carried out to determine the effect of dopamine or norepinephrine on  $D_{2S}$ ,  $D_{2L}$ , and  $5-HT_{2B}$  in the presence of SPN-810M. Specifically, we wanted to evaluate whether these neurotransmitters 1) increased or decreased the antagonistic effect of SPN-810M, and/or 2) caused a reversal in function of SPN-810M from an antagonist to an agonist. The results from the in vitro pharmacology studies demonstrate that the antagonist effects of SPN-810M on the  $D_{2S}$ ,  $D_{2L}$ , and  $5-HT_{2B}$  receptors were not altered by the presence of dopamine or norepinephrine, as very little change in potency was observed. Furthermore, the presence of dopamine or norepinephrine did not lead to SPN-810M reversing from an antagonistic to an agonistic effect.

## Theorized mechanism of action

Our in vitro assays provide some insight into the potential mechanisms of SPN-810M. The results of the in vitro pharmacology studies indicate that SPN-810M is a potent antagonist for  $D_{2S}$ ,  $D_{2L}$ , and  $5-HT_{2B}$ . In children (aged 6–12 years) diagnosed with ADHD and presenting with IA, SPN-810 (extended-release molindone) use at doses up to 36 mg/day resulted in significant improvements in aggressive behavior based on the Retrospective-Modified Overt Aggression Scale (R-MOAS).<sup>21,22</sup> The exact mechanism of action by which SPN-810 improved aggressive behavior is not completely understood. We hypothesize that by acting as a  $5-HT_{2B}$  receptor antagonist, SPN-810M can influence the mesoaccumbens dopamine pathway by reducing the outflow of dopamine.<sup>38</sup> Although we found that SPN-810M is an antagonist for  $5-HT_{2B}$ , it was a more potent antagonist for the  $D_{2S}$  and  $D_{2L}$  receptors, suggesting that SPN-810M

may affect the presynaptic D<sub>2S</sub> autoreceptors to adequately regulate the synthesis and release of dopamine into the synaptic cleft and block the postsynaptic D<sub>2L</sub> receptors to reduce phasic DA signaling in regions of the brain that are likely to be associated with aggression.<sup>12,13,39</sup> At therapeutic doses, antagonistic effects of SPN-810M for D<sub>2S</sub> and D<sub>2L</sub>, as well as for 5-HT<sub>2B</sub>, may work in concert to synergistically contribute to the clinical effects. Indeed, further in vivo neuropharmacological studies including fMRI studies may enable better understanding of the mechanism of action of this drug, with the goal of improving care for patients with IA.

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## Disclosure

CY and GG are employees of Supernus Pharmaceuticals, Inc. The authors report no other conflicts of interest in this work.

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## Supplementary material

**Table S1** Source and reference information for all utilized cell lines

| Assay                    | Eurofins assay reference number | Cell line | Provider                   |
|--------------------------|---------------------------------|-----------|----------------------------|
| <b>D<sub>2S</sub></b>    | 1322                            | HEK-293   | University of Oregon (USA) |
|                          | 46                              | HEK-293   | University of Oregon (USA) |
| <b>D<sub>2L</sub></b>    | 1405                            | HEK-293   | University of Oregon (USA) |
| <b>5-HT<sub>2B</sub></b> | 1333                            | CHO-K1    | ACS                        |
|                          | 1609                            | CHO-K1    | ACS                        |
|                          | G183                            | CHO-K1    | ACS                        |
| <b>5-HT<sub>2A</sub></b> | G181                            | HEK-293   | CEREP                      |
| <b>D<sub>1</sub></b>     | G014                            | CHO       | Inserm                     |
| <b>D<sub>3</sub></b>     | G016                            | CHO       | Inserm                     |
| <b>D<sub>5</sub></b>     | G018                            | GH4CI     | University of Oregon (USA) |
| <b>5-HT<sub>2C</sub></b> | G184                            | 293h      | CEREP                      |
| <b>5-HT<sub>6</sub></b>  | G050                            | CHO-s     | CEREP                      |
| <b>5-HT<sub>7</sub></b>  | G051                            | CHO-s     | CEREP                      |

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