Posiphen as a candidate drug to lower CSF amyloid precursor protein, amyloid-β peptide and τ levels: target engagement, tolerability and pharmacokinetics in humans

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ABSTRACT
Aim A first in human study to evaluate tolerability and pharmacokinetics followed by an early proof of mechanism (POM) study to determine whether the small orally available molecule, Posiphen tartrate (Posiphen), lowers secreted (s) amyloid-β precursor protein (APP) α and -β, amyloid-β peptide (Aβ), tau (τ) and inflammatory markers in CSF of patients with mild cognitive impairment (MCI).

Study design Posiphen single and multiple ascending dose phase 1 randomised, double blind, placebo-controlled safety, tolerance, pharmacokinetic studies were undertaken in a total of 120 healthy volunteers to define a dose that was then used in a small non-randomised study of five MCI subjects, used as their own controls, to define target engagement.

Main outcome measures Pharmacodynamic: sAPPα, sAPPβ, Aβ42, τ (total (t) and phosphorylated (p)) and inflammatory marker levels were time-dependently measured over 12 h and compared prior to and following 10 days of oral Posiphen treatment in four MCI subjects who completed the study. Pharmacokinetic: plasma and CSF drug and primary metabolite concentrations with estimated brain levels extrapolated from steady-state drug administration in rats.

Results Posiphen proved well tolerated and significantly lowered CSF levels of sAPPα, sAPPβ, t-τ, p-τ and specific inflammatory markers, and demonstrated a trend to lower CSF Aβ42.

Conclusions These results confirm preclinical POM studies, demonstrate that pharmacologically relevant drug/metabolite levels reach brain and support the continued clinical optimisation and evaluation of Posiphen for MCI and Alzheimer’s disease.

INTRODUCTION
The treatment of Alzheimer’s disease (AD), the most common dementing disorder of the elderly, remains an unmet medical need.1 Its hallmarks are neurodegeneration, brain atrophy and abnormal protein depositions, particularly of amyloid plaques and neurofibrillary tangles deriving from amyloid-β peptide (Aβ) and hyperphosphorylated τ, respectively,2–4 resulting in progressive cognitive decline. Current approved AD drugs provide symptomatic relief and temporarily delay loss of cognition, but do not halt or modify disease progression.5

The key AD drug target, Aβ, is a proteolytic product of amyloid-β precursor protein (APP) cleavage: an integral transmembrane protein concentrated at the synapse of neurons.4 APP is cleaved by β- and γ-secretases to generate Aβ2,6 which assemble into oligomers that cause inflammation and target synapses to induce cellular dysfunction and impair memory.7–9 Aβ is additionally cleaved into a number of other bioactive N- and C-terminal fragments, including N-APP10 and C-S11–12 (figure 1). These fragments, likewise, may contribute to AD pathogenesis, making Aβ an interesting AD drug target to regulate. Posiphen tartrate (Posiphen (figure 2), also known as (+)-phenserine), an APP synthesis inhibitor,13 interacts via the 5′-untranslated region of APP mRNA14 to inhibit ribosomal access and block APP translation.14–16 Posiphen is the chirally pure positive enantiomer of (−)-phenserine (phenserine).17 However, whereas phenserine is an acetylcholinesterase inhibitor, Posiphen lacks acetylcholinesterase activity; instead, it inhibits the translation of APP. In neuronal cultures and brain of wild type and AD transgenic mice, Posiphen lowered APP and Aβ levels15–18 in a dose-dependent manner and hence represents an interesting candidate drug to reduce APP toxic products in humans.

We describe, herein, three phase 1 studies conducted under an active investigational new drug application. Initially, Posiphen’s safety was assessed in healthy volunteers in a single ascending dose study and then a multiple ascending dose study (SAD and MAD, respectively). Thereafter, using a well-tolerated dose from the former investigations, an early proof of mechanism (POM) study was conducted in patients with mild cognitive impairment (MCI), wherein time-dependent plasma and cerebrospinal fluid (CSF) samples were obtained prior to and following 10 days of Posiphen administration to permit analysis of drug-induced changes in CSF levels of secreted (s) APPα and APPβ, Aβ42, τ (total (t) and phosphorylated (p)) and inflammatory markers. Additionally, human brain levels of Posiphen and metabolites were estimated from measured plasma and CSF samples of MCI patients in light of steady-state Posiphen plasma–brain–CSF pharmacokinetic studies performed in rats.
DESIGN AND METHODS

Drug substance

Posiphen, (SaK)-1, 3a, 8-trimethyl-1, 2, 3, 3a, 8, 8a-hexahydropyrrolo (2, 3-b) indol-5-yl phenyl-carbamate tartrate (investigational new drug #72 654) was manufactured to good manufacturing process requirements by Rhodia (Boulogne-Billancourt, France) (See online Supplemental information SI-1).

Standards

Posiphen and its metabolites, N1- and N8-norposiphen and N1, N8-bisnorposiphen, were synthesised (National Institute on Aging, Baltimore, Maryland, USA) to >99.5% purity.19 20

Animal studies

To aid extrapolation of drug and metabolite concentrations in human brain from those measured in human plasma and CSF in the POM MCI study, Posiphen (75 mg/kg/day continuous infusion) was administered to (male adult Fischer-44) rats under steady-state conditions and samples were simultaneously collected and drug/metabolite concentrations quantified in each compartment (See online Supplemental information SI-2).

Clinical studies

Phase I SAD

A randomised, double blind, placebo-controlled safety, tolerance and pharmacokinetic study (first in human) was performed with six groups of male and female healthy volunteers receiving serially increasing single doses of Posiphen or placebo, followed by monitoring of safety (vital signs, ECGs, clinical laboratory tests, capture of adverse events) and collection of blood and urine samples at regular intervals up to 24 h for preliminary pharmacokinetic analyses. The escalating doses to be studied were 10, 20, 40, 80, 160 and 240 mg (and placebo). Due to the limiting side effects (nausea and vomiting), the highest dose studied was 160 mg of Posiphen; the 240 mg dose was not administered (See online Supplemental information SI-3).

The study was conducted by the PRACS Institute (East Grand Forks, Minnesota, USA) and was fully approved by their Institutional Review Board.

Phase I MAD

A randomised, double blind, placebo-controlled safety, tolerance, pharmacokinetic study was performed with six of eight male and six of eight female healthy volunteers in each of three successive groups being administered one of three serially increasing, multiple dose regimens of Posiphen and two male and two female subjects in each treatment group receiving placebo. Safety (vital signs, ECGs, clinical laboratory tests, capture of adverse events) was monitored throughout the study and blood and urine samples were collected. The escalating regimens were 20, 40 and 60 mg, and placebo four times a day for 7 or 10 days. Plasma obtained from blood samples was analysed as described above (See online Supplemental information SI-4).

For inclusion/exclusion criteria see online Supplemental information SI-5.

The study was likewise conducted by the PRACS Institute and was fully approved by their Institutional Review Board.

Phase I early POM with pharmacokinetics and pharmacodynamics (ClinicalTrials.gov Identifier: NCT01072812)

An open-label study was performed in which five healthy male and female MCI patients (three male and two female patients) received Posiphen at 4×60 mg/day (total 240 mg/day) for 10 days. This dosing regimen was well tolerated in the earlier MAD study. To avoid potential inter-subject variability, subjects were used as their own controls. Specifically, serial plasma and
lumbar CSF samples were collected via an indwelling catheter over 12 h (at 0, 1, 1.5, 2, 3, 4, 6, 8 and 12 h) initiated at the same time of day 1 day prior to the start of dosing to obtain time-dependent baseline control data, and then at the exact same times immediately after the last dose was administered.\(^{21, 22}\) This paradigm was chosen to control for potential circadian alterations in pharmacodynamics markers within each subject. Samples were frozen and then stored at \(-80\)°C. CSF and plasma samples were matched and were analysed for (i) pharmacokinetics of Posiphen and metabolites (N1-norpiphen, N8-norpiphen and N1, N8-bisnorpsiphen) (figure 2) and (ii) pharmacodynamic studies involving measurement of the following proteins: \(s\text{APP}\), \(s\text{APP}^{\beta}\), \(A\beta_{42}\), t-\(\tau\), p-\(\tau\), complement 3, factor H, monocyte chemotactic protein-1 (MCP-1), the inflammation marker and chitinase-like protein YKL-40, and soluble cluster of differentiation 14 (sCD14). One MCI subject withdrew from the POM study on day 1 (table 1 legend); hence analyses were undertaken on four subjects in relation to pharmacokinetic and pharmacodynamics measures.

Subjects were male or postmenopausal females, between 55 and 80 years of age, with self-reported memory complaints that were corroborated by spouse or companion or caregiver as appropriate, and memory difficulties as measured on neuropsychological tests. MCI was determined according to Petersen’s criteria\(^{23}\) with a Mini Mental Status Examination score ≥24, cut-off score on the logical memory II delayed paragraph recall subtest of the Wechsler Memory Scale Revised, Clinical Dementia Rating of 0.5 with a memory box score of 0.5 or 1.0. Inclusion/exclusion criteria were in accordance with those described in the primary compound in each compartment, with the N1- and p-\(\text{APP}\) levels, providing high brain to plasma ratios (Posiphen: 6.8, N1-norpiphen: 3.8, N8-norpiphen: 5.8 and N1, N8-bisnorpsiphen: 1.5) with CSF levels reaching only approximately 1% of brain levels.

### RESULTS

#### Rat pharmacokinetics of Posiphen/metabolites

The comparative plasma, brain and CSF levels of Posiphen and three primary metabolites in rats following steady-state Posiphen infusion are shown in table 2 and figure 2A. Posiphen was the primary compound in each compartment, with the N1- and N8-metabolites reaching 39.1% and 25.8% of Posiphen levels in plasma, respectively, and N1, N8-bisnorpsiphen 20.5%. In accordance with their high lipophilicity (ClogP value, table 2), substantial brain entry of Posiphen and metabolites was evident whereas aqueous CSF levels were low. Specifically, steady-state brain concentrations were greater than concomitant plasma levels, providing high brain to plasma ratios (Posiphen: 6.8, N1-norpiphen: 3.8, N8-norpiphen: 5.8 and N1, N8-bisnorpsiphen: 1.5) with CSF levels reaching only approximately 1% of brain levels.

#### SAD study (healthy volunteers)

### Safety

Posiphen was well tolerated by healthy male and female volunteers at single doses from 10 to 80 mg. A 160 mg dose was associated with an increased incidence of nausea and vomiting (four subjects were nauseous and three vomited). Adverse events were either mild or moderate; none were severe. No higher doses were administered. Posiphen 80 mg was determined as the no observed adverse effect level (table 1).

### Pharmacokinetics

Posiphen, at all doses, was absorbed rapidly (mean \(T_{\text{max}}\): 1.3–1.6 h) and cleared from the circulation biphasecally (terminal half life: 3.7–4.5 h), independent of dose. Posiphen...
Systemic availability increased more than linearly with increasing dose, resulting in a disproportionately large increase in Cmax and AUC<sub>0-1</sub>. Mean Cmax ranged from 1.29 to 288 ng/ml (male subjects) and 6.22 to 480 ng/ml (female subjects) as the dose increased from 10 to 160 mg. Comparable increases were determined for mean AUC<sub>0-1</sub>, 1.63–998 and 12.1–1530 ng·h/ml, respectively, over the same dose range (data not shown).

### MAD study (healthy volunteers)

#### Safety

Posiphen doses up to 4×60 mg daily ×10 days were well tolerated. This 4×60 mg dose produced a small but statistically insignificant difference from placebo regarding gastrointestinal side effects and dizziness, and hence 4×60 mg four times a day was determined the no observed adverse effect level (table 1).

#### Table 1  Summary of adverse events (AEs). Treatment-related AEs that occurred in more than one subject in the Posiphen or placebo groups or in the entire cohort are summarised by dose. In all three (AX-PO-101, AX-PO-102, QR-12001) studies, males and female subjects were combined, as there was no apparent difference between the sexes regarding their tolerance to Posiphen. Of note, markers of hepatic and renal function were additionally analysed and were unaltered by drug.

<table>
<thead>
<tr>
<th>AE in healthy male and female volunteers</th>
<th>10 mg (n=10)</th>
<th>20 mg (n=20)</th>
<th>40 mg (n=10)</th>
<th>80 mg (n=10)</th>
<th>160 mg (n=10)</th>
<th>Placebo (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All AEs, moderate</td>
<td>0 (0)</td>
<td>2 (10.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (40.0)</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>All AEs, severe</td>
<td>0 (0)</td>
<td>1 (5.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>Gastrointestinal symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>0 (0)</td>
<td>2 (10.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>4 (40.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (30.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nervous system symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dizziness</td>
<td>0 (0)</td>
<td>3 (15.0)</td>
<td>1 (10.0)</td>
<td>3 (30.0)</td>
<td>4 (40.0)</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td>Fainting</td>
<td>0 (0)</td>
<td>1 (5.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>General symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feeling hot</td>
<td>0 (0)</td>
<td>2 (10.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Heart rate increased</td>
<td>2 (20.0)</td>
<td>1 (5.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td>Orthostatic hypotension</td>
<td>0 (0)</td>
<td>1 (5.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (8.3)</td>
</tr>
</tbody>
</table>

#### Table 1  Summary of adverse events (AEs). Treatment-related AEs that occurred in more than one subject in the Posiphen or placebo groups or in the entire cohort are summarised by dose. In all three (AX-PO-101, AX-PO-102, QR-12001) studies, male and female subjects were combined, as there was no apparent difference between the sexes regarding their tolerance to Posiphen. Of note, markers of hepatic and renal function were additionally analysed and were unaltered by drug.

<table>
<thead>
<tr>
<th>AE in healthy male and female volunteers</th>
<th>4×20 mg (n=12)</th>
<th>4×40 mg (n=12)</th>
<th>4×60 mg (n=12)</th>
<th>Placebo (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All AEs, moderate</td>
<td>2 (16.7)</td>
<td>0 (0)</td>
<td>1 (8.3)</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td>All AEs, severe</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Gastrointestinal symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>1 (8.3)</td>
<td>0 (0)</td>
<td>2 (16.7)</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>2 (16.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nervous system symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dizziness</td>
<td>2 (16.7)</td>
<td>2 (16.7)</td>
<td>3 (25.0)</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>General symptoms</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>1 (8.3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Headache</td>
<td>2 (16.7)</td>
<td>3 (25.0)</td>
<td>1 (8.3)</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td>Other</td>
<td>5 (41.2)</td>
<td>1 (8.3)</td>
<td>1 (8.3)</td>
<td>6 (50.0)</td>
</tr>
</tbody>
</table>

#### AEs in MCI patients

<table>
<thead>
<tr>
<th>AE</th>
<th>4×60 mg (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All AEs, moderate</td>
<td>0 (0) 1*</td>
</tr>
<tr>
<td>All AEs, severe</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Gastrointestinal symptoms</td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>1 (20) 1*</td>
</tr>
<tr>
<td>Vomiting</td>
<td>0 (0) 1*</td>
</tr>
<tr>
<td>Nervous system symptoms</td>
<td></td>
</tr>
<tr>
<td>Dizziness</td>
<td>1 (20.0)</td>
</tr>
<tr>
<td>General disorders</td>
<td></td>
</tr>
<tr>
<td>General pain</td>
<td>1 (20.0)</td>
</tr>
<tr>
<td>Headache</td>
<td>5 (100.0)</td>
</tr>
<tr>
<td>Other</td>
<td>5 (100.0)</td>
</tr>
</tbody>
</table>

The AEs, dizziness/fainting and orthostatic hypotension, were the most frequently observed safety observations during the course of this study. Orthostatic hypotension was observed in Posiphen treated people, but occurred with comparable frequency in placebo subjects. Some subjects experienced orthostatic effects at multiple measurement points postdose, but there was no apparent correlation between time of occurrence and plasma concentration of Posiphen. Dizziness, nausea and vomiting increased with Posiphen doses and at 160 mg there was a statistically significant effect of Posiphen on these two measures. In the multiple ascending dose studies there was a trend, but no statistically significant effect, in the 4×60 mg/day group.

*One person had leg cramps and was nauseous during the catheterisation (predrug). This subject vomited and dropped out after the second and before the third 60 mg dose on day 1. MCI, mild cognitive impairment.

The systemic availability increased more than linearly with increasing dose, resulting in a disproportionately large increase in Cmax and AUC<sub>0-</sub>1. Mean Cmax ranged from 1.29 to 288 ng/ml (male subjects) and 6.22 to 480 ng/ml (female subjects) as the dose increased from 10 to 160 mg. Comparable increases were determined for mean AUC<sub>0-</sub>1, 1.63–998 and 12.1–1530 ng·h/ml, respectively, over the same dose range (data not shown).
Table 2: Mean pharmacokinetic parameters for Posiphen and primary metabolites in rat and MCI patients

<table>
<thead>
<tr>
<th>Posiphen and metabolites</th>
<th>Parameters</th>
<th>Human plasma (ng/ml) ± SD</th>
<th>Human CSF (ng/ml) ± SD</th>
<th>Rat plasma (ng/ml) ± SD</th>
<th>Rat brain (ng/g) ± SD</th>
<th>Rat CSF (ng/ml) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posiphen</td>
<td>Cmax</td>
<td>118.5 ± 24.8</td>
<td>1.6 ± 0.6</td>
<td>144 ± 69.5</td>
<td>979 ± 429</td>
<td>9.5 ± 4.6</td>
</tr>
<tr>
<td>*ClogP=2.22</td>
<td>AUC0–inf</td>
<td>570 ± 235.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1-Norposiphen</td>
<td>Cmax</td>
<td>25.6 ± 6.7</td>
<td>1.7 ± 0.7</td>
<td>56.3 ± 8.7</td>
<td>213 ± 27.9</td>
<td>2.8 ± 1.1</td>
</tr>
<tr>
<td>ClogP=1.25</td>
<td>AUC0–inf</td>
<td>214.4 ± 77.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N8-Norposiphen</td>
<td>Cmax</td>
<td>31 ± 7.1</td>
<td>3.2 ± 1.2</td>
<td>37.2 ± 9.1</td>
<td>216 ± 28.6</td>
<td>2.8 ± 5.8</td>
</tr>
<tr>
<td>ClogP=1.00</td>
<td>AUC0–inf</td>
<td>261.3 ± 91.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1, N8-bisnorposiphen</td>
<td>Cmax</td>
<td>3.8 ± 1.2</td>
<td>Not detected</td>
<td>29.5 ± 10.8</td>
<td>37.2 ± 10.5</td>
<td>Not detected</td>
</tr>
<tr>
<td>ClogP=0.53</td>
<td>AUC0–inf</td>
<td>36.9 ± 12.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A comparison is shown of plasma, brain and CSF levels of Posiphen and metabolites undertaken under steady-state conditions, which was achieved by continuous administration of Posiphen 75 mg/kg/day for 10 days by osmotic mini-pump. In rat plasma (ng/ml) and brain (ng/g wet weight), Posiphen proved to be the primary drug compound, with the two major metabolites, N1- and N8-norposiphen, comprising up to 39.1% of Posiphen in plasma and up to 22% of Posiphen in brain at the Cmax. The third metabolite, N1, N8-bisnorposiphen, reached 20.4% of Posiphen in plasma and 3.8% of Posiphen in brain.

Mean Cmax plasma concentrations of Posiphen and metabolites are shown in Table 2 and figure 2B, and the time-dependent profiles are provided in figure 3. Similar to rat, Posiphen was the primary drug species, with the N1- and N8-metabolites initially accounting for 21.6% and 26.2% of Posiphen’s Cmax. Total time-dependent levels (AUC0–inf, table 2) were approximately 30% of Posiphen. The majority of these biomarkers were analysed by two different techniques within two independent institutions to cross-validate the data. In all cases, the direction

Pharmacokinetics

Posiphen, at all doses, was absorbed rapidly (Tmax = 1.2 to 1.7 h) and cleared from the circulation biphasically (terminal half-life of 4.5–4.7 h). As with the SAD study, the systemic availability of Posiphen increased more than linearly with increasing dose, resulting in disproportionately large increases in Cmax and AUC values.

Safety

Posiphen (4×60 mg daily ×10 days) in MCI subjects showed a similar safety profile as found in the healthy volunteers (table 1).

Pharmacokinetics

Mean Cmax plasma concentrations of Posiphen and metabolites are shown in Table 2 and figure 2B, and the time-dependent profiles are provided in figure 3. Similar to rat, Posiphen was the primary drug species, with the N1- and N8-metabolites initially accounting for 21.6% and 26.2% of Posiphen’s Cmax. Total time-dependent levels (AUC0–inf, table 2) were approximately 30% of Posiphen. Contrasting with rat studies, N1, N8-bisnorposiphen reached 20.4% of Posiphen in plasma and 3.8% of Posiphen in brain.

The ClogP value is an established measure of a compound’s lipid versus water solubility, with a positive value associated with a preference for the lipid phase.

Pharmacodynamics

Drug-induced differences in CSF biomarkers for MCI patients are shown in Table 3A, determined by comparing the predrug and postdrug biomarker levels at each time point within the same subject to control for both circadian changes and inter-subject variability. The majority of the biomarkers were analysed by two different techniques within two independent institutions to cross-validate the data. In all cases, the direction

*POM study (MCI patients)

**Rat**: A comparison is shown of plasma, brain and CSF levels of Posiphen and metabolites undertaken under steady-state conditions, which was achieved by continuous administration of Posiphen 75 mg/kg/day for 10 days by osmotic mini-pump. In rat plasma (ng/ml) and brain (ng/g wet weight), Posiphen proved to be the primary drug compound, with the two major metabolites, N1- and N8-norposiphen, comprising up to 39.1% of Posiphen in plasma and up to 22% of Posiphen in brain at the Cmax. The third metabolite, N1, N8-bisnorposiphen, reached 20.4% of Posiphen in plasma and 3.8% of Posiphen in brain.

*Human*: The pharmacokinetic parameters of Posiphen and metabolites are shown in plasma and CSF of MCI patients after 10 days of 4×60 mg/day repeat dose oral administration, male and female subjects combined. As expected from the rodent data, the two primary metabolites N1- and N8-norposiphen constitute approximately 20% of Posiphen at the Cmax, with the third metabolite N1, N8-bisnorposiphen being a minor component and reaching only 3% of Posiphen Cmax.

The time-dependent pharmacokinetic profiles of Posiphen and metabolites are provided in figure 3.

Calculated pharmacokinetic parameters for Posiphen in plasma of MCI patients were similar to those in healthy volunteers (SAD and MAD). Posiphen mean Tmax was 1.3–1.6 h, mean terminal half-life 4.0–5.5 h, apparent volume of distribution: 2171±539 l and total body clearance 310±72 l/h. The four times a day regimen resulted in some accumulation of Posiphen in plasma and accumulation of Posiphen/metabolites in CSF (figure 3).

Illustrated in figure 3 are Posiphen and metabolite plasma, CSF and estimated brain levels for MCI patients following 10 day Posiphen dosing. Extrapolated brain levels for MCI patients were determined by applying the rat plasma/CSF brain data (figure 2A) to the human plasma and CSF data (figure 2B). This estimate predicts Posiphen brain levels approaching 1 ug/g or approximately 3.5 μM for a dose of 4×60 mg/day.
of change was the same. Specifically, Posiphen lowered sAPP\(\alpha\) and sAPP\(\beta\) levels by \(-59.9\%\) and \(-57.7\%\), respectively, assessed by the AlphaLisa assay, and by \(-34.1\%\) and \(-34.0\%\), respectively, assessed by the MSD assay, in accordance with Posiphen’s proposed mechanism of action to inhibit APP expression.\(^{13} 14 18\)

In line with this, \(A\beta_{42}\) demonstrated a trend to reduction (\(-45.2\%\), as assessed by the AlphaLisa assay) and \(-51.4\%\), as assessed by the Innogenetics assay). In addition, Posiphen significantly reduced levels of \(\tau\) (\(-74.1\%\), as assessed by the Innogenetics assay) and \(-46.2\%\), as assessed by the AlphaLisa assay) and \(p\)-\(\tau\) (\(-61\%\), as assessed by the Innogenetics assay).

Posiphen’s actions on CSF inflammation markers are shown in table 3B. A significant lowering of pro-inflammatory, C3 (\(-86.9\%\)) and microglial activation markers, MCP-1 (\(-87.5\%\)) and YKL-40 (\(-72.7\%\)), was evident. By contrast, sCD14, associated with early innate immune response to bacterial and viral infection,\(^{26}\) and the complement control protein, factor H, were unaffected by Posiphen (\(-26.1\%\) and \(+25.7\%\), respectively).

Analysis of CSF samples obtained from four healthy Posiphen naive volunteers under conditions similar to those for the MCI subjects permitted comparison between untreated and treated MCI patients and healthy untreated volunteers. Figure 4 demonstrates that Posiphen administration to MCI patients lowers sAPP\(\alpha\), sAPP\(\beta\) and \(\tau\)-\(\tau\) to levels present in healthy volunteers.

**DISCUSSION**

Posiphen was shown in 72 healthy volunteers to be safe in SAD and 55 subjects in MAD and POM studies of 7–10 day administration up to levels determined as greater than fivefold the effective dose. This effective dose was determined both by comparing the extrapolated molar concentration in brain with the 50% effective concentration to inhibit APP in neuronal cultures, as well as from animal studies.\(^{13}\) The human pharmacokinetics of Posiphen/metabolites and extrapolation of data determined from rodents suggests that Posiphen readily enters the brain and achieves levels 6.8-fold higher than in plasma at steady-state, in accordance with its high lipophilicity (ClogP value 2.2). Its hydrophobicity and protein binding capacity (96% of Posiphen/metabolites bind to brain proteins) likely limit levels of Posiphen/metabolites found in CSF of both rodents and MCI patients. Interestingly, Posiphen half life in CSF of MCI patients proved to be longer than its half life in plasma, >12 h versus approximately 5 h, respectively (figure 3). Consistent with its longer half life within the central nervous system, Posiphen’s \(A\beta\), \(\tau\) and inflammation lowering activity lasted longer than the recorded 12 h of sampling. Recent studies in neuronal cultures indicate that Posiphen’s APP lowering actions extend for numerous hours following wash-off and, additionally, are likewise maintained within the brain of transgenic AD mice for over 9 h after cessation of dosing (Sambamurti K, Medical University of S. Carolina, personal communication). The extended duration of Posiphen/metabolites in CSF/brain together with the prolonged inhibition of APP and \(\tau\) expression may permit once a day dosing and is a focus of current studies.

Our early POM study in MCI patients focused on evaluating target engagement demonstrates that Posiphen lowers both sAPP\(\alpha\) and sAPP\(\beta\) in CSF consistent with its preclinical actions\(^{13} 18 27\) and ability to inhibit the translation of APP mRNA via an iron response element within its 5’-untranslated region.\(^{14} 16 28\) The trend of a Posiphen-induced reduction in CSF \(A\beta_{42}\) in MCI subjects is likewise in line with its known action to inhibit APP synthesis, as \(A\beta_{42}\) is a downstream product and is in accordance with the described decline in CSF sAPP\(\beta\) in MCI subjects as well as of \(A\beta_{42}\) levels in preclinical studies.\(^{15–18} 27 28\)

A separate more limited analysis of \(A\beta_{40}\) (AlphaLisa) in CSF collected at 3 and 8 h prior to and following Posiphen administration in MCI subjects provided reduction trends of \(-52\%\) and \(-37\%\), respectively. A caveat of early CNS target engagement investigational studies is the small patient number required to, on one hand, adequately demonstrate pharmacologically driven biological activity in the brain as a result of drug interaction with its intended target to provide proof-of-concept and, on the other hand, protect patients from exposure to potentially inactive or toxic drugs.\(^{29}\) In our Posiphen study, this was undertaken on five MCI subjects, one of whom withdrew, allowing biomarker analyses on four. However, as patients were used as their own controls at 0 day (prior to Posiphen treatment) and as

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**Table 3** (A) AD biomarkers and (B) inflammatory biomarkers in CSF of MCI subjects after 10 days of Posiphen treatment

<table>
<thead>
<tr>
<th>Human biomarker</th>
<th>CSF % of time 0</th>
<th>SE</th>
<th>(p) Value</th>
<th>Assay</th>
<th>Laboratory</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) AD biomarkers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sAPP(\alpha)</td>
<td>-59.9%</td>
<td>0.231</td>
<td>0.0006</td>
<td>AlphaLisa</td>
<td>V. John/Buck Institute</td>
</tr>
<tr>
<td>sAPP(\beta)</td>
<td>-34.1%</td>
<td>0.659</td>
<td>0.0661</td>
<td>MSD</td>
<td>MY Chan/QF Pharma</td>
</tr>
<tr>
<td>MCP-1</td>
<td>-87.5%</td>
<td>4.813</td>
<td>0.0007</td>
<td>R&amp;D Systems</td>
<td>H. Zetterberg/Univ. Go¨teborg</td>
</tr>
<tr>
<td>YKL-40</td>
<td>-72.7%</td>
<td>2.2</td>
<td>0.0113</td>
<td>R&amp;D Systems</td>
<td>H. Zetterberg/Univ. Go¨teborg</td>
</tr>
<tr>
<td>sCD14</td>
<td>-26.1%</td>
<td>1.7</td>
<td>0.1159</td>
<td>R&amp;D Systems</td>
<td>H. Zetterberg/Univ. Go¨teborg</td>
</tr>
<tr>
<td>(B) Inflammatory biomarkers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complement C3</td>
<td>-86.9%</td>
<td>0.139</td>
<td>0.0007</td>
<td>Millipore</td>
<td>C. Pan/Inarian</td>
</tr>
<tr>
<td>Factor FH</td>
<td>23.7%</td>
<td>1.237</td>
<td>0.4988</td>
<td>Millipore</td>
<td>C. Pan/Inarian</td>
</tr>
<tr>
<td>MCP-1</td>
<td>-87.5%</td>
<td>4.813</td>
<td>0.0007</td>
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<td>H. Zetterberg/Univ. Go¨teborg</td>
</tr>
</tbody>
</table>

Biomarkers were assessed in the same MCI subjects prior to and after Posiphen treatment, matched to the same time of day. ‘CSF % of Time 0’ hence represents the mean difference of each subject matched to the timed sample on day 0 (prior to treatment). Whenever possible, determinations were quantified by at least two independent laboratories using different assays (N=4 MCI subjects, nine time points/subject (0, 1, 1.5, 2, 3, 4, 6, 8 and 12 h) assayed in duplicate prior to and post Posiphen treatment. The data were evaluated by repeated mixed model analysis, with a significance of \(p=0.05\). AD, Alzheimer’s disease; MCI, mild cognitive impairment.
test subjects following 10 days Posiphen treatment, the POM study design limited the potential effect of often large inter-subject variability thereby permitting statistical analyses on data derived from this small patient number. In this regard, individual patient data analyses are shown in figure 5. Clearly evident is the inter-subject difference in biomarker levels under naive (day 0) conditions (determined as the mean value ± SD of the nine timed samples across the 12 h sampling period). Evident also is the sometimes high variance around the mean biomarker value for each individual related to the time-dependent change (consistent with the circadian pattern reported by others) in biomarker levels over the 12 h study. Consistently across all individuals within figure 5, 10 day Posiphen administration lowered mean levels of sAPPα, sAPPβ, t-t and p-t and C3, but not factor H. Importantly, the time-dependent analysis of biomarker levels within the same individual, by matching exact same times predrug versus postdrug (table 3A, B), allowed determination of Posiphen-induced differences in such a small patient number (N=4) in the presence of large inter-subject and time-dependent biomarker differences. Of significance, the

Figure 4  Comparison of sAPPα, sAPPβ and t-t between untreated and treated mild cognitive impairment (MCI) patients and healthy volunteers. Posiphen (4×60 mg/day ×10 days) administered to MCI patients for 10 days lowers their CSF levels of sAPPα, sAPPβ and t-t to those determined in healthy (Posiphen naive) volunteers. Biomarker concentrations are expressed as a percent of MCI predrug values (assigned as 100%) and combined all data across assays (AlphaLisa and MSD for sAPPα and sAPPβ, and AlphaLisa and Innogenetics for t-t) in Table 3. Whereas there were significant Posiphen-induced declines in MCI subjects (see Table 3 for statistical analyses), no dramatic differences were evident between Posiphen-treated MCI subjects and healthy volunteers.

Figure 5  Difference in biomarkers between day 0 (naive) and 10 day Posiphen (4×60 mg/day) administration. As the effect of Posiphen on the analysed biomarkers remained present over 12 h, we were able to calculate the mean of the nine samples (collected 0–12 h) on day 0 (naive) and compare this with the mean of the nine samples (collected over the same 0–12 h) after 10 day Posiphen administration. Each bar hence represents the mean of repeat measures over 12 h with SD for each of the four patients (A, B, C, D) who completed the study. Whereas the absolute biomarker level differs between subjects, for each individual subject the post Posiphen values are consistently lower than the predrug (naive) values for all shown markers with the exception of factor H, consistent with its regulatory and not pro-inflammatory role. For this figure, data are derived from the AlphaLisa for sAPPα and sAPPβ, Innogenetics for Aβ42, p-t and t-t, and Millipore for C3 and factor H. In general, assay values are in agreement with literature values. MCI, mild cognitive impairment.
pattern of the changes was remarkably alike between the different assays employed blindly to measure the same CSF analyte at different independent institutions (whether AlphaLisa vs MSD in the quantification of sAPPα and sAPPβ, or AlphaLisa vs Innogenetics for Aβ42 and t-t). Albeit, the percent of the Posiphen-induced inhibition and variance differed between the assay techniques (table 3); similar data deriving from the use of two independent assays provide a valuable level of cross-validation to help guard against unforeseen systematic errors. Clearly, without the potential to match predrug and postdrug time-dependent biomarker levels within the same patient, a far greater number of subjects would have been required to support statistical analyses. Nevertheless, a larger patient number, which is often limiting in early POM studies, would have provided greater statistical power to discriminate drug-induced biomarker actions, as would the inclusion of a placebo group.

Recent reports suggest that CSF elevations in sAPPα and, in particular, sAPPβ may be clinically useful and superior to assessing Aβ42, in the early and differential diagnosis of incipient AD.22, 23 Hence, as APP represents Posiphen’s immediate target, CSF levels of sAPPα and sAPPβ, rather than, simply, Aβ42, were measured and found to be elevated in our MCI patients compared with healthy controls (figure 4), in accordance with others.39–44 Posiphen’s reduction in CSF sAPPα and sAPPβ in MCI patients brought their values in line with healthy controls. A preliminary analysis of Aβ42, analysed by two techniques (table 3A), suggests reductions in the same order as sAPPα and sAPPβ.

Posiphen treatment led to statistically significant reductions in CSF levels of other key AD biomarkers, in particular t-t and p-t. As illustrated in figure 4 and in accordance with others,32, 35–36 CSF t-t and p-t levels were elevated in our MCI patients versus healthy controls37–38 and were normalised by Posiphen. The relevance of these actions and mechanisms through which they are mediated are a focus of current studies. In this regard, resembling the action of Posiphen to impact the translational regulation of APP mRNA,19 t-t and p-t could also be regulated at the level of its RNA stability,45 potentially by Posiphen. Alternatively, reductions in t-t may be secondary to other actions or a combination of primary effects on translational regulation and secondary actions. Nevertheless, similar Posiphen-induced time-dependent reductions in t-t have recently been found in neuronal cell cultures and preclinical AD models (Sambamurti K, personal communication). Posiphen, likewise, induced statistical declines in MCI CSF t-t, a pro-inflammatory factor reportedly elevated in both AD transgenic mice41 and the CSF of AD subjects,42 together with key biomarkers (MCP-1, YKL-40) of microglial activation.24–26 In contrast, Posiphen treatment did not alter levels of the innate immune response protein sCD14 or factor H. Likewise, the decline in specific inflammatory markers may be a secondary effect to the described reductions of APP and t-t.

In synopsis, our pharmacokinetic studies in humans and rodents permitted us to estimate levels of Posiphen/metabolites in human brain after Posiphen (4×60 mg/day, 10 days) to be in the order of 3.5 μM Posiphen, associated with the described biomarker changes. This drug level is greater than the determined 50% effective concentration of Posiphen to lower APP levels in neuronal cultures.15 Recent studies have demonstrated that each Posiphen metabolite, likewise, has APP lowering actions.43 We conclude that Posiphen appears to be a promising experimental drug for MCI and AD as it can effectively lower CSF levels of APP, its primary target in brain, and in addition lower t-t, p-t and key inflammatory markers, and may hence impact disease progression at a number of levels.

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Contributors MLM contributed to the conception and design of the study, oversaw analyses (both experimental and statistical) and provided input into manuscript writing. MYC undertook experimental studies and analyses associated with CSF Aβ42 and sAPPβ quantification. CP undertook experimental studies and analyses associated with CSF Aβ42, p-t, t-t, C3 and factor H. VJ aided in study design, was responsible for assays related to CSF Aβ42, sAPPα and sAPPβ quantification, and provided input into manuscript writing. HZ aided in study design, was responsible for assays related to CSF MCP-1, YKL-40 and sCD14, and provided input into manuscript writing. NHG contributed to the study conception and design, the generation of Posiphen and metabolites, rodent studies, and the manuscript.

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Competing interests MLM and MYC are employees of QR Pharma, Inc. NHG is an inventor on the original Posiphen patent. Having assigned all rights to the US government, he declares that he has no ownership, financial interest or any other competing interests. All other authors declare no competing interests.

Ethics approval The human studies were conducted at and approved by the IRB and Ethics Committees of Cedraa/World Wide Clinical Trials (King of Prussia, PA) and the PRACS Institute (East Grand Forks, MN).

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

Neurodegeneration


Supplemental Information

SI-1, Posiphen Clinical Product: For all clinical studies, single batches of Posiphen capsules were formulated without any excipients and each contained, depending on the study, 20, 40, 60 or 80 mg of Posiphen. The capsules were manufactured by ACE Pharmaceuticals BV (Zeewolde, The Netherlands), and a Certificate of Analysis for the drug product was issued no earlier than 3 months before the start of each study indicating that the capsules met all relevant specifications, and were suitable for use in clinical studies. Placebo capsules that were identical in appearance were similarly prepared. The same drug product was utilized in rodent studies.

SI-2, Posiphen Steady-State Animal Studies to Define the Relationship Between Brain, CSF and Plasma Concentrations of Primary Drug and Metabolites: Five male adult Fischer-44 rats per group (Taconic, Hudson, NY), weighing 210-285 g, were anesthetized (50 mg/kg pentobarbital) and an Alzet micro-osmotic pump (model 2ML2, Alza Corp., Cupertino, CA), freshly filled with Posiphen at a concentration to achieve steady-state 75 mg/kg/day administration, was aseptically inserted into the peritoneal cavity. Animals were euthanized 5 and 10 days thereafter, and plasma, CSF (cisterna magna) and brain (right cerebral hemisphere) samples were simultaneously collected and immediately frozen and stored at -80° C for later analysis. Prior analysis demonstrated that Posiphen was stable (>95%) in saline at 37° C for over 10 days. These studies were conducted under an approved Institutional Animal Care and Use Committee protocol.

SI-3, Phase I, Single Ascending Dose (SAD): A randomized, double blind, placebo-controlled safety, tolerance and pharmacokinetic study (first-in-human) was performed in which six groups of male and female subjects received serially increasing single doses of Posiphen or placebo, followed by monitoring of safety (vital signs, ECGs, clinical laboratory tests, capture of adverse events) and collection of blood and urine samples at regular intervals up to 24 hr for preliminary pharmacokinetic analyses. On the presumption of the absence of limiting side effects, the escalating doses to be studied were 10, 20, 40, 80, 160, and 240 mg (and placebo). Since at 160 mg limiting side effects (nausea and vomiting) were observed, the study was halted at 160 mg and the 240 mg dose was not administered. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes that were placed immediately on wet ice and centrifuged within 10–15 minutes. Collected plasma was frozen to and stored at -80° C for later analysis of Posiphen using a specific LC-MS/MS assay. These data were used to calculate Posiphen pharmacokinetic parameters that were then compared among treatment groups to determine the overall pharmacokinetic profile of Posiphen in normal human volunteers.

A total of 36 men and 36 women, 6 in each treatment group (6 men and 6 women per group for a total of 6 groups = 72 people), were recruited from the general population and were enrolled and completed the study. All subjects were healthy normal individuals between the ages of 18 and 40. They were evaluated according to standard inclusion/exclusion criteria and randomly assigned to one arm of the study. The study was conducted by PRACS Institute (East Grand Forks, MN).

SI-4, Phase I, Multiple Ascending Dose (MAD): A randomized, double blind, placebo-controlled safety, tolerance, pharmacokinetic study was performed in which 6 of 8 male and 6
of 8 female subjects in each of three successive groups were administered one of three serially increasing, multiple dose regimens of Posiphen and 2 males and 2 females in each treatment group received placebo. Safety (vital signs, ECGs, clinical laboratory tests, capture of adverse events) was monitored throughout the study and blood and urine samples were collected. The escalating regimens were 20, 40, and 60 mg (and placebo), administered as a single dose on the first day and QID during the intervening days. The first two treatments were administered for 7 days, and the third, for 10 days. Plasma obtained from blood samples was analyzed as described above.

In this study a total of 24 men and 24 women, 8 per sex per treatment group, were recruited from the general population and were enrolled in the study. They were randomized between Posiphen and placebo treatments in a 3:1 ratio. All subjects were healthy normal individuals between the ages of 18 and 40, with inclusion criteria / exclusion criteria as in the SAD study. The study was likewise conducted by PRACS Institute (East Grand Forks, MN).

SI-5, Inclusion/Exclusion Criteria for SAD and MAD: Criteria required normal age-related findings at physical examination, weight within 30–35% of ideal according to Metropolitan Height/Weight tables, normal laboratory tests (complete blood count (CBC) with differential, platelet count, urinalysis, and blood chemistry panel), normal electrocardiogram (ECG), normal chest x-ray, and no clinically significant pulmonary disease based on spirometry results. Participants were also required to test negative in a urine drug screen for ethanol, amphetamines, barbiturates, benzodiazepines, cannabinoids, cocaine metabolites, methadone, opiates, and propoxyphene. Female subjects were required to be on birth control. Subjects were excluded from the study if they had any clinically significant concomitant disease or a history of clinically significant urinary tract obstruction, cardiovascular disease, or decreased gastric motility. Other exclusion criteria included a history of bronchial asthma, the current routine use of tobacco in any form, the concurrent use of anti-cholinesterase drugs.

Subjects were free to withdraw from the study at any time without prejudice to the quality of further treatment. Participants could also be withdrawn from the study at any time at the discretion of the investigator if, in the investigator’s opinion, continuing in the study was jeopardizing their health.

SI–6, Inclusion/Exclusion Criteria for POM Study:
Inclusion Criteria: Males or post-menopausal females aged 55 to 80 years with self-reported memory complaints that were corroborated by spouse or companion, as appropriate, and memory difficulties as measured on neuropsychological tests. Subject had MCI (amnestic subtype) according to Petersen’s criteria (2004). Progressive cognitive decline fulfilling the Petersen’s criteria for MCI:

a. Memory complaint, corroborated by immediate family.
b. Objective memory impairment measured by neuropsychological tests.
c. Normal or sufficiently preserved daily living activities are essentially normal.
d. General levels of cognition and functional performance sufficiently preserved such that a
Diagnostic and Statistical Manual of Mental Disorders, Vol. IV diagnosis for any type of dementia including Alzheimer’s disease cannot be readily made by the site physician at the time of the screening visit
Subject’s Mini Mental Status Examination (MMSE) score should be ≥24 and score below a pre-determined cut-off score on the logical memory II delayed paragraph recall sub-test of the Wechsler Memory Scale Revised (WMS-R):
   a) less than or equal to 8 for 16 or more years of education;
   b) less than or equal to 4 for up to 15 years of education;
Clinical Dementia Rating of 0.5 with a memory box score of 0.5 or 1.0; general cognition and functional performance sufficiently preserved that the patient can provide written informed consent. Hachinski score of less than or equal to 4. Hamilton Depression rating scale (HAMD17) score of less than or equal to 12 with a score of 0 on items 1, 2 & 3 (depressed mood, feelings of guilt and suicidal ideation). No evidence of current suicidal ideation or previous suicide attempt in past 2 years as evaluated in the Columbia Suicidality Checklist.

MRI scans within 12 months prior to screening, or per screening MRI and a complete medical history, electrocardiogram (ECG), and a physical examination at screening. The physical examination, including orthostatic blood pressure and pulse changes during a provocative maneuver and ECGs (screening and serial ECGs completed prior to dosing), must be normal.

Exclusion Criteria: Any significant neurologic disease other than amnestic MCI, was excluded, such as major depression; major psychiatric disorders as described in the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR); history of alcohol or substance abuse or dependence within the past 2 years (DSM-IV-TR criteria); history of schizophrenia (DSM-IV-TR criteria) and any significant systemic illness or unstable medical condition.

A number of medications excluded participation: beta-blockers, narcotics, methyldopa, anti-Parkinsonian medications, neuroleptics, cholinergic or anticholinergic drugs, anti-convulsants, long-acting benzodiazepines or barbiturates, short-acting anxiolytics or sedative hypnotics and warfarin - all within 4 weeks prior to screening.

SI–7, Posiphen and metabolite pharmacokinetic assays:
Concentrations of Posiphen, N1-norposiphen, N8-norposiphen, and N1,N8-bisnorposiphen in human plasma and CSF samples, as well as rat plasma, brain and CSF samples were determined by LC-MS/MS. Analysis was conducted on an HPLC system consisting of two Perkin Elmer Series 200 micropumps (Wellesley, MA) and a CTC Leap auto-sampler (Carrboro, NC) connected to an Applied Biosystems API4000 triple quadrupole mass spectrometer (Foster City, CA), operated in the MRM mode with a turbo ion spray interface. Chromatographic separation was achieved on a Phenomenex Synergi Polar RP, 100 x 2.0 mm id, 2.5 µm column (Torrance, CA). The mobile phases were 0.1% formic acid in water (A) or 0.1% formic acid in methanol (B). Stable deuterated (d5) internal standards were used for each analyte, except in the case of N1-norposiphen where N8-norposiphen-d5 was used as the internal standard.

Plasma samples were prepared for analysis by acetonitrile precipitation and centrifuged, the supernatant was dried under nitrogen and the dried samples were reconstituted with 10:90:0.1 methanol:water:formic acid, vortexed and analyzed. Brain samples were sonicated in acetonitrile, and, thereafter, treated as described for plasma. CSF samples were prepared for analysis by dilution in 10:90:0.1 methanol:water:formic acid, vortexed and analyzed.
Calibration ranges for each analyte ranged from 1000 ng/mL to 1 ng/mL, in plasma, brain and CSF matrices. The detection limit was 0.025 ng/mL.