RESEARCH PAPER

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

Maria L Maccecchini,1 Mee Young Chang,1 Catherine Pan,2 Varghese John,3 Henrik Zetterberg,4 Nigel H Greig5

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, Aβ1-40, 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 Aβ1-42.

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 assembles into oligomers that cause inflammation and target synapses to induce cellular dysfunction and impair memory.7–9 APP is additionally cleaved into a number of other bioactive N- and C-terminal fragments, including N-APP10 and C311 12 (figure 1). These fragments, likewise, may contribute to AD pathogenesis, making APP 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 levels13 14 in a dose-dependent manner and hence represents an interesting candidate drug to reduce APP toxic products in humans.

We describe, herein, three phase I 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, Aβ1-42, t-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.

1Ori Pharma, Inc., Benvyn, Pennsylvania, USA
2Iranian Neurodiagnostics, LLC, Mercer Island, Washington, USA
3Alzheimer’s Drug Discovery Network, Buck Institute for Research on Aging, Novato, California, USA
4Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Mölndal, Sweden
5Drug Design & Development Section, Laboratory of Neurosciences, Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, Maryland, USA

Correspondence to
Dr Nigel H Greig, Drug Design & Development Section, Laboratory of Neurosciences, Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, Maryland, USA
maccecchini@qrpharma.com

Received 23 February 2012
Revised 21 May 2012
Accepted 23 May 2012
Published Online First
11 July 2012

This paper is freely available online under the BMJ Journals unlocked scheme, see http://jnnp.bmj.com/site/about/unlocked.xhtml

894 J Neurol Neurosurg Psychiatry 2012;83:894–902. doi:10.1136/jnnp-2012-302589
Drug substance
Posiphen, (3aR)-1, 3α, 8-trimethyl-1, 2, 3, 3α, 8, 8α-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.\textsuperscript{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).

Figure 1  Schematic of amyloid-β precursor protein (APP) processing pathway leading to AD. Amyloidogenic processing of APP generates Aβ, a hydrophobic, neurotoxic, self-aggregating 40–42 amino acid peptide that accumulates preferentially within amyloid plaques.\textsuperscript{4, 6} Recent research suggests that APP processing can result in a number of toxic fragments, including N- and C-terminal peptides\textsuperscript{10–11} that may induce neuronal dysfunction, degeneration and death leading to the hypothesis that a number of APP fragments are toxic to nerve cells.

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 4×360 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 obtaining to obtain time-dependent baseline control data, and then at the exact same times immediately after the last dose was administered. 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-norposiphen, N8-norposiphen and N1, N8-bisnorposiphen) (figure 2) and (ii) pharmacodynamic studies involving measurement of the following proteins: sAPPβ, sAPPα, Aβ42, t-t, p-t, 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 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 online Supplemental information SI-6.

To provide comparative healthy control data, a CSF sample was similarly acquired by spinal tap at 08:00 AM from four healthy volunteers who were not treated with Posiphen. This CSF was analysed for the same key pharmacodynamic factors as those described for the MCI patients prior and following Posiphen treatment.

The study was conducted by the CEDRA/World Wide Clinical Trials (King of Prussia, Pennsylvania, USA) and was fully approved by their Institutional Review Board.

Biochemical pharmacodynamic assays
Four independent laboratories and multiple assay methods were used to quantify the CSF biomarkers analysed in this study.

Meso Scale Discovery (MSD) assays were used for quantification of sAPPβ and sAPPβ (human Alzheimer’s panel three sAPPβ/sAPPβ assay kit (catalogue#: K15120E-1, MSD, Gaithersburg, Maryland, USA) from 25 μl of CSF. The plates were measured by multiplayer analyser (SI2400A) and data evaluated by MSD discovery workbench data analysis toolbox software. The same platform was used to measure MCP-1 (also called CCL2) concentrations in CSF, as previously described.

AlphaLISA kits from Perkin Elmer were used to quantify sAPPβ (catalogue#: AL254C, Perkin Elmer, Waltham, Massachusetts, USA), sAPPβ (catalogue#: AL252C), Aβ42 (catalogue#: AL276C), Aβ30 (catalogue#: AL275C) and t-t (catalogue#: AL217C) from 5 μl of an undiluted CSF sample which was added to an AlphaPlate-384 (catalogue#: 6005350).

 Innogenetics kits were used to measure CSF t-t and p-t levels by INN0-BIA AlzBio3 kit (Innogenetics, Gent, Belgium) with CSF samples diluted 1:4 in diluent. Samples were analysed using a LiquiChip Luminex 200 Workstation (Qiagen, Valencia, California, USA).

The proteins C3 and FH in CSF were measured with a kit made by Millipore Corporation (Billerica, Massachusetts, USA), following the manufacturer’s instructions with procedures similar to those described for synuclein assay.

CSF levels of YKL-40 and sCD14 were analysed with commercial ELISAs (R&D Systems, Minneapolis, Minnesota, USA). The CSF was diluted 100 times for the YKL-40 and sCD14 analyses.

Posiphen and metabolite pharmacokinetic assays
Concentrations of Posiphen, N1-norposiphen, N8-norposiphen and N1, N8-bisnorposiphen in human plasma and CSF as well as rat plasma, brain and CSF samples were determined by LC-MS/MS at Absorption Systems (Exton, Pennsylvania, USA) (See online Supplemental information SI-7). Calibration ranges for each analyte ranged from 1000 ng/ml to 1 ng/ml (or ng/g for brain) in plasma, brain and CSF matrices. The detection limit was 0.025 ng/ml.

Statistical analysis
All assay data collected were analysed using a repeated measures mixed model analysis of variance. The model included Day (Day 11/Day 0) as a fixed effect, Time (nine time points per person between 0 and 12 h) as a repeated measure effect and Patient (18 samples per person in total) as a random effect. Compound symmetry was assumed as an appropriate covariance pattern between observations on the same patient, which provided a reasonable model fit. Assumptions of constant variance, normality or residuals and parallelism were used to assess acceptability of the statistical model. Data are presented as means ± SEs, unless otherwise stated. The statistical evaluations were undertaken by Data Magik (Salisbury, UK).

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-bisnorposiphen 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-norposiphen: 3.8, N8-norposiphen: 5.8 and N1, N8-bisnorposiphen: 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 Tmax: 1.3–1.6 h) and cleared from the circulation biphasically (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 AUC0-last. 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 AUC0-last, 1.63 to 1998 and 12.1 to 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).
The semi-log plot of serial time-dependent Cmax and AUC values, increasing dose, resulting in disproportionately large increases in availability of Posiphen increased more than linearly with dose. The third metabolite, N1,N8-bisnorposiphen, reached 20.4% of Posiphen in plasma and 3.8% of Posiphen at Cmax 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. 

*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.

MCI, mild cognitive impairment.

### 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.

### POM study (MCI patients)

**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–last, table 2) were approximately 50% of Posiphen. Contrasting with rat studies, N1, N8-bisnorposiphen represented a minor metabolite in humans, accounting for 3.2% of Cmax and 6.5% total time-dependent Posiphen levels.

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 2B 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 μg/g or approximately 3.5 μM for a dose of 4×60 mg/day.

### 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

---

**Table 2** Mean pharmacokinetic parameters for Posiphen and primary metabolites in rat and MCI patients

| Posiphen and metabolites | Analyte | Parameters | Human plasma (ng/ml) ± SD | Human CSF (ng/ml) ± SD | Rat plasma (ng/ml) ± SD | Rat brain (ng/ml) ± SD | Rat CSF (ng/ml) ± SD |
|--------------------------|---------|------------|---------------------------|-----------------------|------------------------|-----------------------|----------------------|
| Posiphen                 | Cmax    |            | 118.5±24.8                | 1.6±0.6               | 144±69.5               | 979±429               | 9.5±4.6              |
| *ClogP*=2.22             |         | AUC0–last  | 570±235.4                 |                       |                        |                       |                      |
| N1-Norposiphen           | Cmax    |            | 25.6±6.7                  | 1.7±0.7               | 56.3±8.7               | 213±27.9              | 2.8±1.1              |
| *ClogP*=1.25             |         | AUC0–last  | 214.4±77.1                |                       |                        |                       |                      |
| N8-Norposiphen           | Cmax    |            | 31.7±7.1                  | 3.2±1.2               | 37.2±9.1               | 216±28.6              | 2.8±5.8              |
| *ClogP*=1.00             |         | AUC0–last  | 261.3±91.3                |                       |                        |                       |                      |
| N1, N8-bisnorposiphen    | Cmax    |            | 3.8±1.2                   | Not detected          | 29.5±10.8              | 37.2±10.5             | Not detected         |
| *ClogP*=0.53             |         | AUC0–last  | 36.9±12.5                 |                       |                        |                       |                      |

**Figure 3** Time-dependent (A) plasma and (B) CSF Posiphen and metabolite levels following the final dose of 10 day Posiphen administration (4×60 mg). Semi-log plot of serial time-dependent Posiphen and metabolite (N1- and N8-norposiphen and N1, N8-bisnorposiphen) concentrations in plasma and CSF obtained following the final dose of a 10 day Posiphen (4×60 mg) schedule (n=4 MCI subjects, mean ± SD).
of change was the same. Specifically, Posiphen lowered sAPPα and sAPPβ levels by −59.9% and −57.7%, respectively, assessed by the AlphaLisa assay, and by −34.1% and −34%, respectively, assessed by the MSD assay, in accordance with Posiphen’s proposed mechanism of action to inhibit APP expression.13 14 18

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 associated with early innate immune response to bacterial and viral infection,26 and the complement control protein, factor H, were not affected 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 sAPPα, sAPPβ and t-tau to levels present in healthy volunteers.

| Human biomarker | (A) AD biomarkers | (B) Inflammatory biomarkers |
|----------------|------------------|-----------------------------|
|                | CSF % of time 0  | SE        | p Value | Assay      | Laboratory          |
| sAPPα          | −59.9%           | 0.231     | 0.0006  | AlphaLisa | V. John/Buck Institute |
| sAPPβ          | −34.1%           | 0.659     | 0.0661  | MSD       | MY Chan/QR Pharma    |
| Aβ42           | −57.7%           | 0.361     | 0.0001  | AlphaLisa | V. John/Buck Institute |
| t (total)      | −34.0%           | 1.516     | 0.0901  | MSD       | MY Chan/QR Pharma    |
| t (Phosphorylated) | −51.4%        | 1.119     | 0.0533  | Innogenetics | C. Pan/Inarian |
|                | −46.2%           | 0.538     | 0.0020  | AlphaLisa | V. John/Buck Institute |
| sCD40          | −74.1%           | 0.259     | 0.0150  | Innogenetics | C. Pan/Inarian |
|                | −61.0%           | 0.195     | 0.0005  | Innogenetics | C. Pan/Inarian |

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.

**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 APP, t 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 t 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 sAPPα and sAPPβ in CSF consistent with its preclinical actions13 18 27 and ability to inhibit the translation of APP mRNA via an iron response element within its 5’-untranslated region.14 16 20 The trend of a Posiphen-induced reduction in CSF Aβ42 in MCI subjects is likewise in line with its known action to inhibit APP synthesis, as Aβ42 is a downstream product and is in accordance with the described decline in CSF sAPPβ in MCI subjects as well as of Aβ42 levels in preclinical studies.15–18 20 27 28 A separate more limited analysis of Aβ40 (AlphaLisa) in CSF collected at 3 and 8 h prior to and following Posiphen administration in MCI subjects provided reduction trends of −32% 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
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, Aβ42, t-t, 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...
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.24–34 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,25–28 CSF t-t levels were elevated in our MCI patients versus healthy controls27,29 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,14 15 26 t-t can also be regulated at the level of its RNA stability.33–36 It is 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 and preclinical AD models (Sambamurti K, personal communication). Posiphen, likewise, induced statistical declines in MCI and preclinical AD models (Sambamurti K, personal communication).

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.13 Recent studies have demonstrated that each Posiphen metabolite, likewise, has APP lowering actions.42 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.

Acknowledgements The authors are grateful to the following: (i) Qian-sheng Yu, Intramural Research Program, National Institute on Aging, NIH, for synthesis and chemical characterisation of highly pure samples of Posiphen, N1-norposiphen, N8-norposiphen, and N1, N8-bisnorposiphen that were used as standards for analytical chemistry in pharmacokinetic studies. (ii) Harold W Holloway, Intramural Research Program, National Institute on Aging, NIH, for aid with pharmacokinetic studies. (iii) Karen Pocksay and Olivier Descamps, Buck Institute for Research on Aging, for performing AlphaLisa analyses of CSF samples and (iv) David Fleet, Data Magik, for statistical analyses.

Contributors MLM contributed to the conception and design of the study, overview analyses (both experimental and statistical) and provided input into manuscript writing. MYC undertook experimental studies and analyses associated with CSF sAPPα and sAPPβ quantification. OP undertook experimental studies and analyses associated with CSF Aβ, p-t-t, t-t and factor H. VJ aided in study design, was responsible for assays related to CSF Aβ, 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.

Funding This work was supported in part by QR Pharma, Inc. Henrik Zetterberg was supported by the Swedish Research Council (grant numbers K2010-35P-21562-01-4 and K2011-16X-20401-05-6) and the Swedish State Support for Clinical Research. Nigel H Greg was supported by the Intramural Research Program, National Institute on Aging, NIH.

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 CEDERA/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

1. Stone JG, Casadesus G, Gustaw-Rothenberg K, et al. Frontiers in Alzheimer’s disease therapeutics. Ther Adv Chronic Dis 2011; 29–33.
2. Sambamurti K, Greig NH, Lahiri DK. Advances in the cellular and molecular biology of the beta-amyloid protein in Alzheimer’s disease. Neuronomolecular Med 2002; 1:1–31.
3. Sambamurti K, Suram A, Venugopala C, et al. A partial failure of membrane protein turnover may cause Alzheimer’s disease: a new hypothesis. Curr Alzheimer Res 2006; 3:81–90.
4. Selkoe DJ. Alzheimer’s disease. Cold Spring Harb Perspect Biol 2011; 3: pii: a004537.
5. Popp J, Arti S. Pharmacological treatment of dementia and mild cognitive impairment due to Alzheimer’s disease. Curr Opin Pharmacol 2011; 26:556–61.
6. Selkoe DJ. Defining molecular targets to prevent Alzheimer disease. Arch Neurol 2005; 62:192–5.
7. Asaha KH, Zahn KR. Probing the biology of Alzheimer’s disease in mice. Neuron 2010; 66:631–45.
8. De Felice FG, Velasco PT, Lambert MP, et al. Aβ oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. J Biol Chem 2007; 282:11590–601.
9. Shankar GM, Li S, Mehta TH, et al. Amyloid-beta protein dimers isolated directly from Alzheimer’s brains impair synaptic plasticity and memory. Nat Med 2009; 15:837–42.
10. Nikolaev A, McLaughlin T, O’Leary DG, et al. APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. Nature 2009; 457:981–9.
11. Galvan V, Gorostiza OF, Banwait S, et al. Reversal of Alzheimer’s-like pathology and behavior in human APP transgenic mice by mutation of Asp664. Proc Natl Acad Sci U S A 2006; 102:7130–5.
12. Harris JA, Davidon N, Halabisky B, et al. Many neuronal and behavioral impairments in transgenic mouse models of Alzheimer’s disease are independent of caspase cleavage of the amyloid precursor protein. J Neurosci 2010; 30:372–81.
13. Lahiri DK, Chen D, Maloney B, et al. The experimental Alzheimer’s disease drug Posiphen lowers Aβ peptide levels in cell culture and mice. J Pharmacol Exp Ther 2007; 320:388–96.
14. Shaw K, Utsuki T, Rogers JT, et al. Phenserine regulates translation of β-amyloid precursor protein mRNA by a putative interleukin-1 responsive element: a novel target for drug development. Proc Natl Acad Sci U S A 2001; 98:7605–10.
15. Rogers JT, Randall JD, Cahill CM, et al. An iron-responsive element type II in the 5’-untranslated region of the Alzheimer amyloid precursor protein transcript. J Biol Chem 2002; 277:45518–28.
16. Cho HH, Cahill CM, Vanderburg CH, et al. Selective translational control of the Alzheimer amyloid precursor protein transcript by iron regulatory protein-1. J Biol Chem 2010; 285:31217–22.
17. Greig NH, Sambamurti K, YU GS, et al. An overview of phenserine tartrate, a novel acetylcholinesterase inhibitor for the treatment of Alzheimer’s disease. *Curr Alzheimer Res* 2005; 2:281–93.

18. Maroteaux A, Dhmritu M, Nilbratt M, et al. Modulation of human neural stem cell differentiation in Alzheimer (APP23) transgenic mice by phenserine. *Proc Natl Acad Sci U S A* 2007; 104:12506–11.

19. Yu QS, Greig NH, Holloway HW, et al. Syntheses and anticholinesterase activities of (3aS)-N(1), N(8)-bisnorphenserine(3aS)-N(1), N(8)-bisnorphysostigmine, their antipodal isomers, and other potential metabolites of phenserine. *J Med Chem* 1998; 41:2371–9.

20. Yu QS, Pei XF, Holloway HW, et al. Total syntheses and anticholinesterase activities of (3aS)-N(8)-norphysostigmine, (3aS)-N(8)-norphenserine, their antipodal isomers, and other N(8)-substituted analogues. *J Med Chem* 1997; 40:2895–901.

21. Soares H, Raha N, Sikpi M, et al. Aβ variability and effects of γ-secretase inhibition on plasma and CSF levels of Aβ in healthy volunteers. *Poster P1—264. International Conference on Alzheimer’s Disease*, Vienna, Austria: Alzheimer’s Association, 2009.

22. Ereshefsky L, Jhee S, Yen M, et al. Cerebrospinal fluid beta-amyloid and dynamin bridging in Alzheimer’s disease drug development. *Biomark Med* 2008; 3:711–21.

23. Petersen RC. Mild cognitive impairment as a diagnostic entity. *J Intern Med* 2004; 256:183–94.

24. Mattsson N, Tabatabaei S, Johansson P, et al. Cerebrospinal fluid microglial markers in Alzheimer’s disease: elevated chitinotidase activity but lack of diagnostic utility. *Neuromolecular Med* 2011; 13:151–9.

25. Hong Z, Shi M, Chung KA, et al. DJ-1 and alpha-synuclein in human cerebrospinal fluid as biomarkers of Parkinson’s disease. *Brain* 2010; 133:713–26.

26. Yin GN, Jeon H, Lee S, et al. Role of soluble CD14 in cerebrospinal fluid as a regulator of glial functions. *J Neurosci Res* 2009; 87:2576–80.

27. Salehi M, Fersi R, Taktimoto J, et al. Examining the effects of (±)-phenserine in Tg65N mouse model of Down syndrome. *Alzheimer’s Dement* 2008; 4:1462–3.

28. Rogers JT, Mikkilineni S, Cantuti-Castelvetri I, et al. The alpha-synuclein 5’-untranslated region targeted translation blockers: anti-alpha synuclein efficacy of cardiac glycosides and Posiphen. *J Neural Transm* 2011; 118:499–507.

29. Bacchetti P, Deeks SG, McCune JM. Breaking free of sample size dogma to perform innovative translational research. *Sci Transl Med* 2011; 3:87ps24.

30. Bateman RJ, Wen G, Morris JC, et al. Fluctuations of CSF amyloid-beta levels: implications for a diagnostic and therapeutic biomarker. *Neurology* 2007; 68:566–9.

31. Huang Y, Patter R, Sigurdson W, et al. Effects of age and amyloid deposition on Aβ dynamics in the human central nervous system. *Arch Neural* 2012; 69:51–8.

32. Lewczuk P, Kamrowski-Kruck H, Peters O, et al. Soluble amyloid precursor proteins in the cerebrospinal fluid as novel potential biomarkers of Alzheimer’s disease: a multicenter study. *Mol Psychiatry* 2010; 15:138–45.

33. Pernecky R, Tsolakidou A, Arnold A, et al. CSF soluble amyloid precursor proteins in the diagnosis of incipient Alzheimer disease. *Neurology* 2011; 77:35–8.

34. Alexopoulos P, Tsolakidou A, Rosell F, et al. Clinical and neurobiological correlates of soluble amyloid precursor proteins in the cerebrospinal fluid. *Alzheimers Dement*. Published Online First: 4 November 2011.

35. Shaw LM, Korecka M, Clark CM, et al. Biomarkers of neurodegeneration for diagnosis and monitoring therapeutics. *Nat Rev Drug Discov* 2007; 6:295–303.

36. Sepplä TT, Koivisto AM, Hartikainen P, et al. Longitudinal changes of CSF biomarkers in Alzheimer’s disease. *J Alzheimers Dis* 2011; 25:583–94.

37. Buchhave P, Minthon L, Zetterberg H, et al. Cerebrospinal fluid levels of β-amyloid 1-42, but not of tau, are fully changed already 5 to 10 years before the onset of Alzheimer dementia. *Arch Gen Psychiatry* 2012; 69:98–106.

38. Shaw LM, Vandersteichele H, Knapp-Cajka M, et al. Cerebrospinal fluid biomarker signature in Alzheimer’s disease neuroimaging initiative subjects. *Ann Neural* 2009; 65:403–13.

39. Bottley A, Phillips NM, Webb TE, et al. eIF4A inhibition allows translational regulation of mRNAs encoding proteins involved in Alzheimer’s disease. *PLoS One* 2010; 5:pii: e13030.

40. Vee BL, Krushel IA. Translation initiation of the human tau mRNA through an internal ribosomal entry site. *J Alzheimers Dis* 2009; 16:271–8.

41. Reichwald J, Danner S, Wiederhold KH, et al. Expression of complement system components during aging and amyloid deposition in APP transgenic mice. *J Neuroinflammation* 2009; 6:35.

42. Wang Y, Hancock AM, Bradner J, et al. Complement 3 and factor h in human cerebrospinal fluid in Parkinson’s disease, Alzheimer’s disease, and multiple-system atrophy. *Am J Pathol* 2011; 178:1509–16.

43. Mikkilineni S, Cantuti-Castelvetri I, Cahill C, et al. The anti-cholinesterase phenserine and its enantiomer Posiphen as 5’-untranslated region directed translation blockers of the Parkinson’s alpha synuclein expression. *Parkinson’s Dis* 2012; 2012:142372.
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 ethylenediamine tetraacetic 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 and last 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.