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^{o} C for later analysis. Prior analysis demonstrated that Posiphen was stable (>95%) in saline at 37^{o} 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^{o} 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).[23] 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.