In-vivo Pharmacology Assays - Efficacy

1. Addiction Assay

1.1 Schedule Induced Ethanol Polydipsia

In order to evaluate antiaddictive property of NCE under evaluation, four groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be subjected to schedule induced ethanol polydipsia and alcohol consumption will be estimated.

2. Anxiety Assays

2.1 Elevated Plus maze

In order to evaluate anxiogenic or anxiolytic property of NCE under evaluation, four groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be subjected to elevated plus maze assay and time spent in the open arms, % time in open arm, time in closed arm, number of open arm entries and % open arm visit will be analyzed.

2.2 Hole Board

In order to evaluate anxiolytic property of NCE under evaluation, four groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be subjected to hole board assay and latency of head dips, number of head dips and cumulative time of head dips will be analyzed.

2.3 Vogel conflict

In order to evaluate anxiogenic or anxiolytic property of NCE under evaluation, six groups of animals (Control, Positive Control Group I, Positive Control Group II, Three dose levels of test compound; n=10) will be subjected to conflict test assay and number of shocks received will be analyzed.

2.4 Novelty induced hypophagia

In order to evaluate anxiogenic or anxiolytic property of NCE under evaluation, six groups of animals (Control, Positive Control Group I, Positive Control Group II, Three dose levels of test compound; n=10) will be subjected to experimentation and latency and the amount of food consumed in a novel environment will be analyzed.

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3. Cognition Assays

3.1 Water maze – Spatial memory

In order to evaluate cognition-enhancing property of NCE under evaluation, five groups of animals (Control, Positive Control Group, Three dose levels of test compound; n=12) will be subjected to test and latency to reach the platform will be the dependant measure during acquisition trials. Swim speed and path length will be measured in acquisition trials. Latency to reach the target, time spent in the target quadrant and latency to the quadrant, which previously contained the platform, will be measured in probe trial will be analyzed.

3.2 NORT – Scopolamine induced

In order to evaluate cognition-enhancing property of NCE under evaluation, three groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=12) will be subjected to test and time spent with the novel, familiar object and discriminative index will be analyzed.

3.3 NORT – Time induced

In order to evaluate cognition-enhancing property of NCE under evaluation, four groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=12) will be subjected to test and time spent with the novel, familiar object and discriminative index will be analyzed.

3.4 Radial arm maze

In order to evaluate cognition-enhancing property of NCE under evaluation, three groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=12) will be subjected to test and choice accuracy and the total error will be analyzed.

3.5 T-maze

In order to evaluate cognition-enhancing property of NCE under evaluation, three groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=12) will be subjected to test and choice accuracy will be analyzed.

3.6 Fear conditioning

In order to evaluate cognition-enhancing property of NCE under evaluation, four groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=12) will be subjected to test and time spent freezing will be analyzed.

4. Depression Assays

4.1 Apomorphine induced hypothermia

In order to evaluate antidepressant property of NCE under evaluation, four groups of animals (Control, Chemical challenger, Positive Control Group, one dose levels of test compound; n=10) will be subjected to test and change in body temperature will be analyzed.

4.2 Dominant submissive assay

In order to evaluate antidepressant property of NCE under evaluation, three groups of animals (Control, Two dose levels of test compound; n=10) will be subjected to test and dominance level and feeding scores will be analyzed.

4.3 DRL-72s assay

In order to evaluate antidepressant property of NCE under evaluation, four groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=12) will be subjected to test and response efficiency, IRT, response and reinforces will be analyzed.

4.4 Forced swim test

In order to evaluate antidepressant property of NCE under evaluation, three groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be subjected to test and immobility time will be analyzed.

4.5 Tail suspension test

In order to evaluate antidepressant property of NCE under evaluation, three groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be subjected to test and immobility time will be analyzed.

5. Schizophrenia Assays

5.1 Prepulse Inhibition

In order to evaluate antipsychotic property of NCE under evaluation, five groups of animals (Control, Chemical challenge Group , Positive Control Group, Two dose levels of test compound; n=12) will be subjected to Prepulse inhibition test and % prepulse inhibition and acoustic startle response will be analyzed.

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5.2 MK-801 antagonism

In order to evaluate antipsychotic property of NCE under evaluation, four groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be subjected to open field test and distance traveled will be analyzed.

6. Pain Models

6.1 Formalin Induced Nociception.

In order to evaluate analgesic property of NCE under evaluation, four groups of animals (Control, Positive Control Group, Three dose levels of test compound; n=10) will be subjected to formalin induced pain and hind paw licking and biting will be analyzed.

6.2 Spinal Nerve (L5) Ligation Model of Neuropathic Pain.

In order to evaluate analgesic property of NCE under evaluation, four groups of spinal nerve ligated animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be subjected to tactile allodynia, measured by assessing rat hind paw withdrawal thresholds in response to mechanical stimulation using a dynamic plantar Aesthesiometer or Von Frey Monofilaments.

6.3 Chronic Constriction Injury (CCI) Model of Neuropathic Pain.

In order to evaluate analgesic property of NCE under evaluation, four groups of chronic constriction injury animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be subjected to Mechanical allodynia, measured by assessing rat hind paw withdrawal thresholds in response to mechanical stimulation using Von Frey Monofilaments.

6.4 Partial Sciatic Nerve Ligation (PSNL) Model of Neuropathic Pain.

In order to evaluate analgesic property of NCE under evaluation, four groups of partial sciatic nerve ligated animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be subjected to Mechanical allodynia, measured by assessing rat hind paw withdrawal thresholds in response to mechanical stimulation using Von Frey Monofilaments.

6.5 Inflammatory Mechanical hyperalgesia

In order to evaluate analgesic property of NCE under evaluation, four groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be subjected to mechanical hyperalgesia, measured by assessing rat hind paw withdrawal thresholds in response to mechanical stimulation using Randall–Selitto (after administration of Freund's complete adjuvant).

6.6 Diabetes - Induced Neuropathic Pain

In order to evaluate analgesic property of NCE under evaluation, four groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be

subjected to mechanical hyperalgesia, measured by assessing rat hind paw withdrawal thresholds in response to mechanical stimulation using Randall–Selitto (after administration of streptozotocin).

6.7 Chemotherapy- Induced Neuropathic Pain

In order to evaluate analgesic property of NCE under evaluation, four groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be subjected to mechanical hyperalgesia, measured by assessing rat hind paw withdrawal thresholds in response to mechanical stimulation using Randall–Selitto (after administration of Vincristine).

6.8 Capsaicin-Induced Allodynia

In order to evaluate analgesic property of NCE under evaluation, four groups of animals (Control, Positive Control Group, Two dose levels of test compound; n=10) will be subjected to tactile allodynia, measured by assessing rat hind paw withdrawal thresholds in response to mechanical stimulation using a dynamic plantar Aesthesiometer (after administration of capsaicin).

7. In- Vivo Microdialysis Assays

7.1 Modulation of Neurotransmitter Levels using Brain Microdialysis Assay (Rats and Guinea-pigs)

In order to evaluate modulation of neurotransmitter levels in specific brain region due to administration of NCE under evaluation, four groups of animals (Control, Positive Control Group, minimum two dose levels of test compound; n=8), subjected to stereotaxic surgery for implantation of a semi-permeable membrane into a specific brain region (hippocampus, cortex, striatum, etc), perfused with aCSF and dialysates will be collected (four- five basal samples and for desired time post treatment) will be quantitated for neurotransmitter concentrations using specific and sensitive analytical method. % change in neurotransmitter post treatment will be compared with vehicle group using suitable statistical method.

Following analytical methods are employed for quantification of neurotransmitters:

- Acetylcholine : LC-MS/MS (API-4000)
- Amino acids : Glutamate and GABA – HPLC + Fluorescence
- Histamine : HPLC + Fluorescence
- Monoamines : LC-MS/MS (API-4000 Q-Trap)
- Monoamine metabolites : HPLC-ECD (BASi Epsilon)
- Test compounds : LC-MS/MS (API-4000 Q-Trap)

7.2 *In- Vivo* Spinal Microdialysis for modulation of inflammatory biomarkers and neurotransmitters

Levels of inflammatory biomarkers and neurotransmitters monitoring in specific spinal region like dorsal horn helps in evaluating the efficacy of NCEs acting on the inflammation/ pain centrally. A semi-permeable membrane will be implanted into spinal region of interest (dorsal horn). Animal will be allowed to recover from surgery and the probe is perfused with aCSF. Dialysates will be collected (four- five basal samples and for desired time post treatment) and quantified for neurotransmitter concentrations using specific and sensitive analytical method. % change in neurotransmitter post treatment will be compared with vehicle group using suitable statistical method.

Following analytical methods are employed for quantification of neurotransmitters/ biomarkers:

- Acetylcholine : LC-MS/MS (API-4000)
- Amino acids : Glutamate and GABA – HPLC + Fluorescence
- Monoamines : LC-MS/MS (API-4000 Q-Trap)
- PGE2 : ELISA

8. *In-vivo* receptor occupancy

A test item at series of doses will be dosed through specific route (p.o or s.c or i.p or i.v) followed by an administration of specific i.v. tracer at Tmax time point of test item. Animals (Rats) of will be sacrificed at pre-defined time points and specific and non-specific brain regions will be scooped out. Brain regions will be subjected for the quantification of tracer using LC-MS/MS method to evaluate the receptor occupancy.

List of validated targets for receptor occupancy are:

- Adenosine A2A
- Nicotinic acetylcholine receptors; α4β2 and α7
- Cannabinoid CB1
- Dopamine; D2
- $GABA_A$
- Histamine H_3
- Serotonin; 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_4 and 5-HT_{2C}
- Phosphodiesterase 10 (PDE10)
- Serotonin transporter (SERT)
- Dopamine transporter (DAT)
- Norepinephrine transporter (NET)

8.1 Receptor Occupancy Study–simultaneous for two receptors

One test item, three doses, oral or sc or ip administration followed by specific combination of *i.v.* tracers (D_2/5-HT_{2a} or 5-HT2A/SRI) administration. Sacrifice rats at predetermined time points to scoop specific and nonspecific brain regions and quantifying tracer concentration simultaneously using LC-MS/MS method to evaluate the receptor occupancy.

8.2 Receptor Occupancy Study–simultaneous for three receptors

One test item, three doses, oral or sc or ip administration followed by specific combination of *i.v.* tracers (D_2/5-HT_{2A}/5-HT_{1A}) administration. Sacrifice rats at predetermined time points to scoop specific and nonspecific brain regions and quantifying tracer concentration simultaneously using LC-MS/MS method to evaluate the receptor occupancy.

9. *Ex-vivo* Assay

9.1 5-HT_{2B} Rat Fundus Assay

In order to evaluate functional activity of NCE under evaluation at 5-HT_{2B} receptors, four tissues will be used for each group (Control, Positive Control Group, Two dose levels of test compound; n=10), contractile response will be recorded.

9.2 5-HT_4 Guinea Pig Colon Assay

In order to evaluate functional activity of NCE under evaluation at 5-HT_4 receptors, four tissues will be used for each group (Control, Positive Control Group, Two dose levels of test compound; n=10), contractile response will be recorded.