4.0 MATERIALS AND METHODS:

Swiss albino male mice weighing 25±2g obtained from Punjab University, Chandigarh, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana and Central Research Institute, Kasauli, India, maintained on a normal laboratory diet procured from Kisan feeds Ltd., Mumbai, India. Animals used in present study having free access to water. They were put up in the departmental animal house and were debunked to a regular 12 hour cycle of illumination and dark. The experiments were taken in a semi-sound proof laboratory.

The experimental protocol was approved by the Chitkara institutional animal ethical committee, Chitkara College of Pharmacy under the registration number 1181/ab/08/CPCSEA and the upkeep of the animals were done as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests, Government of India.

4.1 DRUGS AND CHEMICALS:

SU-6656, Ro 32-0432 hydrochloride, ammonium pyrrolidine dithiocarbamate (APD), RS 102895 and FTI-276 trifluoroacetate, Mecamylamine hydrochloride and nicotine tartrate (Sigma-Aldrich Chemicals Pvt. Ltd., St. Louis, USA), were dissolved / diluted in DMSO or sterile saline or prepared in triple distilled water. The chemicals used were of analytical grade.

4.2 INDUCTION OF NICOTINE WITHDRAWAL SYNDROME IN MICE:

Sub-acute administration of nicotine followed by a single injection of mecamylamine was used to induce nicotine withdrawal in mice (Damaj et al., 2003; Biala and Weglinska, 2005; Rehni et al., 2012; Singh et al., 2013a, b). Nicotine dependence was induced by repeated s.c. injections, four times daily, at an interval of 4 h starting at 08:00, for 7 days (2.5 mg/kg, s.c). The control groups were treated with saline following the same agenda. On the test day (day 8), mice received one nicotine injection in the break of the day. In an effort to precipitate nicotine abstinence mice were moved over the nicotine receptor antagonist.
mecamylamine (3 mg kg⁻¹, i.p.), 1 h after the last nicotine injection. The somatic signs of drawing were appraised for 30 min, immediately after mecamylamine administration.

Transparent perspex chamber with dimensions of 30 cm X 30 cm X 30 cm was used for making all observations. Two observers blind to the treatment schedule simultaneously observed each animal for all the withdrawal measures and the mean value of both the observations was recorded as data in the study. Direct systemic delivery of nicotine has been reported to most closely mimic the pharmacokinetics of inhaled nicotine from the cigarette smoke (Russell and Feyerabend, 1978). Therefore, the route of nicotine administration was selected sub cutaneously in the present study (Figure 1).

4.3 ASSESSMENT OF WITHDRAWAL SEVERITY SCORE (WSS):

A set of withdrawal severity score was employed to quantitate the magnitude of withdrawal syndrome in mice in terms of the earlier reported characteristic behavioral patterns seen in mice suffering from experimental nicotine withdrawal syndrome viz., grooming, scratching, chewing, fore paw tremor, wet dog shake, cage scratching, head nodding, paw licking, all in a composite manner (Damaj et al., 2003; Biala and Weglinska, 2005; Rehni et al., 2012; Singh et al., 2013a, b). The severity of the withdrawal phenomenon was graded on a scale of 0–24 (normal score, 0; maximal withdrawal severity score, 24). In each of the individual behavioral components of severity scores of withdrawal, 0 score point is awarded for no change in the normal behavior of mice with respect to each observation criteria, 1 score point is awarded for a mild increase in the respective observation criteria in mice, 2 score point is awarded for a moderate increase in the respective observation criteria in mice, 3 score point is awarded for a severe increase in the respective observation criteria in mice. Thus, the higher the score, the more severe is the withdrawal syndrome. The test was performed immediately after mecamylamine administration and the results were based on observations spanning first 30 minutes (Table 1; Figure 1).
4.4 ASSESSMENT OF NICOTINE WITHDRAWAL SYNDROME IN TERMS OF JUMPING FREQUENCY:

Administration of nicotine receptor antagonist mecamylamine (3 mg kg\(^{-1}\), i.p.) 1 h after the nicotine injection, induced stereotyped jumps. This has been considered as a major sign for quantification of Nicotine withdrawal syndrome in mice (Biala and Weglinska, 2005; Singh et al., 2013a, b). Jumping frequency observed in a period of 30 min was used as a quantitative symptom of Nicotine withdrawal immediately after mecamylamine administration.

4.5 ASSESSMENT OF NICOTINE-INDUCED HYPERALGESIA USING TAIL FLICK LATENCY:

Subcutaneous (s.c.) administration of nicotine produced an antinociceptive effect (i.e., an increase in pain thresholds) (Kiguchi et al., 2008; Singh et al., 2013a, b). Nociceptive inception was measured by the tail flick (D'Amour and Smith, 1941; Singh et al., 2013a, b). Time between tail exposure to radiant heat and its withdrawal is considered as tail flick latency. Radiant heat in the analgesiometer was obtained through the electrically heated nichrome wire. In order to obtain pretreatment latency between 2 and 3 sec in the animals the intensity of radiant heat was regulated. A cut off latency time was fixed at 10 sec. Therefore, the tail flick latency was observed five minutes afterwards, after mecamylamine administration.

4.6 ASSESSMENT OF ANXIETY LIKE BEHAVIOUR USING ELEVATED PLUS MAZE TEST:

Anxiety like behavior was monitored in mice using an elevated plus maze test (Navarro et al., 2006; Singh et al., 2013a, b). The plus maze consists of two open arms, 16 X 5 X 12 cm and two enclosed arms, 16 X 5 X 16 cm with an open roof, set up so that the two open arms are opposite to each other. The maze was elevated to a peak of 25 centimeter. The mouse is identified in the middle of the maze, facing one of the enclosed arms. During a 5 min observation, the following measures were taken in consideration: the number of entries into and time spent in
the central platform, open and enclosed arms of the maze. Along with these, frequency of stretched attend posture (SAP) and head-dipping (HD) were also measured.

A number of classical parameters were collected during the session (a) Open arm duration: the total amount of time the mouse spent in the open arms; (b) Closed arm duration: the total amount of time the mouse spent in the closed arms; and (c) Central platform duration: the total amount of time the mouse spent in the central platform. Likewise, different ethological measures were also quantified: (a) Stretched attend posture (SAP): a body posture in which the mouse stretches forward and then retracts to its original position without moving the feet, and (b) Head-dipping (HD): movement of the head over the side of the maze and down towards the floor.

The elevated plus maze test was performed 30 minutes after the administration of mecamylamine on day 8 of the nicotine dependence procedure to assess the level of anxiety like behaviour in mice before and after the treatment schedule is over.

4.7 ASSESSMENT OF NICOTINE WITHDRAWAL SYNDROME IN TERMS OF PILOERECTION FREQUENCY:

Piloerection frequency observations were taken in for a full stop of 30 min to quantitate the severity of the experimental withdrawal phenomenon immediately after mecamylamine administration. This parameter has been observed to be suggestive of the intensity of withdrawal syndrome as also described earlier (Damaj et al. 2003; Biala and Weglinska, 2005).

4.8 ASSESSMENT OF NICOTINE WITHDRAWAL SYNDROME IN TERMS OF BODY TREMOR FREQUENCY:

Body tremor frequency observations were taken in for a full stop of 30 min to quantitate the severity of the experimental withdrawal phenomenon immediately after mecamylamine administration. These parameters have been
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observed to be suggestive of the intensity of withdrawal syndrome as also described earlier (Damaj et al., 2003; Biala and Weglinska 2005).

4.9 EXPERIMENTAL PROTOCOL:

Twenty five groups were employed in the present study, with each group comprising of 08 animals out of which half were males and half females.

Group I-II (Vehicle-vehicle control): Vehicle (Saline, 10 ml/kg, i.p.) for Nicotine was administered four times daily for a period of seven days. Vehicles for the respective test compounds (10% DMSO in water or Saline for group I & II respectively, 10 ml/kg, i.p., once daily) for the same period of seven days. Vehicle (10 ml/kg, i.p.) for Mecamylamine was then injected on the morning of day 8, 1 hr after administering vehicle (10% DMSO in water or Saline for group I & II respectively, 10 ml/kg, i.p.) for nicotine.

Group III-IV (Vehicle-mecamylamine control): Vehicle (Saline, 10 ml/kg, i.p.) for Nicotine was administered four times daily for a period of seven days. Vehicles for the respective test compounds (10% DMSO in water or Saline for group III & IV respectively, 10 ml/kg, i.p., once daily) for the same period of seven days. Mecamylamine (3 mg/kg, i.p.) was then injected on the morning of day 8, 1 hr after administering vehicle (Saline, 10 ml/kg, i.p.) for nicotine.

Group V (Nicotine–Mecamylamine control): Nicotine (2.5 mg/ kg, s.c.) was administered four times daily for a period of seven days. Vehicle (10% DMSO in water, 10 ml/kg, i.p.) for respective test compound was simultaneously injected once daily for the same period of seven days. Mecamylamine (3 mg/kg, i.p.) was then injected on the morning of day 8, 1 hr. after administering Nicotine (2.5 mg/kg, s.c.)

Group VI (APD treatment + Vehicle- Mecamylamine control): Vehicle (Saline, 10 ml/kg, i.p.) for nicotine was administered four times daily for a period of 7 days. APD (100 mg/kg, i.p.) was simultaneously injected once daily for the same period of 7 days. Mecamylamine (8 mg/kg, i.p.) was then injected on the
morning of day 8, 1 hr. after administering vehicle (Saline, 10 ml/kg, i.p.) for Nicotine.

**Group VII-IX (APD treatment + nicotine-mecamylamine):** nicotine (2.5 mg/kg, s.c.) was administered four times daily for a period of seven days. APD (at a dose level of 10, 30 and 100 mg/kg/d, i.p. for groups number VII, VIII, and IX, respectively) was simultaneously injected once daily for the same period of seven days. Mecamylamine (3 mg/kg, i.p.) was then injected on the morning of day 8, 1 hr. after administering nicotine (2.5 mg/kg, s.c.).

**Group X (RS 102895 treatment + Vehicle- Mecamylamine control):** Vehicle (Saline, 10 ml/kg, i.p.) for nicotine was administered four times daily for a period of 7 days. RS 102895 (10 mg/kg, i.p.) was simultaneously injected once daily for the same period of 7 days. Mecamylamine (8 mg/kg, i.p.) was then injected on the morning of day 8, 1 hr. after administering vehicle (Saline, 10 ml/kg, i.p.) for Nicotine.

**Group XI-XIII (RS 102895 treatment + nicotine-mecamylamine):** nicotine (2.5 mg/kg, s.c.) was administered four times daily for a period of seven days. RS 102895 (at a dose level of 1, 3 and 10 mg/kg/d, i.p. for groups number XI, XII, and XIII, respectively) was simultaneously injected once daily for the same period of seven days. Mecamylamine (3 mg/kg, i.p.) was then injected on the morning of day 8, 1 hr. after administering nicotine (2.5 mg/kg, s.c.).

**Group XIV (SU-6656 treatment + Vehicle- Mecamylamine control):** Vehicle (Saline, 10 ml/kg, s.c.) for nicotine was administered four times daily for a period of 7 days. SU-6656 (1.0 mg/kg, i.p.) was injected once daily for the same period of 7 days. Mecamylamine (8 mg/kg, i.p.) was then injected on the morning of day 8, 1 hr. after administering vehicle (Saline, 10 ml/kg, s.c.) for nicotine.

**Group XV-XVII (SU-6656 treatment + nicotine-mecamylamine):** Nicotine (2.5 mg/kg, s.c.) was administered four times daily for a period of seven days. SU-6656 (at a dose level of 0.3, 1 and 3 mg/kg/d, i.p. for groups number XV, XVI, and XVII, respectively) was injected once daily for the same period of seven
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days. Mecamylamine (3 mg/kg, i.p.) was then injected on the morning of day 8, 1 hr. after administering nicotine (2.5 mg/kg, s.c.).

**Group XVIII (Ro 32-0432 hydrochloride treatment + Vehicle-Mecamylamine control):** Vehicle (Saline, 10 ml/kg, i.p.) for nicotine was administered four times daily for a period of 7 days. Ro 32-0432 hydrochloride (1 mg/kg, i.p.) was simultaneously injected once daily for the same period of 7 days. Mecamylamine (8 mg/kg, i.p.) was then injected on the morning of day 8, 1 hr. after administering vehicle (Saline, 10 ml/kg, i.p.) for Nicotine.

**Group XIX-XXI (Ro 32-0432 hydrochloride treatment + nicotine-mecamylamine):** Nicotine (2.5 mg/kg, s.c.) was administered four times daily for a period of seven days. Ro 32-0432 hydrochloride (at a dose level of 0.1, 0.3 and 1 mg/kg/d, i.p. for groups number XIX, XX, and XXI, respectively) was simultaneously injected once daily for the same period of seven days. Mecamylamine (3 mg/kg, i.p.) was then injected on the morning of day 8, 1 hr. after administering nicotine (2.5 mg/kg, s.c.).

**Group XXII (FTI-276 trifluoroacetate hydrochloride treatment + Vehicle-Mecamylamine control):** Vehicle (Saline, 10 ml/kg, i.p.) for nicotine was administered four times daily for a period of 7 days. FTI-276 trifluoroacetate hydrochloride (1 mg/kg, i.p.) was simultaneously injected once daily for the same period of 7 days. Mecamylamine (8 mg/kg, i.p.) was then injected on the morning of day 8, 1 hr. after administering vehicle (Saline, 10 ml/kg, i.p.) for Nicotine.

**Group XXIII-XXV (FTI-276 trifluoroacetate hydrochloride treatment + nicotine-mecamylamine):** Nicotine (2.5 mg/kg, s.c.) was administered four times daily for a period of seven days. FTI-276 trifluoroacetate hydrochloride (at a dose level of 0.1, 0.3 and 1 mg/kg/d, i.p. for groups number XXIII, XXIV, and XXV, respectively) was simultaneously injected once daily for the same period of seven days. Mecamylamine (3 mg/kg, i.p.) was then injected on the morning of day 8, 1 hr. after administering nicotine (2.5 mg/kg, s.c.).
Figure 10: **TEMPORAL SEQUENCE OF EVENTS FOR NICOTINE WITHDRAWAL SYNDROME ASSESSMENT IN MICE.**

Mecamylamine Administration

Assessment of withdrawal severity score, jumping frequency, Tremor & Piloerection (30')

Tail Flick Test

Tail Flick Test

Elevated Plus Maze Test

5'  5'  25'  5'
4.10 STATISTICAL ANALYSIS:

The results were shown as mean ± standard error of mean (S.E.M.). Data of the results were analyzed using ANOVA followed by post-hoc comparison using Sheffe’s test. For elevated plus maze, the numbers of entries and time (in seconds) spent in both arms was compared in vehicle- and nicotine-treated mice. A value of \( P<0.05 \) was considered to be statistically significant. The statistical analysis was performed using the Sigma Stat 6.0 software.