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Original Article

In vivo pharmacological profile of substituted (3-pyridyl)-2-phenylisoxazolidine analogues of nicotine as novel antinociceptives

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Abstract

Nicotinic ligands have been studied as novel therapeutic interventions in pain therapeutics since a long time. Several nicotinic agonists have been withdrawn from later stages of clinical trials due to lack of efficacy or narrow therapeutic window. These have been documented to act in the central nervous system and produce a wide range of pharmacological effects, including memory enhancing and analgesic actions, antianxiety, antidepressant and muscle coordination. Taking cognizance of the wide pharmacological profile of nicotine and its ligands, it was decided to evaluate some novel isoxazolidine analogues of nicotine for their potential as analgesics, using animal models like Eddy's hot plate and Tail immersion method. The compounds showed marked decrease in hyperalgesic response as compared to pentazocine at a wide range of doses. They were well tolerated as none of the compounds was found to have any seizure potential or mortality even at the highest doses. Thus, these compounds can be developed as potent antinociceptives with better safety profile than nicotine and other currently available pain therapeutics.

Keywords: Nicotine; Isoxazolidine; Antinociceptive activity; Opioids; Hyperalgesia

INTRODUCTION

Managing pain is one of the most serious unmet problems having enormous impact on both the individual and society. Pain is an unpleasant feeling caused by actual or perceived injury to body tissues and produces physical and emotional reactions. It has evolved to protect our bodies from harm by causing us to perform certain actions and avoid others. Thus, pain might simply be called a protector, a forecaster, or a scuffle (1). Currently, research in pain therapy concerns development of new potent antinociceptives having high efficacy comparable to morphine and other centrally acting analgesics and low adverse effects normally associated with opioids (respiratory depression, constipation, tolerance, and physical dependence liability) and non-opioids such as NSAIDS (gastro-intestinal lesions and nephrotoxicity) (2). Over the years, nicotine (1) has attracted tremendous interest on account of its ability to interact with acetylcholine receptors and for its potential in the treatment of a variety of conditions such as Alzheimer's disease, anxiety, adult attention deficit hyperactivity disorder (ADHD), depression, Parkinson's and Tourette's syndromes, ulcerative colitis, inflammatory bowel disorder, Schizophrenia and as antinociceptive (3,4).

Literature reports have shown that epibatidine (**2**), a naturally occurring chloro-pyridine derivative, isolated from the venom of the poison arrow frog *(Epipedobates tricolor*), is amongst the most potent antinociceptive nicotinic acetylcholinergic receptor (nAChR) ligands identified to date. Both of its isomers are potent full agonists of $\alpha4\beta2$, $\alpha3\beta2$, $\alpha3\beta4$, $\alpha7$, $\alpha8$ nAChRs and are found to be equally potent analgesics. However, this desirable activity is also accompanied by high toxicity. ABT-594 (**3**) and ABT-089 (**4**) are 3-pyridyl ethers, acting as nAChR agonist with potent antinociceptive and anxiolytic effects in rodent models (3, 5-7). This prompted considerable interest in identifying agents lacking the side effect liabilities of these compounds as analgesics.

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Exploiting the role of nicotine and other analogues as analgesic (Fig. 1), it was decided to evaluate the structurally resembling substituted (3-pyridyl)-2-phenylisoxazolidine analogues previously synthesised by us for their analgesic activity. In this study, the compounds were tested for their tolerability using rotarod apparatus and analgesic activity was evaluated using Eddy's Hot Plate and Tail immersion test, taking pentazocine (mixed agonist-antagonist analgesic of benzomorphan derivative having agonistic action at the κ and σ opioid receptors and weak antagonist action at the μ receptor) as standard (3,8,9).

MATERIALS AND METHODS

Animals

Young Swiss albino mice (3-4 months old), weighing 20-30 g from our own breeding stock were used. The animals were housed in groups in standard cages (1-5 per cage) and maintained at room temperature with natural day and night cycles.

Animals had free access to food (standard laboratory rodent's chow) and water during the study. All experiments were carried out between 07:00 to 16:00 h. A one-week habituation period was given to the animal room before testing. They were acclimatized with the laboratory conditions by handling them at least once a day during the period. Each group contained 6 mice. The experimental protocol was approved by the Institutional Animal Ethical Committee (IAEC) of CPCSEA.

Drugs and chemicals

Carboxymethylcellulose (CMC, s.d. Fine Chem Ltd.), pentazocine (Neon Laboratories Ltd), nicotinic analogues synthesized in the laboratory (10); *syn* – 5-hydroxymethyl-2- phenyl -3 -(3 -pyridyl) isoxazolidine (**6**), *anti*-5-methoxycarbonyl-2-phenyl-3-(3-pyridyl) isoxazolidine (**7**), *syn*-5-methoxycarbonyl-5- methyl- 2- phenyl - 3-(3-pyridyl) isoxazolidine (**8**) and *endo*-2,5-diphenyl-3-pyridin-3-yl-tetrahydro-pyrrolo (3,4-d) isoxazole-4,6-dione (**9,** Fig. 2). A suspension of various doses (5, 10, 15, 50, 100 and 150 mg/kg) of the compounds was prepared in 0.1% CMC solution. 0.9% saline was used as vehicle control. Dose of pentazocine injection (30 mg/kg) was made using distilled water. All the drugs were administered intraperitoneally (i.p.).

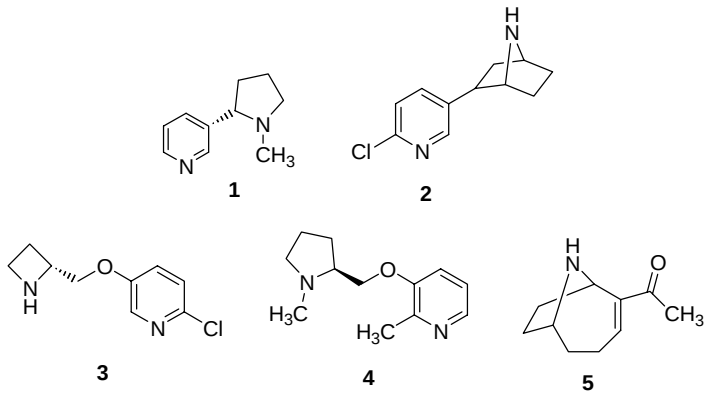

Fig. 1. Some antinociceptive nAChR ligands.

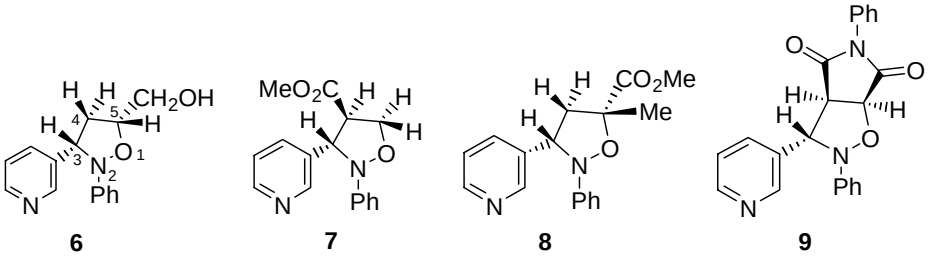

Fig. 2. Isoxazolidine analogues.

Tolerability testing

Neurotoxicity test using rotarod apparatus

Neurotoxicity at different doses of compounds was determined using rotarod apparatus. Mice which were able to remain on the rotating rod at 10 rpm for 5 min or more were selected and divided into different groups and received varying doses of compounds **6, 7, 8** and **9**. All animals were placed on the rotating rod after 30 min of treatment with different doses of compounds. Neurotoxicity was assessed as inability of the animal to maintain equilibrium on rotating rod for at least 3 min. A speed (10 rpm) of rotating rod was set to evaluate the neurotoxic effect of compounds affecting normal minimum muscle movement required to maintain the posture on rotating rod (11,12).

Seizure threshold and acute toxicity testing

The compounds were administered at different doses, up to ten times of the effective doses i.e. 50, 100 and 150 mg/kg of all the compounds, to different groups of mice. Occurrence of seizure, mortality percent and gross behavioral changes were observed during 24 h after administration of the compounds (11,12).

Antinociception assessment

Eddy's Hot Plate

The animals divided into 8 groups (containing 6 animals each) were individually placed on the hot plate maintained at constant temperature of 55 ± 0.5°C. The response time was noted as the time at which animals reacted to the pain stimulus either by paw licking or jump response, whichever appeared first. A cut-off period of 15 sec was observed to avoid damage to the paws (2,9). The mice were administered varying doses of compounds **6, 7, 8, 9**, pentazocine and vehicle as mentioned earlier. Paw licking/jumping response in mice was observed in every group, 5, 15, 30, 45, 60, 120 min after the injection on the analgesiometer. Increase in reaction time was considered as index of analgesia in treated groups as compared to that of control group.

Tail immersion test

Tail immersion test was used to assess hyperalgesic effect in mice. Similarly, animals were divided into 8 groups with 6 animals each.

The terminal 1-2 cm part of the tail was immersed in a water bath maintained at 55 ± 0.5°C. The withdrawal latency was defined as the time for the animal to withdraw its tail from hot water (2,9). Cut off time of 10 seconds was maintained to avoid damage to the tail for all groups. The mice were administered varying doses as mentioned earlier of compounds **6, 7, 8, 9,** pentazocine and vehicle. The time required for flicking off the tail, was recorded, to assess response to noxious stimulus in every group, 5, 15, 30, 45, 60, 120 min after the injection. Hyperalgesic response was defined when the average latency of tail flick response was significantly decreased in treated group as compared to that of control group.

Statistical analysis

All the results were expressed as mean ± standard error (S.E.M.). Data was analyzed using Student's t-test or one way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test (InStat Software, 3.10). P-values of <0.05 were considered statistically significant for all comparisons.

RESULTS

Tolerability studies of the compounds

Neurotoxicity testing

In neurotoxicity test, the doses (5, 10, 15 and 50 mg/kg) of all compounds were found to have no neurotoxic effect but at the higher doses (100 and 150 mg/kg), the compounds were found to be neurotoxic as indicated by the earlier fall off time of mice from the rod (Table 1).

Acute toxicity testing

No mortality and no occurrence of seizures were observed in mice treated with all doses of the above compounds. There was no change in behavior of mice during 24 h of administration of the different doses of compounds (Table 1).

Effect on nociceptive activity

Eddy's hot plate

There was significant dose and time dependent initial increase (at lower doses of all the compounds **6, 7, 8** and **9**) and then gradual decrease (at higher doses of all the compounds) in the paw licking/jumping time in mice as compared to control and pentazocine groups. It was found that maximum analgesic response occurred after 15 minutes of administration of the compound **6** (10 mg/kg dose), compound **7** (15 mg/kg dose), compound **8** (15 mg/kg dose) and compound **9** (15 mg/kg dose) when compared to vehicle control and standard drug respectively. However, at higher doses of all the compounds (100 and 150 mg/kg), the action was found to be reversed (Figs. 3-6).

Tail immersion method

There was significant dose and time dependent initial increase (at lower doses of all the compounds **6, 7, 8** and **9**) and then gradual decrease (at higher doses of all the compounds **6, 7, 8** and **9**) in the tail flicking time in mice as compared to control and pentazocine groups.

It was found that maximum analgesic response occurred after 15 min of administration of the compound **6** (10 mg/kg dose), compound **7** (15 mg/kg dose), compound **8** (15 mg/kg dose) and compound **9** (15 mg/kg dose) when compared to vehicle control and standard drug respectively. However, at higher doses of the compounds (100 and 150 mg/kg), the action was found to be reversed (Figs. 7-10).

Table 1: Tolerability testing of different doses of compound **6, 7, 8** and **9**.

Dose (mg/kg)	Neurotoxicity test (time in sec)				Mortality	Seizure potential
	Compound 6	Compound 7	Compound 8	Compound 9	Compounds 6, 7, 8 & 9	Compounds 6, 7, 8 & 9
5	(>180)	- (>180)	- (>180)	- (>180)	-	-
10	- (>180)	- (>180)	- (>180)	- (>180)	-	-
15	- (>180)	- (>180)	- (>180)	- (>180)	-	-
50	- (>180)	- (>180)	- (>180)	- (>180)	-	-
100	+ (102)	+ (97)	+ (82)	+ (128)	-	-
150	+ (139)	+ (113)	+ (99)	+ (157)	-	-

−; negative test, +; positive test

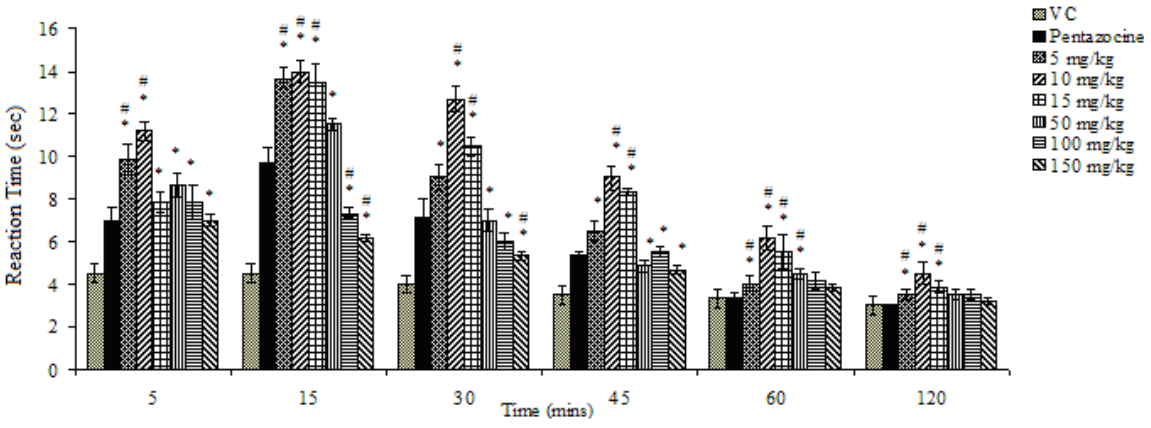

Fig. 3. Effect of varying doses of compound **6** (5, 10, 15, 50, 100 and 150 mg/kg) at various time intervals (5, 15, 30, 45, 60 and 120 min) on the reaction time in mice, *represents P<0.05 as compared to vehicle control (VC), #represents P<0.05 as compared to pentazocine.

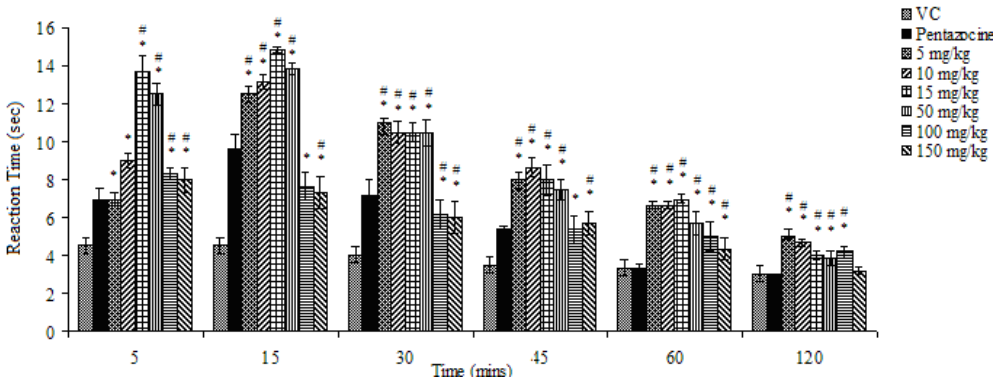

Fig. 4. Effect of varying doses of compound **7** (5, 10, 15, 50, 100 and 150 mg/kg) at various time intervals (5, 15, 30, 45, 60 and 120 min) on the reaction time in mice, *represents P<0.05 as compared to vehicle control (VC), #represents P<0.05 as compared to pentazocine.

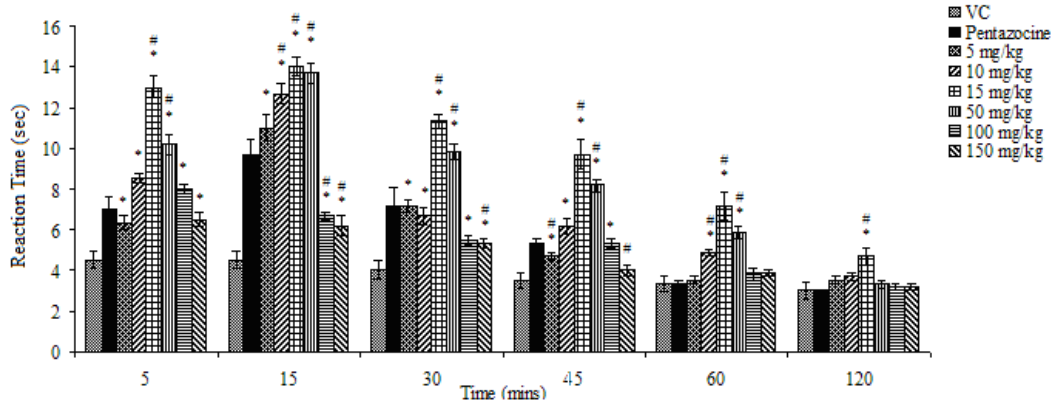

Fig. 5. Effect of varying doses of compound **8** (5, 10, 15, 50, 100 and 150 mg/kg) at various time intervals (5, 15, 30, 45, 60 and 120 min) on the reaction time in mice, *represents P<0.05 as compared to vehicle control (VC), #represents P<0.05 as compared to pentazocine.

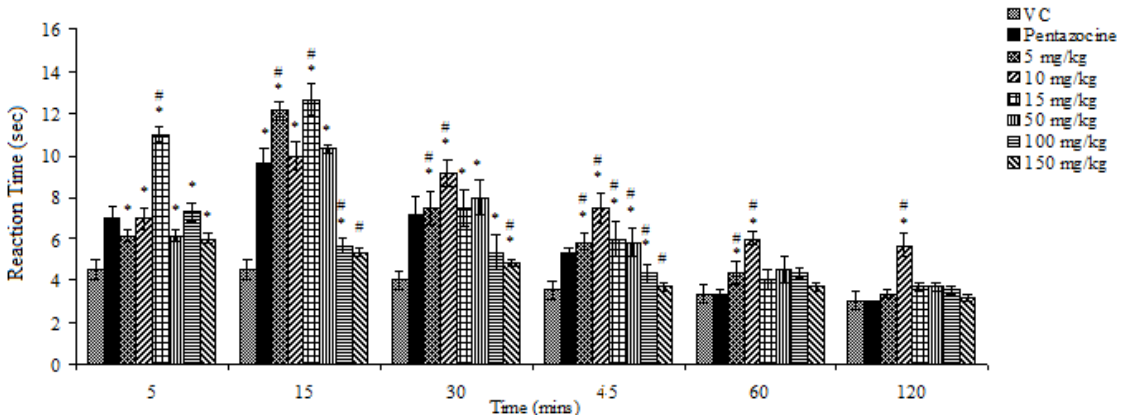

Fig. 6. Effect of varying doses of compound **9** (5, 10, 15, 50, 100 and 150 mg/kg) at various time intervals (5, 15, 30, 45, 60 and 120 min) on the reaction time in mice, *represents P<0.05 as compared to vehicle control (VC), #represents P<0.05 as compared to pentazocine.

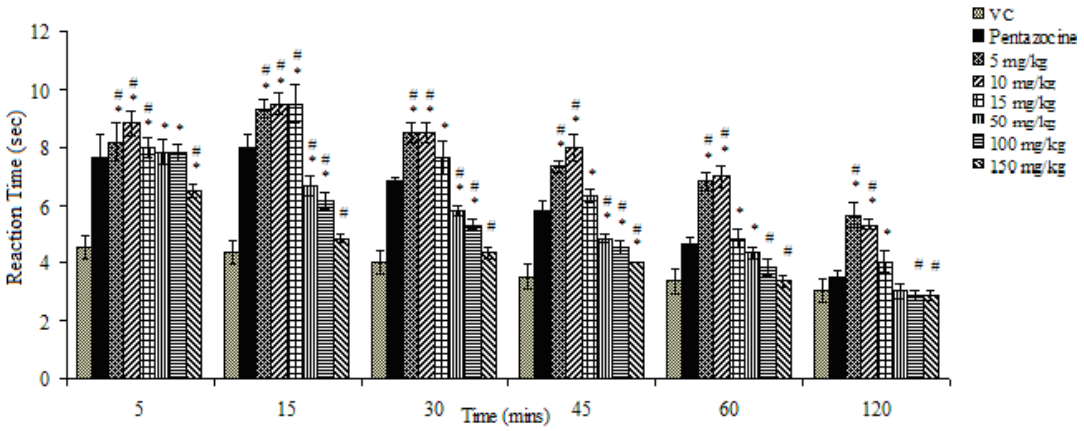

Fig. 7. Effect of varying doses of compound **6** (5, 10, 15, 50, 100 and 150 mg/kg) at various time intervals (5, 15, 30, 45, 60 and 120 min) on the reaction time in mice, *represents P<0.05 as compared to vehicle control (VC), #represents P<0.05 as compared to pentazocine.

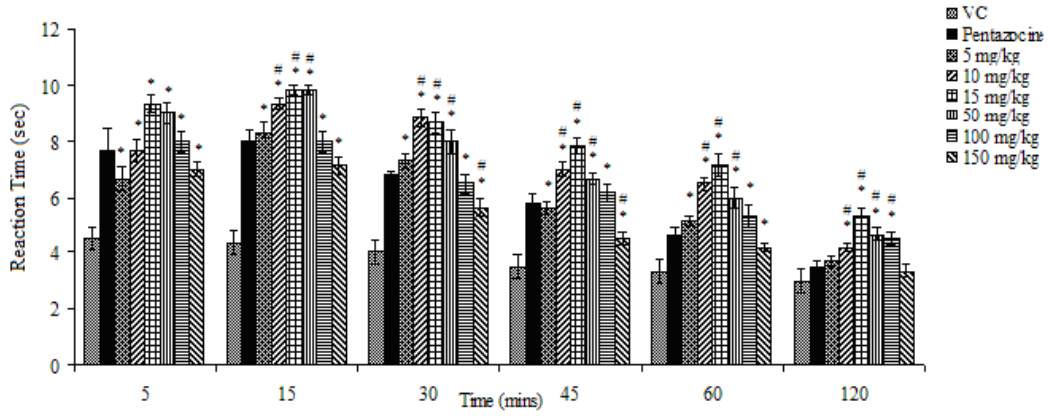

Fig. 8. Effect of varying doses of compound **7** (5, 10, 15, 50, 100 and 150 mg/kg) at various time intervals (5, 15, 30, 45, 60 and 120 min) on the reaction time in mice, *represents P<0.05 as compared to vehicle control (VC), #represents P<0.05 as compared to pentazocine.

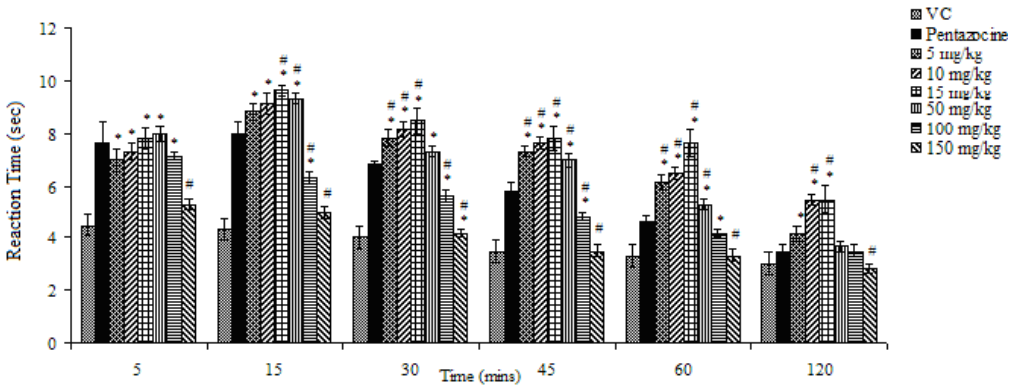

Fig. 9. Effect of varying doses of compound **8** (5, 10, 15, 50, 100 and 150 mg/kg) at various time intervals (5, 15, 30, 45, 60 and 120 min) on the reaction time in mice, *represents P<0.05 as compared to vehicle control (VC), #represents P<0.05 as compared to pentazocine.

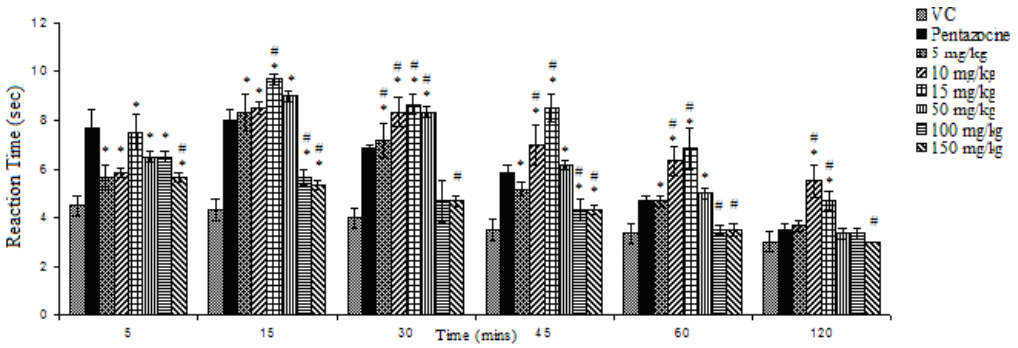

Fig. 10. Effect of varying doses of compound **9** (5, 10, 15, 50, 100 and 150 mg/kg) at various time intervals (5, 15, 30, 45, 60 and 120 min) on the reaction time in mice, *represents P<0.05 as compared to vehicle control (VC), #represents P<0.05 as compared to pentazocine.

DISCUSSION

Despite much effort, there are so far no available pharmacotherapies providing satisfactory pain relief for patients with persistent chronic pain. Multiple mechanisms are involved in the management of pain. Hence, understanding the mechanisms underlying this syndrome is critical to permit the discovery of new molecular targets with the intent to develop effective analgesic drugs. However, with the advancements in the knowledge about nAChRs and their sub types, and their involvement in cognition, learning, memory, arousal, reward, motor control and analgesia, these have become interesting targets for newer compounds.

Further studies based on three dimensional quantitative structure activity relationships (3D-QSAR) methods like comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) carried out by Gohlke and coworker which applied to α2β4 nAChR ligands have shown correlation between their steric, electrostatic, hydrophobic, hydrophilic and hydrogen bonding properties (13). Both CoMFA and CoMSIA have helped to relate differences in molecular structure to differences in binding affinities and the change in protonation state affecting all molecules equally.

The CoMFA contour of the chloro substituted epibatidine indicates that this region is favourable for steric occupancy and CoMSIA contour includes part of the aromatic ring system. It also revealed the importance of *para*-substitution to the aromatic ring as the substituted one has higher binding affinity than the unsubstituted ones. These studies also comply well with the previously postulated 'ensemble' approach applied to distance geometry for deducing the pharmacophore for nAChR ligands. This pharmacophoric model includes a quaternary or protonatable nitrogen (e.g., of the aliphatic bicycle in anatoxin-a, **5**), an electronegative atom as hydrogen bond acceptor (e.g., the pyridine nitrogen of epibatidine or a carbonyl oxygen of the acetyl group in anatoxin-a) and a pair of atoms that form a dipole (13,14). The isoxazolidines of nicotine may fulfil the desirable pharmacophore necessary for binding to receptors.

The current investigation of nicotinic analogues for antinociceptive activity showed that there was significant time dependent initial increase (at lower doses of all the compounds **6, 7, 8** and **9**) and then gradual decrease (at higher doses of all the compounds **6, 7, 8** and **9**) in the paw licking/jumping time and tail flick time in mice compared to control and pentazocine groups and maximum analgesia occurred 15 minutes after the i.p. administration. The responses, however, reversed in the case of the higher doses of all the compounds. This may be attributed to the biphasic/agonist-antagonist action of the nicotinic analogues. This is in complacence with the agonist antagonist action of pentazocine on opioid receptors. The compounds (**6**-**9**) were more potent than pentazocine at lower doses which further validates their use as centrally acting antinociceptives. Antinociceptive activity of

synthetic pyrazolyl isoxazolines and isoxazoles on Tail Flick method using pentazocine as standard drug have been reported and these have shown similar results (15,16). Light tail flick method has also been employed to study the antinociceptive effect of extracts of *Platanus orientalis* leaves and aerial parts of *Stachys lavandulifolia* in mice (17,18).

On further comparison of efficacy of the four compounds, it was found that compound **6** showed maximum analgesia at 10 mg/kg dose with both Eddy's hot plate and tail immersion method. However, compounds **7, 8** and **9** showed maximum activity at 15 mg/kg dose with both the models. The compound **6** was found to be most active amongst the four tested compounds. The di-substituted (**8**) and fused (**9**) compounds were found to be less efficacious than their mono-substituted (**6** and **7**) counterparts.

Also, all the doses of compounds tested for their tolerability did not show any convulsions or death as reported for nicotine (19). The effective doses of the compounds did not show any neurotoxicity. The compounds showed no withdrawal induced anxiogenesis which strengthens the further prospects of development of these compounds as novel antinociceptives. However, further studies are being carried out to know the exact mechanism of action of these analogues.

CONCLUSION

The current study revealed that novel isoxazolidine analogues of nicotine are promising candidates for their use as antinociceptives. The compounds are well tolerated, with no mortality, seizures, anxiogenic behaviour and are more efficacious than pentazocine at their effective doses. Out of the four tested compounds, *syn*-5-hydroxymethyl-2-phenyl- 3 - (3-pyridyl) isoxazolidine (**6**) is more active than the other three *i.e. anti*-5-methoxycarbonyl-2-phenyl-3-(3-pyridyl) isoxazolidine (**7**) *syn*-5-methoxycarbonyl-5-methyl-2-phenyl-3-(3-pyridyl) isoxazolidine (**8**) and *endo*-2, 5-diphenyl-3-pyridin-3-yl-tetrahydro-pyrrolo (3,4-d) isoxazole-4,6-dione (**9**). It may therefore be concluded that the nicotinic analogues could serve as useful leads in the design and synthesis of potent and safe nicotinic analogues for mitigation of analgesic states.

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