SECTION 7:

DISCUSSION
In the present investigation, administration of SU-6656, a selective inhibitor of src family kinases (Blake et al., 2000); ammonium pyrrolidine dithiocarbamate (APD), a selective inhibitor of NF-κB (Schreck et al., 1992); RS 102895, a selective CCR-2 chemokine receptor antagonist (Mirzadegan et al., 2000; Onuffer et al., 2002); tributyrin, a selective inhibitor of histone deacetylase (Chen and Breitman, 1994; Rocchi et al., 2005); trichostatin A, another chemically distinct, potent and selective inhibitor of histone deacetylase (Yoshida et al., 1995); N-Acetyl-Asp-Glu-Val-Asp-al (Ac-DEVD-CHO; ADC), a selective interleukin-1β converting enzyme inhibitor (Margonin, 1997); sodium orthovanadate, a competitive inhibitor of protein tyrosine phosphatase (McLauchlan et al., 2010; Sugano et al., 2004); and SJA 7019, a selective competitive non-peptide inhibitor of calpain (Liu et al., 2002), produced a significant dose dependent attenuation of the development of morphine dependence as observed in the naloxone induced withdrawal syndrome in morphine dependent mice, particularly in terms of stereotyped jumping behavior, withdrawal severity score, rearing activity, forepaw licking and circling behavior. Further, SU-6656; APD; RS 102895; tributyrin; trichostatin A; sodium orthovanadate and SJA 7019 treatment produced a significant dose dependent attenuation of the naloxone induced withdrawal contracture in morphine dependent rat ileum in vitro, particularly in terms of tension ration results. However, SU-6656; APD; RS 102895; tributyrin; trichostatin A; Ac-DEVD-CHO; sodium orthovanadate or SJA 7019 administration did not demonstrate any alteration of CNS activity as assessed in terms of locomotor activity count thus ruling out their per se sedative activity on mice. Moreover, SU-6656; APD; RS 102895; tributyrin; trichostatin A; Ac-DEVD-CHO; sodium orthovanadate or SJA 7019 pretreatment did not alter the acute analgesic effect of morphine.
Src family kinases are non-receptor, cytoplasmic, protein tyrosine kinases that are one of the principal mediators of intracellular signal transduction system inside the cell cytosol (Schlessinger, 2000). Src protein tyrosine kinase family mediates a regulatory influence on cell proliferation, differentiation, survival, metabolism, and other essential functions of the cells. Src phosphorylation has been shown to regulate switching of opioid receptors from an inhibitory to a stimulatory signal which induces a sudden efflux of impulses from locus coeruleus region of brain that emanate to different parts of the central nervous system and ultimately culminate into the precipitation of opioid withdrawal syndrome (Zhang et al., 2009). Moreover, src has been shown to regulate the activity and recycling of opioid receptors in the neurons (Walwyn et al., 2007). Further, src-kinase has been shown to mediate the development of opioid tolerance and is proposed to mediate the progression of opioid dependence (Garzón et al., 2008; Sánchez-Blázquez et al., 2009). This contention is further supported by our investigation with SU-6656, a selective inhibitor of src family kinases (Blake et al., 2000), the treatment of which, markedly suppressed the intensity of the naloxone precipitated withdrawal syndrome in morphine dependent mice. Therefore, it may be deduced that the activation of src family kinases (Src, Yes, Lyn, and Fyn) is involved in the precipitation opioid withdrawal syndrome. The results of the present investigation further demonstrated that the treatment of SU-6656, a selective inhibitor of src-kinase, to the morphine dependent rat ileum was able to effectively suppress the magnitude of the withdrawal contracture. Thus, it is proposed that the withdrawal syndrome induced by morphine treatment followed by a naloxone challenge may involve src-kinase activation.

Various signal transduction systems have been reported to be secondarily linked to the activation of src-kinase. Studies have implicated the importance of a mechanistic link
between of src-kinase and neurotrophin receptor tyrosine kinases in regulating a multitude of diverse biological processes (Huang et al., 2010). Further, cytokines related activation of various signaling cascades have been ascribed to the src-kinase activity as well (Li et al., 2009). It may be put forth that these src-kinase linked biochemical events might be playing a potential role in causing the src—kinase dependent development of opioid withdrawal syndrome.

NF-κB is a transcription factor which causes the activation of multiple upstream signals to the nucleus, resulting in the regulation of a number of NF-κB-dependent genes responsible for the transcription of cytokines, which are in turn implicated in mediating various effects of opioid drugs on the central nervous system (Baeuerle, 1991; Chen et al., 2006). NF-κB and related factors have been reported to be transcribed in various cell types present in the brain (O’Neill and Kaltschmidt, 1997). Moreover, Capasso et al (2001) have shown the inhibitory effect of NF-κB modulator on an in vitro model of opioid dependence. Further, in a previous study we have demonstrated ameliorative effect of diethyl dithiocarbamic acid, a relatively selective NF-κB inhibitor, on opioid withdrawal syndrome in mice (Rehni et al., 2008a). This evidence is further supported by our present investigation in which, the administration of APD, a highly selective NF-κB inhibitor (Schreck et al., 1992), has been observed to abolish the naloxone induced opioid withdrawal syndrome in morphine dependent mice, thus further validating the potential involvement of NF-κB in the opioid withdrawal syndrome. Furthermore, in consonance with the findings of Capasso et al. (2001) we observed that APD dose dependently attenuated naloxone induced response in morphine dependent rat ileum thus adding to the body of data affirming the significance of NF-κB in opioid withdrawal syndrome.
Chemokines are chemotactic cytokines that direct the progression of inflammatory processes by mediating the migration of cells that express the appropriate chemokine receptors. Chemokine C-C motif ligand 2 (CCL2) is a potent attractant protein for inflammatory cells. The biological effects of CCL2 are mediated via interactions with its receptor, chemokine C-C motif receptor 2 (CCR2) which is a G protein-coupled receptor and regulates the migration and infiltration of monocytes, T-lymphocytes and natural killer cells to regions of inflammation (Matsushima et al., 1989; Allavena et al., 1994; Carr et al., 1994). In the central nervous system, CCR2 expression has been demonstrated in endothelial cells, astrocytes, microglia and neurons (Boddeke et al., 1999). In addition, CCR2 expressing cells are observed in multiple brain regions including the hippocampus (Banisadr et al., 2005) and the expression patterns of CCR2 have shown to be altered in various neuropathological conditions viz multiple sclerosis (McManus et al., 1998), HIV encephalopathy (Conant et al., 1998; Kelder et al., 1998), Alzheimer's disease (Sokolova et al., 2009) and epilepsy (Wu et al., 2008). Inflammatory processes have been proposed to mediate the amplification of the effect of basic transduction mechanisms involved in mediating sub-cellular and biochemical changes occurring during the development of opioid abuse related withdrawal syndrome (Capasso, 2001). Moreover, CCR2 activation has been proposed to mediate the development of opioid abuse related complications like human immunodeficiency virus-1 neuropathogenesis (El-Hage et al., 2006). Further, the release of chemokines has been reported to be intrinsically associated with opioid receptor transduction systems (Avdoshina et al., 2010; White and Wilson, 2010). Therefore, chemokine receptor activation might participate in the progression of opioid withdrawal syndrome. Results of the present study demonstrate that treatment of RS 102895, a selective CCR-2 chemokine receptor antagonist, produced a significant dose dependent attenuation of the development of naloxone.

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-precipitated opioid withdrawal syndrome in both in vivo model as well as in vitro model. In addition, the isobolographic study design and analysis affirmed the presence of a synergistic interaction between opioid withdrawal inhibiting effect of APD and RS 102895. A synergistic interaction between APD and RS 102895 is indicative of a potential link between the mechanisms of the two pharmacological agents by which they enhance each other’s opioid withdrawal syndrome inhibiting potential. However, the exact biochemical process, which leads to this observed synergistic interaction between the inhibition of NFκB and the antagonism of CCR-2 chemokine receptors in attenuating the opioid withdrawal syndrome, needs to be explored by future studies.

Studies have implicated the involvement of histone deacetylase in mediating cAMP-CREB-AC8 pathway dependent super activation of adenylyl cyclase and resultant up-regulation of the cAMP pathway (Liu and Anand, 2001; Nestler, 2004). This compensatory increase in AC activity in the locus coeruleus has been classically associated with opioid dependence development and the withdrawal syndrome (Sharma et al., 1977; Liu and Anand, 2001; Nestler, 2004). Further, opioid receptor regulation, an important molecular event leading to the development of opioid dependence, has been reportedly ascribed to the histone deacetylase mediated epigenetic control of transcription promoters (Hwang et al., 2007). Additionally, pharmacological inhibition of histone deacetylase has been shown to enhance the transcription of opioid receptors in neurons (Hwang et al., 2007). Thus, it is suggested that the activation of histone deacetylase plays an important role in mediating the progression of opioid dependence and that the pharmacological manipulation of these enzymes might exert an ameliorative effect on opioid withdrawal induced abstinence syndrome in mice. This contention is further supported by our investigation with tributyrin, a selective inhibitor of histone deacetylase (Chen and Breitman, 1994; Rocchi et al., 2005), as well as trichostatin A,
another chemically distinct, potent and selective inhibitor of histone deacetylase (Yoshida et al., 1995), the treatment(s) of which, markedly suppressed the intensity of the naloxone precipitated withdrawal syndrome in morphine dependent mice. Therefore, it is deduced that the activation of histone deacetylase might be involved in the precipitation opioid withdrawal syndrome induced by sub-chronic morphine administration followed by a naloxone challenge. This observed conclusion was further affirmed by the present in vitro data which showed that tributyrin as well as trichostatin A dose dependently attenuated naloxone induced withdrawal contracture in morphine dependent rat ileum. The present findings are in line with previous studies showing that histone deacetylase inhibition results in the reversal of a number of measures of behavioral plasticity linked with opioid addiction viz., behavioral sensitization associated with single morphine exposure (Jing et al., 2011), morphine-induced locomotor sensitization and conditioned place preference (Sanchis-Segura et al., 2009; Wang et al., 2010). Therefore, the present data in corroboration with the previous studies implicates that histone deacetylase mediated deacetylation of histone protein might be associated with the pathogenesis of the development of opioid withdrawal syndrome in opioid dependent subjects.

Histone deacetylase inhibition is noted to modulate the activity of CREB by an interleukin 1-β converting enzyme (IL-1β) associated pathway (Suzuki et al., 2003). Further, it has been observed that an elevated level of hippocampal expression of IL-1β is associated with opioid withdrawal induced jumping behavior in mice (Liu et al., 2011). Therefore, interleukin-1β converting enzyme over-activation might be put forth to be involved in the potential histone deacetylase linked precipitation of opioid dependence related withdrawal syndrome. This contention is further supported by our investigation with N-Acetyl-Asp-Glu-Val-Asp-al (Ac-
DEVD-CHO; ADC), a selective interleukin-1β converting enzyme inhibitor (Margonin, 1997), the treatment of which, markedly suppressed the intensity of the naloxone precipitated withdrawal syndrome in morphine dependent mice. In addition, the isobolographic study design and analysis affirmed the presence of a synergistic interaction between opioid withdrawal inhibiting effect of ADC and trichostatin A. A synergistic interaction between ADC and trichostatin A is indicative of a potential link between the mechanisms of the two pharmacological agents by which they enhance each other’s opioid withdrawal syndrome inhibiting potential. Therefore, it is deduced that the activation of histone deacetylase might be involved in the precipitation opioid withdrawal syndrome by an IL-1β associated pathway.

O’Connor et al. (1995) has suggested that tyrosine phosphatase induced dephosphorylation is required for the activation of c-SRC, which in turn mediates the biochemical processes, leading thereof to AC super-activation and opioid withdrawal syndrome. Therefore, the tyrosine phosphatases might also be mediating the opioid receptor-Gα_{12}-c-SRC-Src-kinase complex dependent switching of G-proteins and thus can serve as a potential target for pharmacological modulation of opioid withdrawal syndrome. A recent study by Ishiguro et al. (2008) has recently revealed a Ser127Gly polymorphism on the chromosome 12 loci, that is responsible for the transcription of protein tyrosine phosphatase receptor type beta (PTPRB), associated with substance abuse vulnerability in man. Moreover, chronic morphine treatment has been shown to up-regulate the expression of PTPRB in rodents (Ishiguro et al., 2008). Thus, it is suggested that the activation of tyrosine phosphatase might play an important role in mediating the progression of opioid dependence and that the pharmacological manipulation of this enzyme would exert an ameliorative effect on opioid withdrawal induced abstinence syndrome in rodents. This contention is further supported by
our investigation with sodium orthovanadate, a selective inhibitor of tyrosine phosphatase (Sugano et al., 2004; McLauchlan et al., 2010), the treatment of which, markedly suppressed the intensity of the naloxone precipitated withdrawal syndrome in morphine dependent mice. This observed conclusion was further affirmed by the present in vitro data which showed that sodium orthovanadate dose dependently attenuated naloxone induced withdrawal contracture in morphine dependent rat ileum. Moreover, a subtype of PTPases has been reported to be up regulated in the locus coeruleus region of the brain as a result of chronic opioid receptor activation linked superactivation of cAMP pathway or CREB (McClung et al., 2005). Therefore, the pharmacological inhibition of PTPases induced reversal of opioid withdrawal may be ascribed to the blockade of PTPases related end effector pathways that were leading to the excitation of the neurons located in the locus coeruleus region in brain.

Protein tyrosine phosphatases are categorized into four classes: the classical receptor PTPs (RPTPs); the classical non-receptor PTP (nrPTPs); the dual specificity PTP (dsPTPs) and the low Mr PTPs (Alonso et al., 2004). These subtypes of tyrosine phosphatases have been shown to modulate the activity of various cytokines, neurotransmitters, secondary messengers and adhesion molecules which have in turn been shown to modulate the progression of opioid dependence and related opioid withdrawal syndrome (Ishiguro et al., 2008; Zhou et al., 2010). However, the delineation of such tyrosine phosphatase dependent factors in causing the pathological condition requires a systematic study. Tyrosine phosphatases have been shown to modulate the activity of various cytokines, neurotransmitters, secondary messengers and adhesion molecules which might further be involved in modulating the progression of opioid dependence and related opioid withdrawal syndrome (Alonso et al., 2004; Ishiguro et al., 2008; Zhou et al., 2010).
Sato-Kusubata *et al.* (2000) have described that calpain, a calcium activated neutral cysteine endopeptidase, catalyses the proteolytic activation of Gαs proteins which mediate adenylyl cyclase super-activation linked causation of opioid withdrawal syndrome (Trujillo and Akil, 1991; Trujillo and Akil, 1995; Zang *et al.*, 2000; Sweitzer *et al.*, 2004a; Esmaeili-Mahani *et al.*, 2008; Drdla *et al.*, 2009; Zadran *et al.*, 2010). Therefore, calpains may mediate the biochemical progression of opioid dependence based withdrawal syndrome. This point is further supported by our investigation with SJA 7019, a competitive inhibitor of calpains (Liu *et al.*, 2002), the treatment of which, markedly suppressed the intensity of naloxone precipitated withdrawal syndrome in morphine dependent mice. This observed conclusion was further affirmed by the present *in vitro* data which showed that SJA 7019 dose dependently attenuated naloxone induced withdrawal contracture in morphine dependent rat ileum. Therefore, it is deduced that calpain activation might be involved in the development of physiological dependence associated precipitation of opioid withdrawal syndrome.

Phosphatases are one of the substrate proteins activated by calpain system (Goll *et al.*, 2003). Studies have shown that calpains modulate protein-tyrosine phosphorylation by causing a proteolytic stimulation of protein-tyrosine phosphatase α and ε (Ariyoshi *et al.*, 1995; Gil-Henn *et al.*, 2001). Additionally, µ-calpains have been shown to regulate tyrosine dephosphorylation events by a calcium dependent process downstream of αIIb/β3 integrins by causing activation of protein tyrosine phosphatase (PTP)-1B (Ragab *et al.*, 2003).

Therefore, it is hypothesized that calpains, which are potentially involved in the mediation of opioid withdrawal syndrome, might be causing the activation of tyrosine phosphatase which in turn be causing the development of adenylyl cyclase super-activation based mediation of development of physiological dependence associated precipitation of opioid withdrawal.
Conclusion

On the basis of the above discussion, it may be concluded that the inhibition of src-kinase; nuclear factor kappa B; chemokine CCR-2 receptor; histone deacetylase; interleukin-1-β converting enzyme; tyrosine phosphatase and calpain attenuates the development of morphine dependence as observed in the naloxone-induced precipitation of withdrawal symptoms in morphine dependent mice as well as withdrawal response in isolated rat ileum preparation (Figure A).
Figure A: Proposed mechanisms of opioid withdrawal syndrome and its modification by various novel interventions.