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Design and development of 1,3,5-triazine derivatives as protective agent against spinal cord injury in rat via inhibition of NF-κB

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ABSTRACT

Spinal cord injury (SCI) is a chronic disease causing motor and sensory loss in the affected individuals. The SCI has a huge impact on the lives of patients that makes them susceptible to life-long disability. However, the current clinical modalities are ineffective to cope the aftermath of SCI. Thus, in the present study, we aimed to develop a series of 1,3,5-triazine derivatives as a protective agent against SCI. The molecules were developed by facile synthetic route and obtained in excellent yield. The compounds were tested for their efficacy to inhibit the transcription of NF-κB in RAW 264.7 cells, where they displayed mild to potent activity. Compound **8a** was identified as most potent NF-κB inhibitor among the tested analogues. The effect of compound **8a** was further scrutinized against the SCI injury in rats induced by contusion injury. It has been found that compound **8a** improves motor function of rats together with reduction in inflammation and edema in spinal cord of rats. It also showed to inhibit oxidative stress and inflammation in the SCI rats. In a western blot analysis, after SCI induction, compound **8a** inhibited NF-κB and its upstream regulator TLR4 in a dose-dependent manner. Collectively, our study provides a novel class of agent that provide protective action against SCI.

The injury of spine which is better known as spinal cord injury (SCI) is a chronic disease causing motor and sensory loss in the affected individuals. The SCI has a huge impact on the lives of patients that makes them susceptible to life-long disability. This has a serious financial implication on the patients and their families. Accumulating shred of evidences suggest that SCI arises in the course of mechanical insult which initiate cascade of events classified as primary and secondary injury.[1] The primary injury mainly causes damage to the neurons that cannot be regenerated; whereas, secondary injury which subsequently occurs after primary injury considered as more serious but preventable, if treated well in time. Thus, the clinical approach to treat SCI is mainly reliant upon controlling the impact of secondary injury, such as inflammation, oxidative stress and apoptosis of neurons.[2,3]

Studies have suggested that oxidative stress plays a significant role in the patho-physiology of SCI which further aggravate the clinical status of the patients.[4] The over-production of free radicals coupled with low level of antioxidants is considered as a main cause of oxidative stress. It is considered as extremely aggressive to the surrounding tissues by inducing lipid-peroxidation, increased infiltration, activation of the nuclear enzyme PARP, depletion of nicotinamide adenin dinucleotide and ATP, and ultimately cell death.[5] It also promotes inflammation which is deemed as a distinctive feature of SCI. It plays a critical role in the pathogenesis of acute and chronic SCI which leads to tissue damage and neurodegeneration via the activation of innate immune response. This activated immune system causes oligodendrocyte apoptosis, demyelination, axonal de-generation, and neuronal death. The nuclear factor-kappaB (NF-kappaB), an important mediator of inflammatory cascade belongs to a family of transcription factors which govern the activation of a variety of genes. It controls inflammation, proliferation, and cell death. Before the injury in non-inflammatory condition, the NF-κB exists as inactive form in complex with IκB in cytoplasm (cyto). Following the injury, the IκB kinase activates the NF-κB pathway by degrading cyto- IκB. This degradation promotes the rapid translocation of NF-κB from cytoplasm to nucleus which is responsible for the activation of microglia. This activation further recruits various inflammatory cytokines under the influence of oxidative stress.[6] Accumulating shreds of evidences suggest that inhibition of activation of NF-κB and production of free radical provide significant benefit against SCI.[7–9]

Imatinib, a protein tyrosine-kinase inhibitor used for the treatment of chronic myelogenous leukemia (CML) and acute lymphocytic leukemia (ALL) that are Philadelphia chromosome-positive (Ph^+). It acts by inhibiting activation of Bcr-Abl tyrosine kinase caused by a

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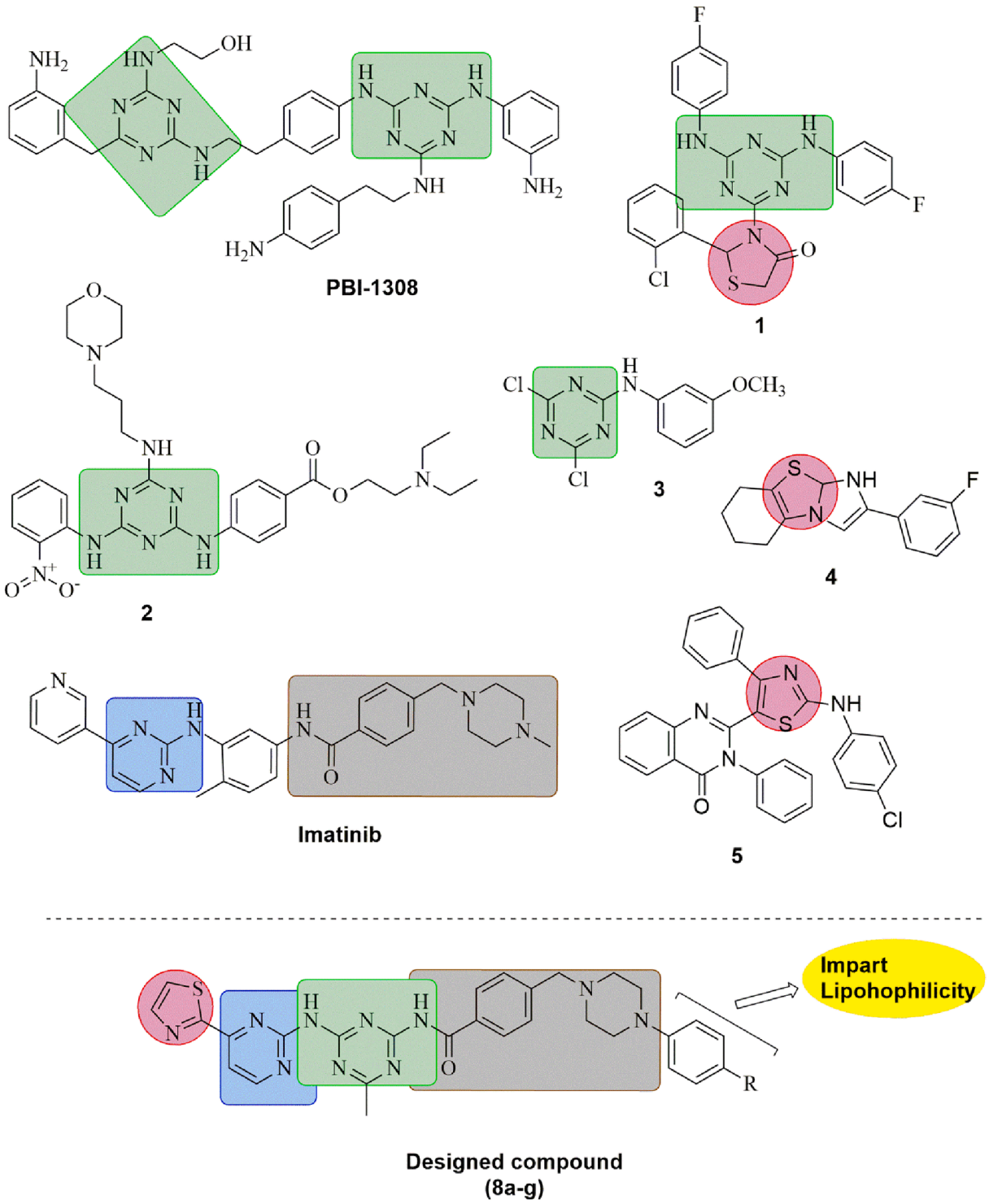
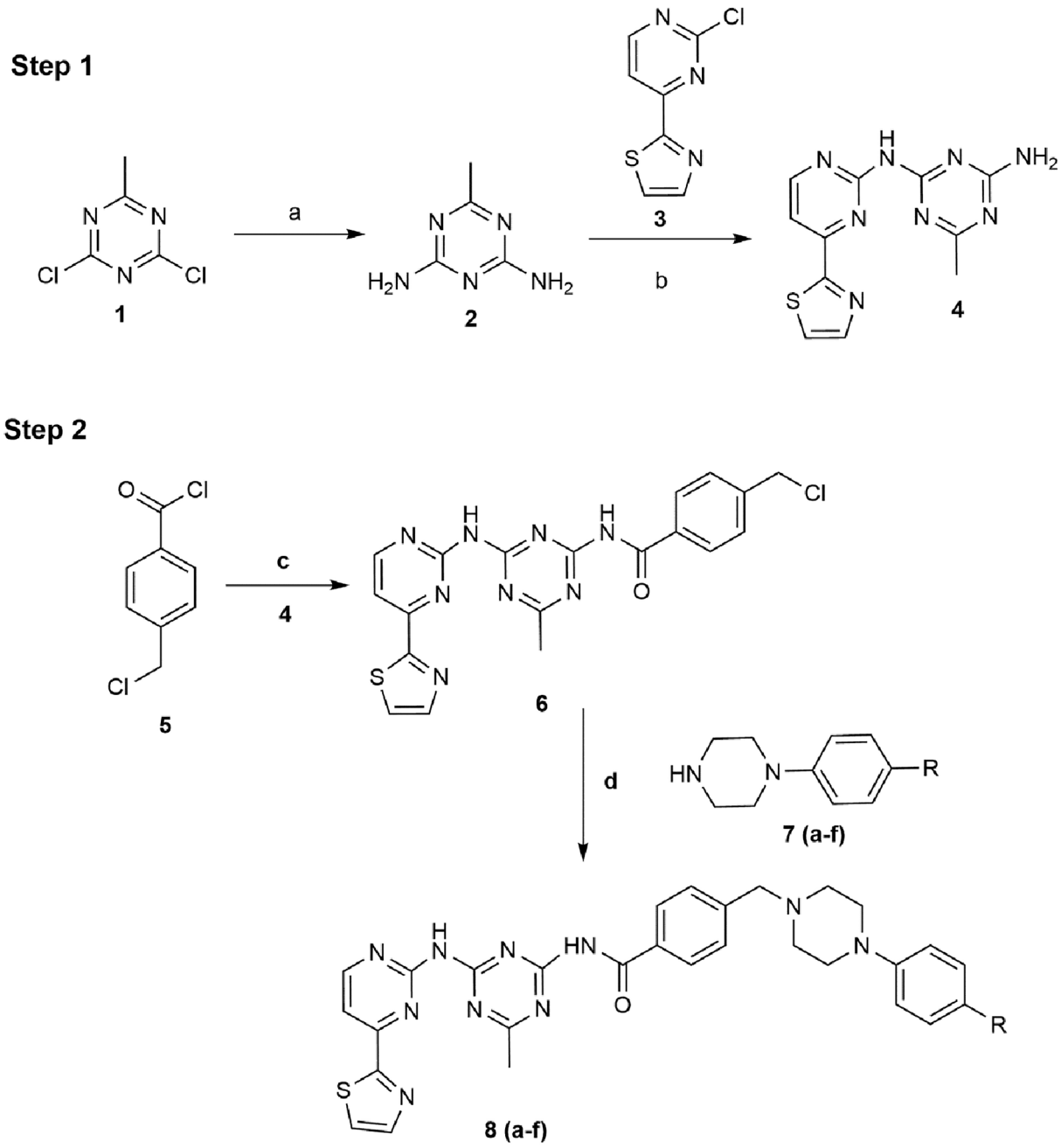


Fig. 1. Structure of some 1,3,5-triazines, thiazole derivatives as inhibitor of NF-κB, imatinib and designed compounds.

chromosomal translocation.[10] Recently, it showed beneficial effects against SCI via improving blood-spinal cord-barrier integrity, hind-limb locomotor function, sensori-motor integration, and bladder function. It also showed to inhibit astrogliosis and deposition of chondroitin sulfate proteoglycans, and increased tissue preservation.[11] In another study, imatinib improves hind limb locomotion and bladder recovery when initiation of treatment was delayed until 4 h after injury and that bladder function was improved with a delay of up to 24 h with no hypersensitivity reaction.[12] It is under Phase II clinical trial (NCT02363361) to assess its uptake, safety and tolerability in acute SCI patients.[13]

1,3,5-Triazine, a highly active pharmacophore found in many of clinically relevant pharmacological agent, such as, antibacterial,[14–19] anti-fungal,[20–22] antimalarial,[23–28] anti-HIV[29] and against diabetes[14] and cystic fibrosis.[30] Various studies have documented the significant inhibitory activity of 1,3,5-triazine derivatives (compound **1**, **2** and **3**, Fig. 1) against the activation of NF-κB. The PBI-1308, a 1,3,5-triazine derivative, inhibits both inflammation and human prostate cancer cell proliferation via inhibition of activation of NF-κB.[31]

Thiazole scaffold is also reported to exhibit strong NF-κB inhibitory activity. Compound **4** (Imidazothiazole derivative) showed potent



Scheme 1. Reagents and conditions: a) Liq·NH_3 in acetone, 40–45 °C, heat, 2–3 h, b) NaOBu-t/THF 10 °C to rt, 2 h, c) THF-TEA, 0 °C, 3 h, d) K_2CO_3, Et_3N, reflux 3–5 h, 120–135 °C.

inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 and prevented translocation of NF-κB from cytoplasm to nucleus by using an immunofluorescent staining method.[32] In an another study, several 2,4-disubstituted-thiazole-5-yl)-3-aryl-3*H*-quinazoline-4-one derivatives (**5**) showed excellent inhibition of NF-κB and AP-1 mediated transcription activation in HEK293 cells and antinflammatory effect in carrageenan induced rat paw edema assay in Sprague-Dawley rats.[33] Singh et al. reported the development of novel 1,3,5-triazine-thiazole derivatives via dual inhibition of NF-κB and EGFR-TKS for possible benefit in rheumatoid arthritis. In molecular docking analysis, the most potent molecule of the series showed strong interaction with Tyr57, Val58, Cys59, His141, and Val142 in DNA binding domain of NF-κB.[34]

The blood-spinal cord barrier (BSCB) is the functional equivalent of the blood–brain barrier (BBB) in the sense of providing a specialized microenvironment for the cellular constituents of the spinal cord. The drugs designed to target SCI need to cross the BSCB and have lipophillic in nature.[35–38] In the present study, we have replaced phenyl instead of methyl substituent of piperazine to provide more lipophilicity to the target molecules.[39–41] Therefore, prompted by the above, in the present study, we have attempted to develop a novel class of hybrid molecule containing imatinib and 1,3,5-triazine as a potential agent against SCI (Fig. 1).

The entire synthetic routes which give rise to final derivatives were bifurcated in two steps as shown in Scheme 1. Initially, the synthesis was achieved by reacting 2,4-dichloro-6-methyl-1,3,5-triazine (**1**) with liq. ammonia to afford corresponding di-amine substituted 1,3,5-triazine (**2**). The 2-(2-chloropyrimidin-4-yl)thiazole (**3**) was allowed to couple with compound **2** in the presence NaO-*t*Bu in THF from 10 °C to room temperature to furnish 6-methyl-N^2-(4-(thiazol-2-yl)pyrimidin-2-yl)-1,3,5-triazine-2,4-diamine (**4**). The step 2 of the synthesis was

Table 1
Inhibitory activity of compounds (**8a-g**) on NF-κB transcriptional activity in LPS-stimulated RAW264.7 cells.[a]

Entry	R	IC_{50} (in μM)[a]
8a	4-Cl	1.04 ± 0.11
8b	4-Br	3.21 ± 0.42
8c	4-CH_3	9.90 ± 1.05
8d	4-NO_2	6.12 ± 0.87
8e	4-OH	54.23 ± 12.61
8f	4-OCH_3	87.32 ± 18.26
8g	H	136.41 ± 28.32
Dexamethasone (Reference)		0.92 ± 0.27
Imatinib (Reference)		1.34 ± 0.17

**P < 0.01 *vs.* Control.
##P < 0.01 *vs.* LPS.
[a] IC_{50} values expressed as mean ± SD of at least three independent assays.

concentrated towards the development of other side chain which was intended to be substituted on the 1,3,5-triazine core. In this step, 4-(chloromethyl)benzoyl chloride (**5**) was act as starting material for the development of 4-(chloromethyl)-N-(4-methyl-6-((4-(thiazol-2-yl)pyrimidin-2-yl)amino)-1,3,5-triazin-2-yl)benzamide (**6**) after reacting with compound **4** in the presence of THF-TEA at 0 °C, 3 h. Finally, the designed compounds **8** (**a-f**) were readily obtained after refluxing compound **6** with substituted piparazine derivatives **7** (**a-f**) in the presence of K_2CO_3 and Et_3N at 120–135 °C for 3–5 h.

In the present study, we have developed a heterocyclic scaffold consisting of imatinib and 1,3,5-triazine as NF-κB inhibitor against SCI. In previous studies, these two scaffolds individually showed protective effect against SCI, thus it is worthwhile to assess their effect when they are used in combination.[13,31,42] The designed hybrid skeleton envisaged to be act by multiple pathways against SCI which is a much sought strategy to counteract the secondary damage in SCI. The NF-κB has been shown to activate, via transcription, the genes encoding pro-inflammatory cytokines, cell adhesion molecules, iNOS and COX-2.[43,44] In SCI, various studies have shown that activation of NF-κB has a detrimental effect on CNS recovery, thus its inhibition offer significant benefit following the traumatic CNS injury.[45–48]

The designed compound showed significant to moderate range of NF-κB transcriptional inhibitory activity in LPS-stimulated RAW264.7 cells. Among the tested derivatives, compound **8a** showed excellent inhibitory activity (IC_{50} = 1.04 μM). It was found more potent than imatinib but less potent than dexamethasome as a standard. The replacement of chloro with bromo (**8b**) or with methyl (**8c**) results in reduction in inhibitory activity with IC_{50} of 3.21 μM and 9.90 μM, respectively. The compound **8d** containing *p*-nitro group showed slight improvement in inhibitory activity (IC_{50} = 6.12 μM) as compared to compound **8c**. However, the introduction of *p*-hydroxy (OH, **8e**) or *p*-methoxy (OCH_3, **8f**) in the place of nitro (**8d**) significantly reduces the inhibitory activity. The unsubstituted phenyl (**8g**) showed least activity among the tested derivatives (Table 1).

The exceptional NF-κB transcription inhibitory activity of compound **8a** has compelled us to determine its effect in traumatic SCI injury in rats to rationalize its potential clinical utility. The SCI injury in Sprague-Dawley rats was induced by contusion injury via a weight drop method where a rod was dropped on the spinal cord of the rats. This method suggested producing human like SCI symptoms in rats. After induction of injury, the rat received compound **8a** in different dose (10, 20, and 30 mg/kg) by intra-peritoneal route (i.p.) after suspending in 2% CMC (carboxy methylcellulose) for once a day for 14 days. Initially, the effect of compound **8a** was determined on the motor function of rats.

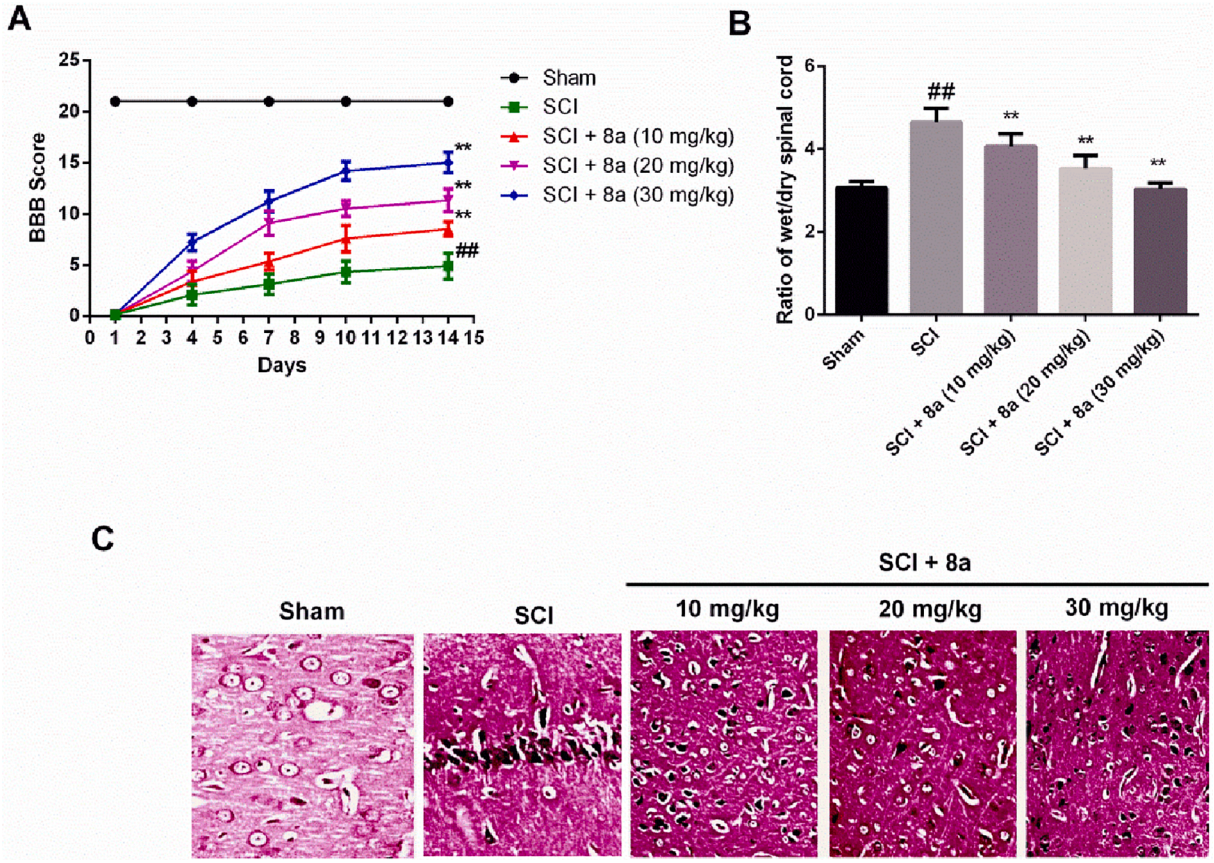


Fig. 2. Effect of compound 8a after SCI in rats. (A) BBB score for determination of motor function, (B) ratio of wet to dry weight of spinal cord, and (C) H & E staining spinal cord. Values represent the mean ± SEM and are representative of three independent experiments. ##P < 0.05 *vs* sham; **P < 0.05 *vs.* SCI, one-way analysis of variance followed by a Tukey's *post hoc* test.

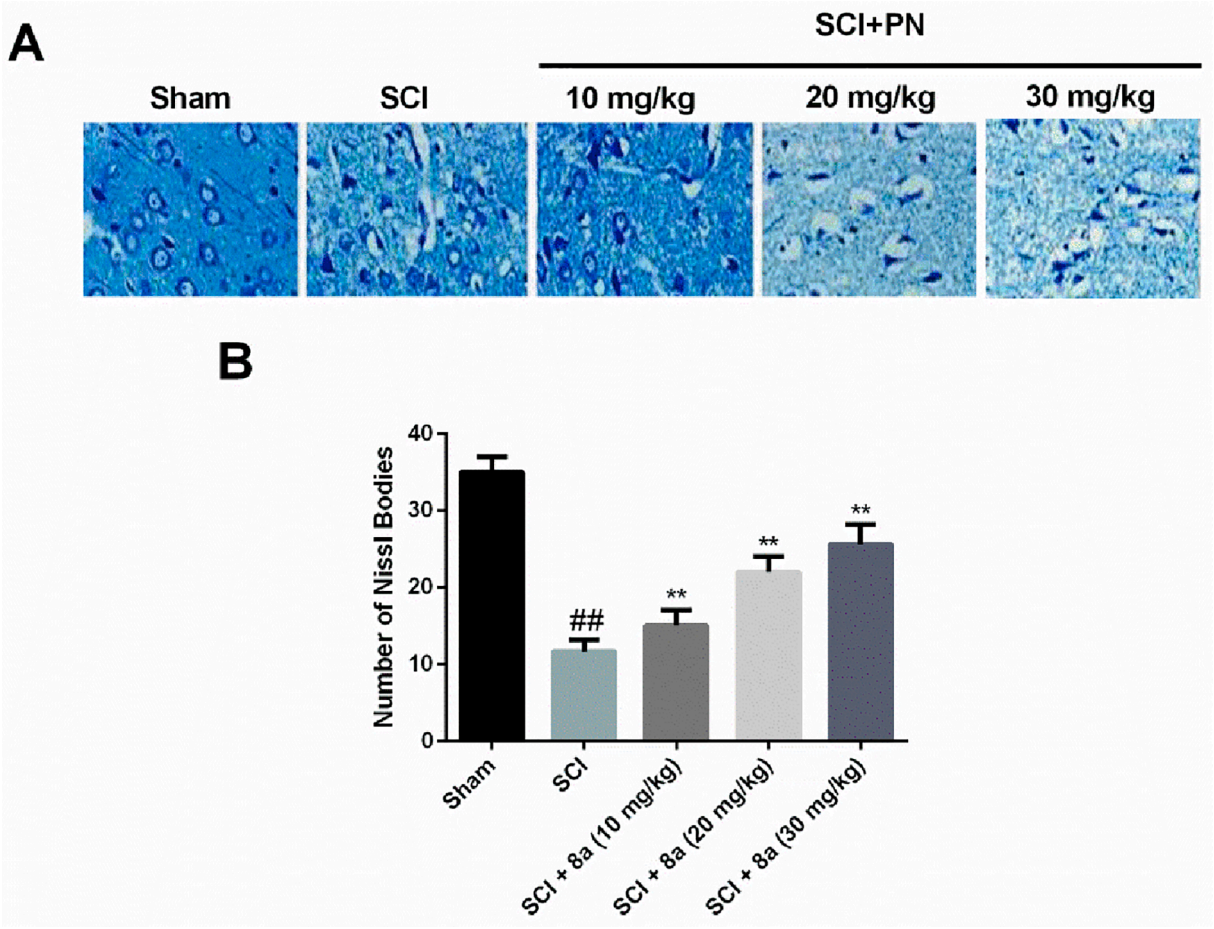


Fig. 3. Effect of compound 8a on the neuronal damage determined by (A) Nissl staining, and (B) quantitative analysis of (B) Nissl bodies. Values represent the mean ± SEM and are representative of three independent experiments. $^{\#\#}P < 0.05$ *vs* sham; $^{**}P < 0.05$ *vs.* SCI, one-way analysis of variance followed by a Tukey's *post hoc* test.

The loss of motor ability is the most frequently altered function of the body after SCI. The Basso-Beattie-Bresnahan (BBB) score is a most widely accepted method to determine function recovery and locomotors testing in chronic SCI study. The scale ranges from 0 to 21 which stands for chronological recovery phases and categorizes alone or together rat functions, such as, rat joint movement, hindlimb movements, stepping, forelimb and hindlimb coordination, trunk position and stability, paw placement and tail position.[49,50] As shown in Fig. 2A, the effect of compound **8a** was determined on the motor function of rats the BBB score of rats on the day 1, 4, 7, 10 and 14 days. The results suggested that SCI group showed altered motor function as compared to sham treated group. The motor function of rats was found significantly restored in the compound **8a** treated group in dose-dependent manner with maximal activity shown in 30 mg/kg treated group. The compound 8a treated rats also showed improved ratio of wet to dry spinal cord weight 24 h post-SCI (Fig. 2B). The effect of compound **8a** was also investigated by histopathological architecture of spinal cord tissues via H and E staining. As shown in Fig. 2C, compound **8a** significantly alleviated the edema in

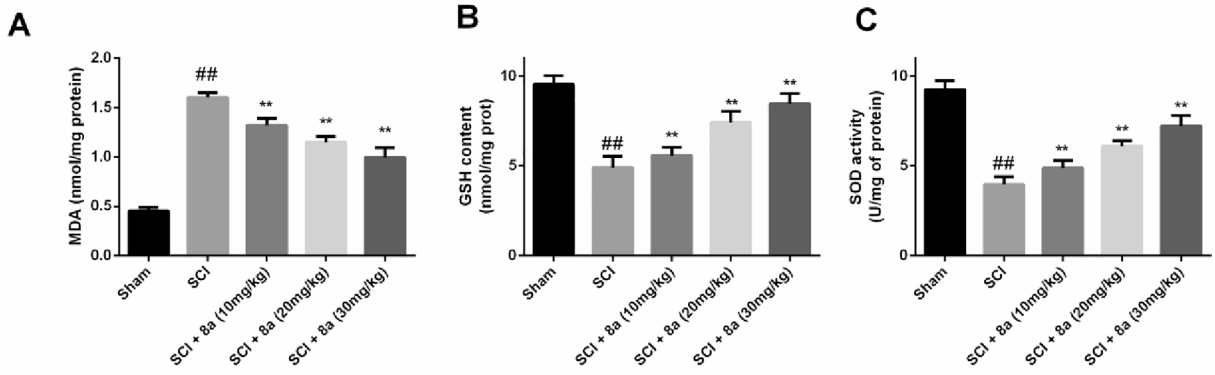


Fig. 4. Effect of compound 8a on the various indices of oxidative stress in spinal cord of rats after SCI induction. (A) MDA, (B) GSH, and (C) SOD. Values represent the mean ± SEM and are representative of three independent experiments. $^{\#\#}P < 0.05$ *vs* sham; $^{**}P < 0.05$ *vs.* SCI, one-way analysis of variance followed by a Tukey's *post hoc* test.

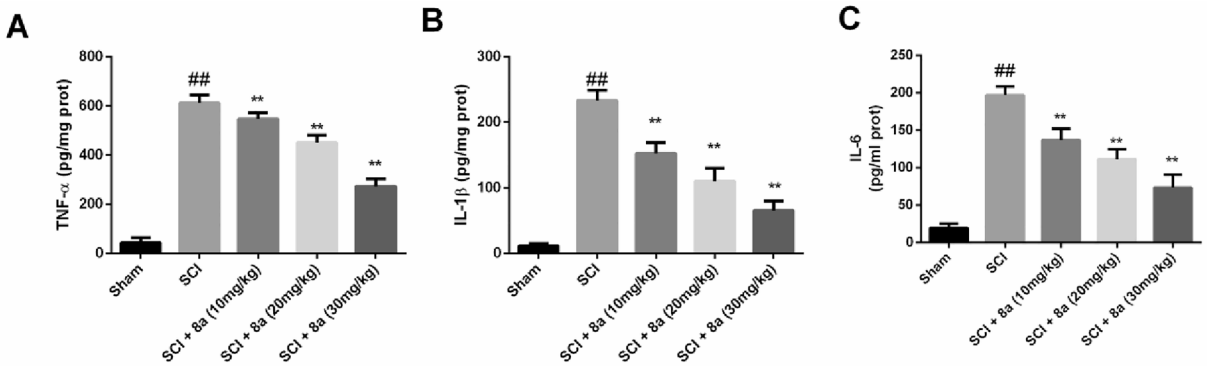


Fig. 5. Effect of compound 8a on the level of pro-inflammatory cytokines as determined by ELISA analysis (A) TNF-α, (B) IL-1β and (C) IL-6. Values represent the mean ± SEM and are representative of three independent experiments. $^{\#\#}P < 0.05$ *vs* sham; $^{**}P < 0.05$ *vs.* SCI, one-way analysis of variance followed by a Tukey's *post hoc* test.

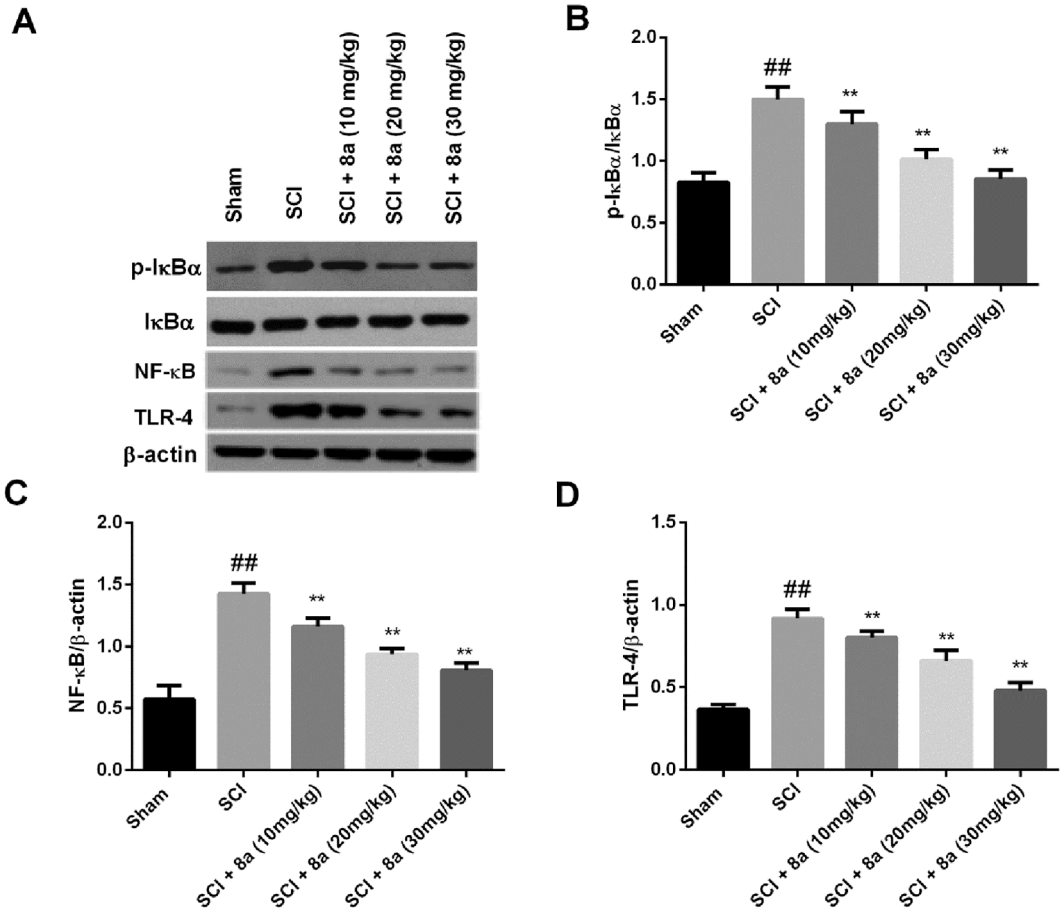


Fig. 6. Effect of compound 8a on the expression of (A) NF-κB, p-IκBα, and TLR4 via western blot analysis, and qualitative analysis of (B) p-IκBα, (C) NF-κB and (D) TLR4 in the spinal cord of rats after SCI induction. Values represent the mean ± SEM and are representative of three independent experiments. $^{\#\#}P < 0.05$ *vs* sham; $^{**}P < 0.05$ *vs.* SCI, one-way analysis of variance followed by a Tukey's *post hoc* test.

spinal cord and inhibited the morphological changes and re-established the histopathology of tissue nears to normal. On the basis of above observation, it could be suggested that that compound **8a** possessed the ability of alleviating SCI-related symptoms. The effect of compound **8a** was investigated on neuronal damage via nissl staining (Fig 3). The SCI treated rats showed reduced nissl granules suggesting significant damage to neurons as compared to sham treated group. Compound **8a** prevented neuronal damage as shown by higher number of nissl bodies as compared to SCI treated rats. The compound **8a** showed dose-dependent neuro-protective effective via averting the neuronal damage. In the next part, we aimed to study the effect of compound **8a** on various biomarkers associated with SCI to elucidate its mechanism of action.

Oxidative stress and inflammation are closely linked and studies have shown their significant role in progression of various neurodegenerative diseases, such as SCI.[51,52] Thus, it is worthwhile to examine the effect of compound **8a** on the mediators of inflammation and oxidative stress. As shown in Fig. 3, the activities of GSH and SOD were found reduced together with increased level MDA in SCI rats in comparison to control. The compound **8a** treated group showed increased level of GSH and SOD with drop in MDA activity as compared to SCI

group. Moreover, the level of tested inflammatory cytokines (IL-1β, IL-6, and TNF-α) was found reduced significantly in **8a** treated group as compared to disease control, Fig 4.

In the above sections, we have discussed the role of NF-κB in SCI, but toll-like receptor 4 (TLR4) is another protein which is a upstream regulator of NF-κB and regulates its activation.[53,54] Various studies have shown that TLR4 regulates inflammation, gliosis, and myelin sparing after spinal cord injury, and its inhibition offers beneficial effects.[55,56] Moreover, in steady state, the NF-κB dimers are sequestered by IκB in the cytoplasm. Upon stimulation following the injury, IκBα phosphorylated (p-IκBα) to release the NF-κB, which then enters into the nucleus where it activates the expression of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α. Studies have shown that blockade of IκB degradation prevented NF-κB activation. Therefore, the last part of the study was aimed to determine the effect of compound **8a** on the expression of NF-κB, TLR4 and p-IκBα protein in the spinal cord tissues of rats. Towards this, a western blot analysis was conducted and results have been shown in Fig 5. It has been found that the expression of NF-κB, TLR4, and IκBα was found significantly elevated in SCI rats, whereas upon administration of compound **8a**, the level of these proteins were found significantly reduced (Fig. 6).

Collectively, our study has demonstrated the pharmacological benefit of imatanib and 1,3,5-triazine hybrid against SCI. These compounds revealed as potent inhibitors of NF-κB transcription activity in RAW264.7 cells. Moreover, the most potent NF-κB inhibitor, compound **8a** showed neuro-protective activity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2021.127964.

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