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Protective effect of Novel Substituted Nicotine Hydrazide analogues against Hypoxic Brain Injury in Neonatal Rats via inhibition of Caspase

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Abstract

In hypoxic-ischemic injury of the brain of neonates, the level of caspase-3 was found to be aberrantly activated. Its overexpression leads to the alteration of cytoskeleton protein fodrin and loss of DNA repair enzyme which ultimately results in neurological impairment and disability. Concerning this, the present study was intended to develop novel nicotine hydrazide analogues as caspase inhibitors via efficient synthetic route. These compounds were subsequently tested for inhibitory activity against caspase-3 and -7 where they exhibit highly potent activity against caspase-3 revealing compound 5k as most potent inhibitor (IC_{50} = 19.4±2.5 μM). In Western blot analysis, 5k considerably inhibits the overexpression of caspase-3. The aryl nicotinate of compound 5k, as indicated by molecular docking was found to engage His121 and critical enzyme thiols, i.e., Cys163 of caspase-3 for its potent activity. Moreover, histopathological examination of brain tissues and hippocampus neurons showed that compound 5k considerably improves the brain injury and exert neuroprotective effects in hypoxic-ischemic (HI). In brain homogenate, 5k significantly improves the activity of MDA, SOD, GSH-Px, CAT and T-AOC to exert its beneficial effect against oxidative stress induced by HI injury.

Keywords: Synthesis, Nicotine hydrazide, docking, ischemic injury.

Due to asphyxia, the newborn babies are more prone to hypoxic-ischemic brain damage (HIBD) which results in neurological impairment and disability.[1–3] It was often coupled with inefficient diagnosis and acute mortality. The etiology of HIBD is highly complex, which cannot be related with single source, instead, it was associated with numerous causes, for instance, alteration of the blood-brain barrier permeability, loss of ion-cell homeostasis, toxicity mediated via free radical, deficiency of growth factor and inflammation of immature brain. According to an estimate, HIBD causes 23% of all neonatal deaths worldwide.[4–6] Therefore, there is an urgent need of new therapeutic agents that can be able to prevent and normalize HIBD.

Caspase belongs to a family of cysteine proteases, which plays an important function in programmed cell death and inflammation. They are considered as a mammalian homologs of the ced-3 gene product and was conveniently divided into three groups depend upon its role, e.g., initiator caspase (caspase-2, -8, -9, -10), effector caspase (caspase-3, -6, -7), and inflammatory caspase (caspase-1, -4, -5, -11, -12).[7–11] It has been found that, during development of brain and acute hypoxic-ischemic (HI) injury, the caspase-3 initiates the execution of neuronal apoptosis.[12,13] Particularly, in hypoxic-ischemic injury of immature brain, the level of caspase-3 was found to be aberrantly activated which leads to the alteration of cytoskeleton protein fodrin and loss of DNA repair enzyme ability. Moreover, it has been found that, mice deficient of caspase-3 die early in the embryonic stage or in the perinatal period. These results suggested the critical role of caspase-3 in the dysfunction of the normal process of apoptosis.[14–16]

Toxicity mediated via generation of reactive oxygen species (ROS) play critical role in the progression of the HIBD. It was initiated via generation of oxygen and nitrogen free radicals at the onset of reperfusion.[17,18] Consequently, several studies also indicated the use of appropriate antioxidants for the normalization of the ROS which exert neuroprotective effects in the HIBD.[19]

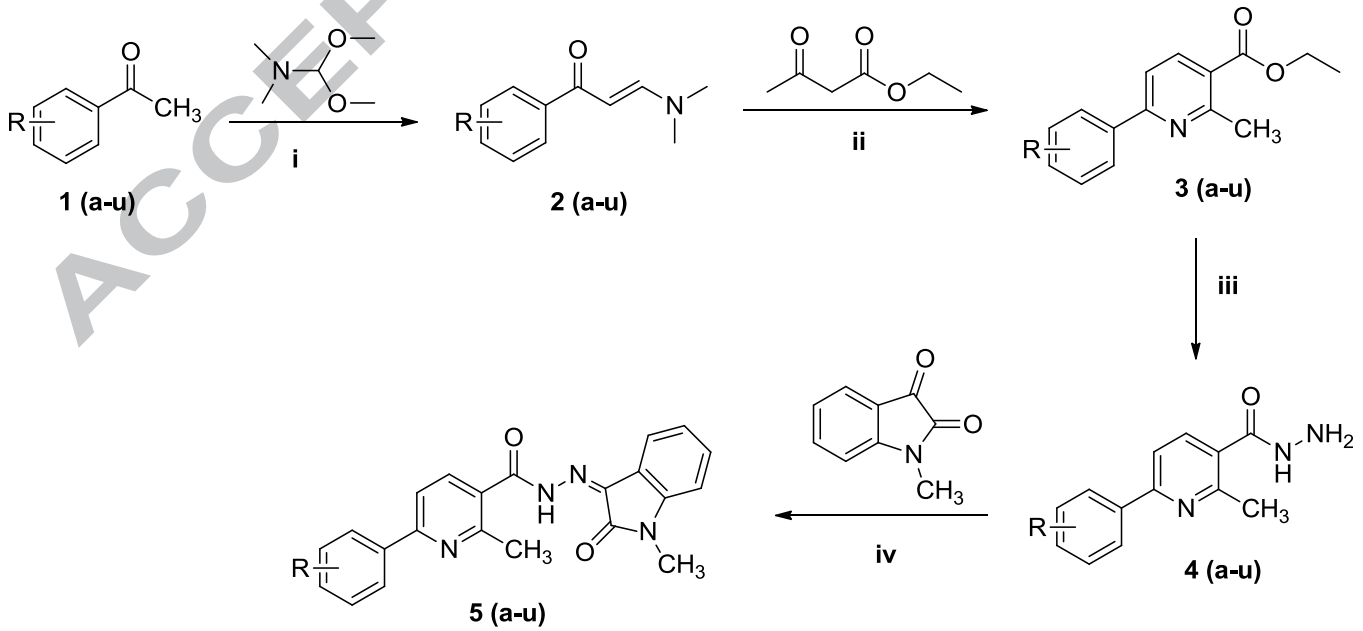
Thus, anti-apoptotic therapies simultaneously targeting caspase-3 and oxidative stress have been considered to be useful in the suppression of HIBD and thereby act as a neuroprotective agent.

Hydrazones, belongs to a highly versatile family of medicinal agents endowed with numerous pharmacological properties. Chemically, it has been characterized by the presence of –NH–N=CH– or –NH–N=CR^1R^2 – structural fragments in the compounds. Various studies have indicated that, it possesses anti-inflammatory, antiplatelet, antidepressant, analgesic, anti-convulsant, anti-tubercular, anticancer, anti-HIV and antimicrobial activity.[20] As a matter of fact, levosimendan, a novel inodilator from the family of hydrazones showed high level of neuroprotection in *in vitro* model of traumatic brain

injury.[21] Moreover, hydrazones are also reported to exhibit tremendous antioxidant activity.[22] Nicotine, an important chemical fragment, has been shown to interfere with variety of biological functions, ranging from gene expression to regulation of hormone secretion and enzyme activities.[23] More recently it has been observed that, it can be able to inhibit the caspases-3 activation and over-expressed anti-apoptotic protein expression.[24]

Prompted by the above, the present study was intended to elucidate the protective role of substituted nicotine hydrazide analogues in HIBD of neonatal rats. In the current work, developed analogues were tested for possible caspase inhibition via enzyme based assay. The hypoxic-ischemic neonatal rat model was also utilized for the determination of mechanism of action.

The synthesis of target compounds has been realized in four-step reaction in excellent yields. The overall synthetic steps has been clearly outlined in the scheme 1. Initially, the synthesis has been started with the condensation of substituted acetophenones 1(a-u) with DMF-DMA (N,N-Dimethylformamide dimethyl acetal) to afford corresponding chalcones 2 (a-u). The resulting enaminones 2 (a-u) was further allowed to react with active methylene compound (ethyl acetoacetate) and ammonium acetate in refluxing acetic acid to yield ethyl 2-methyl-6-arylnicotinates 3 (a-u). The ester fragment of the above synthesized ethyl 2-methyl-6-arylnicotinates was further subjected to hydrazinolysis to furnish nicotinic acid hydrazides 4 (a-u). The last step corresponds to the condensation of 1-methyl isatin with 4(a-u) to afford corresponding target derivatives 5 (a-u).[25,26]



Scheme 1 Synthesis of substituted (2-methylnicotinoyl)diazenyl)-3-methyl-1*H*-inden-2(3*H*)-one 5 (a-u), where reagents and condition: (i) xylene, reflux; (ii) NH_4OAc/CH_3COOH/reflux; (iii) $NH_2NH_2{\cdot}H_2O$/reflux; (iv) ethanol/glacial CH_3COOH/reflux.

Table 1. Inhibitiory effect of compounds 5 (a-u) against caspases-3 and caspases-7 in enzyme based assay

Compound	Structral modification (R)	IC_{50} (in μM)[a]	
		Caspase-3	Caspase-7
5a	H	456.1±11.2	ND
5b	4-NO_2	202.4±26.4	ND
5c	3-NO_2	278.2±32.2	ND
5d	2-NO_2	296.5±21.3	> 200
5e	4-Cl	52.34±1.3	ND
5f	3-Cl	37.2±4.2	> 200
5g	2-Cl	63.2±2.2	ND
5h	4-Br	121.7±11.4	ND
5i	3-Br	129.5±6.7	ND
5j	2-Br	132.4±9.2	ND
5k	4-F	19.4±2.5	> 200
5l	3-F	35.0±6.2	ND
5m	2-F	38.3±9.4	ND
5n	4-CH_3	375.3±23.4	ND
5o	3-CH_3	310.0±30.6	ND
5p	2-CH_3	350.6±19.1	ND
5q	4-OCH_3	487.2±12.5	> 200
5r	3-OCH_3	547.0±25.3	> 200
5s	2-OCH_3	551.5±10.2	> 200
5t	3,4-OCH_3	598.6±18.7	> 200
5u	3,4,5-OCH_3	674.3±23.4	ND
Ac-DEVD-CHO		1.7±0.04nM	60.4±3.3 nM

[a] Values are the mean of at least three independent experiments.

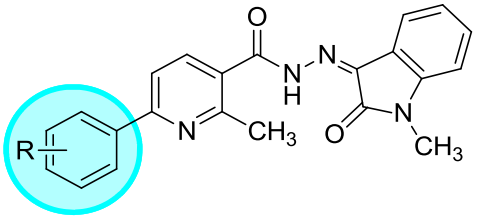
Peptidic inhibitors are well-known for the inhibitory activity against caspase.[27,28] More recently compounds bearing isatin as core scaffold proved to posses excellent in vitro selectivity for the caspase-3 and -7.[29] These derivatives were identified as a competitive nonpeptidyl caspase inhibitors. Moreover, it also efficiently act as a probe for molecular imaging of caspase in apoptosis (radiotracers).[30–34] Concerning this, the compounds synthesized in the present work were designed to contain istain fragment and postulated to have similar inhibitory activity. Therefore, designed compounds developed in the present work were tested for inhibitory activity against caspase-3 and -7 and the results are presented in Table 1. The target compounds showed considerable inhibition of caspase-3 with almost no

inhibitory activity against caspase-7. Initially, compound 5a showed moderate activity against caspase-3 with IC_{50} of 456.1±11.2 μM. The activity was drastically influenced by the presence of the *p-nitro* (5b) which render resulting compound two-fold more active than non-substituted counterpart. Whereas, changing the pattern of substitution, i.e., *para* to *ortho* or *meta*, showed reduction in activity, compound 5c and 5d, respectively.

The inhibitory activity was significantly improved with the introduction of *chloro*, which indicated that, *meta* substituted compound (5f; IC_{50}: 37.2±4.2 μM) found more active than its ortho (5g) and para (5e) counterparts. Whereas, the presence of bromo was not able to influence the activity and compounds substituted with it, (5h, 5i and 5j) showed mild inhibition of caspase-3. The inhibitory efficacy was significantly improved in the case of compounds 5k, 5l and 5m bearing isomeric substitution of fluoro. Among them, compound 5k IC_{50}: 19.4±2.5 μM was indicated as a potent inhibitor than other isomeric analogues, and identified as a most promising inhibitor of the tested series.

We were also interested in evaluating the effect of electron-donating groups on the inhibitory potency against caspase. Markedly, it has been found that, compounds containing electron donating groups were also found to be completely inactive against caspase-7; while exhibiting moderate activity against caspase-3. The results of the comparative inhibitory analysis revealed that, methyl bearing compounds were found more active than methoxy. For instance, compound 5n (IC_{50}: 375.3±23.4 μM) was found to be more active than 5q (IC_{50}: 487.2±12.5 μM). The similar fashion of the activity was observed in the case of rest of the analogues i.e., 5o and 5r; 5p and 5s. Compounds 5t and 5u showed further reduction in the activity.

Based on the inhibitory activity of tested analogues, it was indicated that, none of the synthesized compound found active against caspase-7. Thus, it has been suggested that molecules are selectively inhibit caspase-3 over caspases-7. The structure activity relationship studies has suggested that, the inhibitory potency was strongly influenced by the position of the molecules along with its electronic behaviour. The detailed analysis of results has been depicted in fig. 1.



Order of Group

F > Cl > Br > NO_2 > CH_3 > H > OCH_3 > 3,4-OH_3 > 3,4,5-OCH_3

Position of group

4 > 3 > 2

Figure 1: Structure activity relationship of target analogues.

Caspase are a family of cysteine endoproteases that are involved in apoptosis and inflammation. Based on the molecular mechanism, it was further divided into initiator caspase, effector caspase and inflammatory caspase. As suggested by various studies, caspase-3 belongs to the sub-family of effector caspase, and has been considered as a key factor for the brain injury in neonatal rats. Thus, its selective regulation offers protective effect in brain injury. Therefore, we are interested in determination of the effect of compound 5k on the level of caspase-3 by western blot analysis. As shown in fig 2, the level of caspase-3 was found to be significantly higher at 48 h after HI (p < 0.05). Consequently, the post-treatment of compound 5k and Nimodipine at tested doses causes significant reduction in elevated level of caspase-3 (Fig. 2B; p < 0.05).

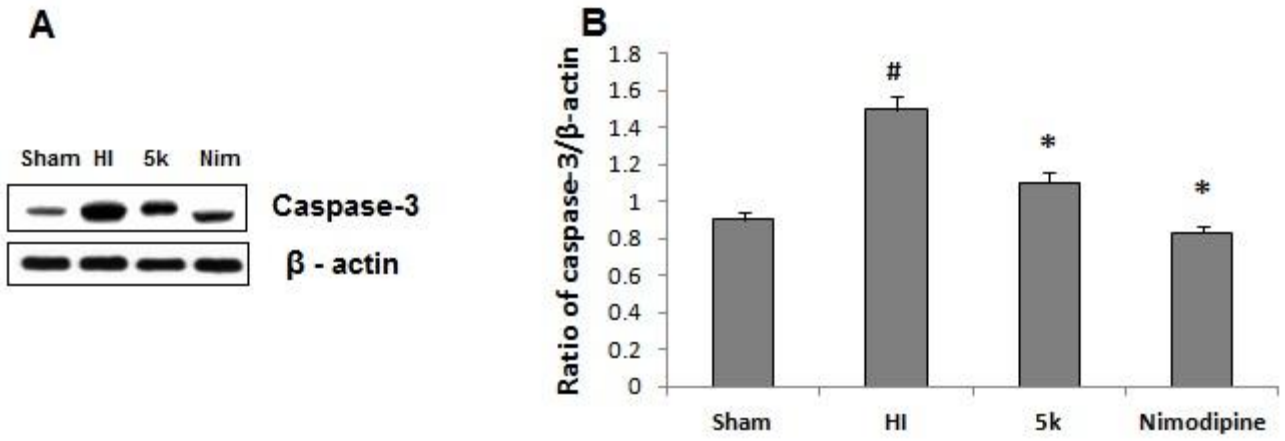


Figure 2. Effects of compound 5k on the expression of caspase-3. (**A**) The corresponding western blot band of caspase-3 activation in the ischemic brain at 48 h after HI. (**B**) Effect of compound 5k (30 mg/kg) on the caspase-3 activation in HI neonatal rats at 48 h. Data are expressed as mean±SEM (n = 6). $^{\#\#}p$ < 0.01 *versus* sham group; *p < 0.05, **p < 0.01 *versus* HI group.

To further exemplify the molecular basis of excellent caspase-3 inhibtion by compound 5k, molecular docking study was undertaken with the help of CDOCKER (Discovery Studio 2.5). The crystal

structure of caspase-3 docked with an isatin sulfonamide inhibitor (pdb code: 1GFW) was retrieved from Protein Data Bank (RCSB-PDB) for further docking analysis. The study revealed that, compound 5k was efficiently docked into the active site of caspase-3 by making proficient interactions. Particularly, the hydrazone fragment was found to engage His121 and critical enzyme thiols, i.e., Cys163 via formation of three H-bonds. Consequently, the efficient binding of catalytic core of Cys163 by compound 5k could be act as possible reason for its excellent inhibitory activity. Moreover, the nicotinate fragment was able to create additional H-bonds with Arg64. These molecular interactions were found in aggrement with the previous study by Wang et al, which suggests the utility of docking and 3-D QSAR based approach for the development of Isatin sulfonamide analogues as caspase-3 Inhibitors.[35] Together with considerable in-vitro results and molecular docking study against caspase-3, it has been corroborated that, compound 5k act as efficient inhibitor.

The effect of compound 5k was also determined on the volume of brain infarction size after Hypoxic-ischemia. As shown in Figure 4, the rats belong to sham operated groups showed no sign of infarction area in the brain. Moreover, the pretreatment of compound 5k at the dose of 10, 20 and 30 mg/kg significantly causes reduction in the percentage of infarction.

To further confirm the activity, Hematoxylin and Eosin (H and E) staining was used to examine the histopathological changes in neurons of brain of newly born rat. As depicted in fig. 5, the morphology of neurons were remained unaltered in Sham operated group and found evenly distributed in the cortex. Moreover, with the help of a histopathological brain injury score, it has been confirmed that almost entire region of the ipsilateral hemisphere of the brain was severely damaged under the influence of HI. The pre-treatment of test compound and Nimodipine significantly improved the arrangement of neurons along with reduction in interstitial edema. The observations were further confirmed by the improvement of injury score as depicted in fig. 5E.

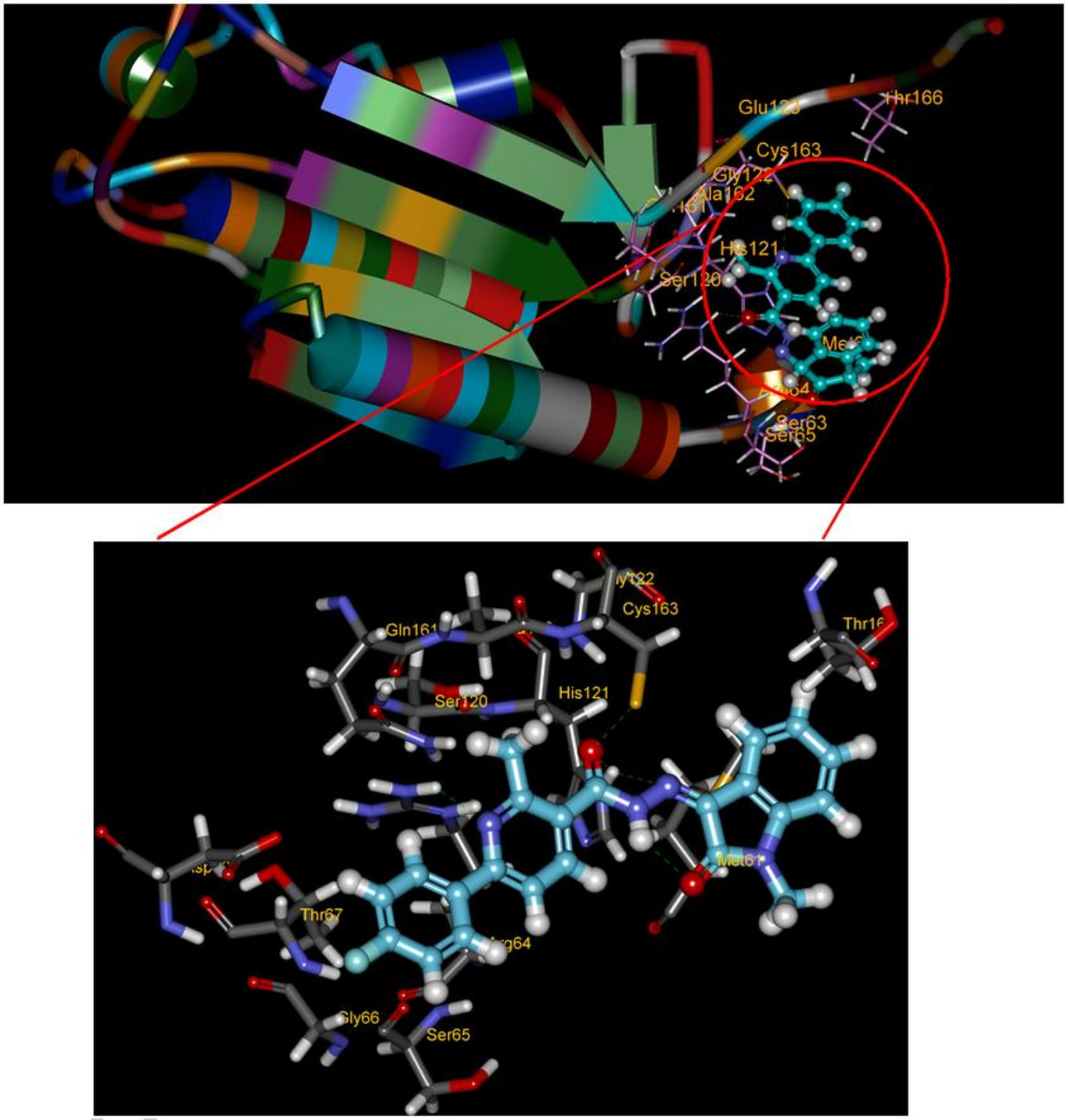


Figure 3 Docked orientation of compound 5k in the active site of caspase-3.

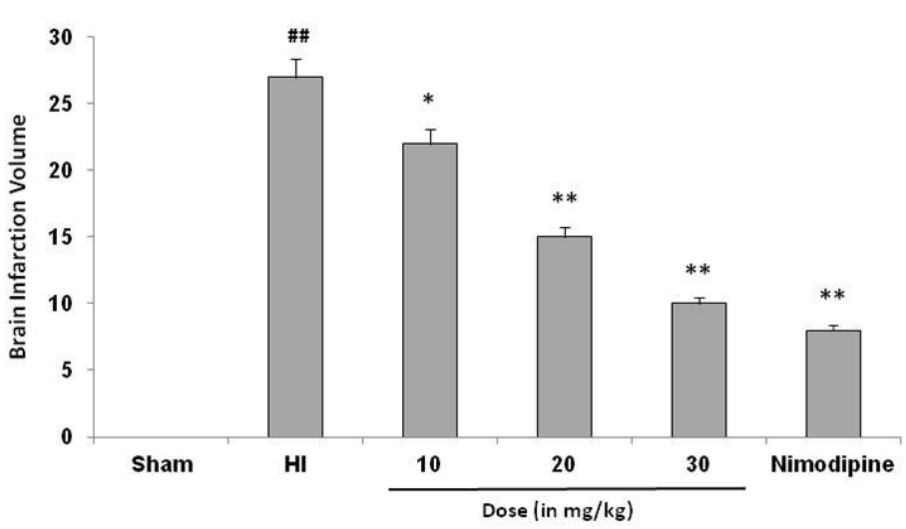


Figure 4: Effect of compound 5k on brain infarct volumes at 48 h after hypoxic-ischemia (HI).Data are expressed as mean ± SEM (n = 6). $^{\#\#}p < 0.01$, versus sham group, *p < 0.05, **p < 0.01 versus HI group

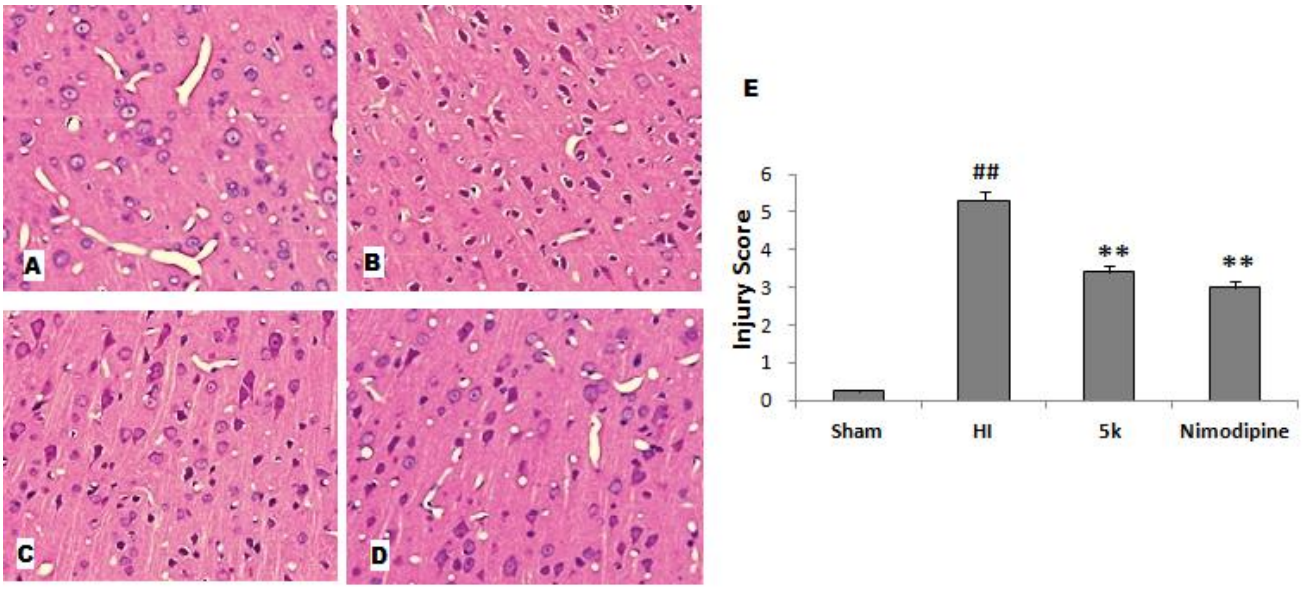


Fig. 5. Effect of compound 5k on histological alterations of ischemic cerebral cortex at 48 h after hypoxic-ischemic determined by hematoxylin-eosin staining (×400). (**A**) Sham operated group. (**B**) 5k treated (30 mg/kg) group. (**D**) Nimodipine treated (1 mg/kg) group. Data are expressed as mean ±SEM (n = 6). $^{\#\#}p < 0.01$ *versus* sham operated group; $^{**}p < 0.01$ *versus* HI group.

The effect of compound 5k on the morphology of hippocampus neurons was determined via electron microscopy and the results are presented in Fig. 6. As found in Sham-operated group, which corresponds to the Normal hippocampus neurons; a well defined large oval nucleus with evenly distributed chromatin along with normal cell organelles were observed in abundant quantity (Fig. 6A). Later, after induction of HI, the neurons present in the hippocampus present severe injury, Fig. 6B. It has been found that, almost entire nucleus was found irregular in shape, suggesting the progression of cell death. It was surprising to note that, after administration of compound 5k, the morphology of the neurons were returned to its original structure, i.e., regular nucleus and vaguely disrupted cell organelles (Fig. 6C). More pronounced recovery was observed in the case of Nimodipine treated group (Fig. 6D). These results suggest that, compound 5k considerably improves the brain injury and exert neuroprotective effect in brain injury induced by hypoxic-ischemia.

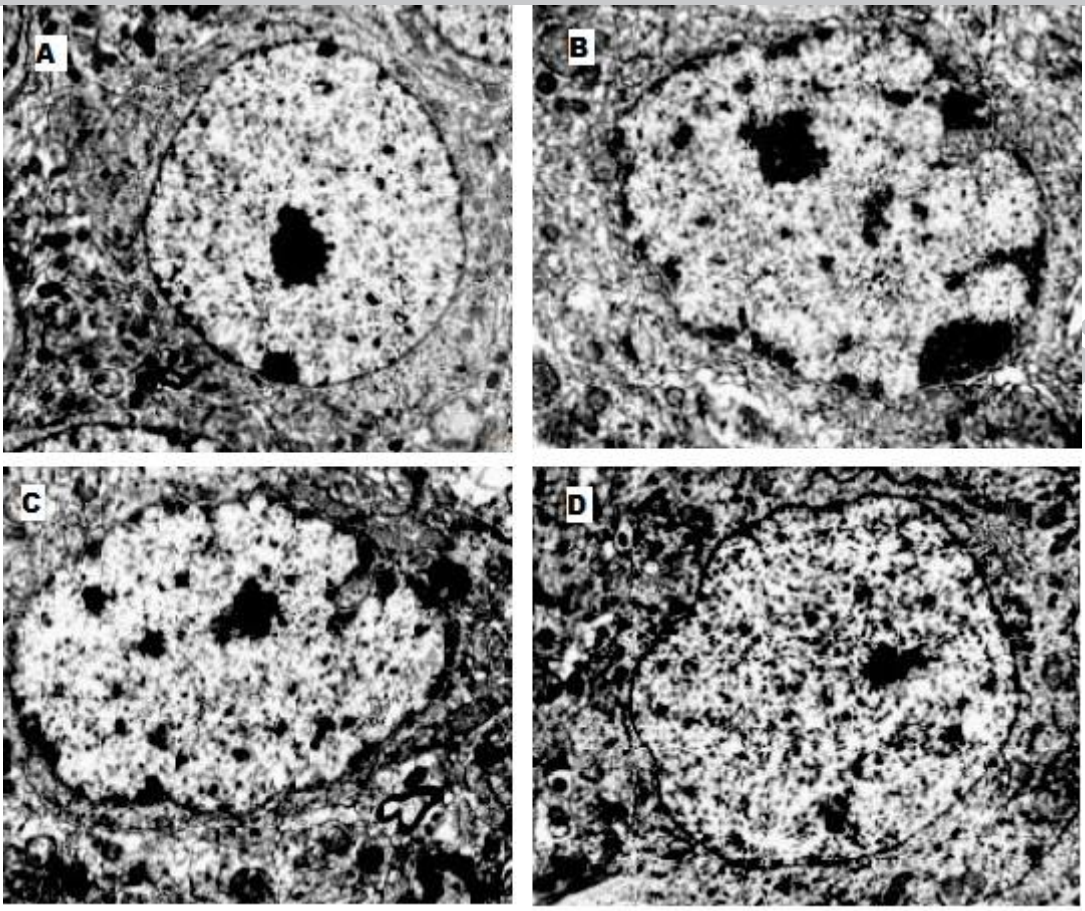


Figure 6 Effect of after treatment of compound 5k in morphological characterstic of hippocampus neuron induced by hypoxic–ischemic (2500x). (A) Nucleolus of sham group. (B) nucleolus at 48 h after hypoxic–ischemic in HI group. (C) nucleolus in 5k treated group (30mg/kg). (D) nucleolus in Nimodipine treated group.

The alteration of endogenous redox mechanism and associated oxidative stress was termed as a major contributing factor for the progression of the HIBD in neonatal rats via generation of reactive oxygen species (ROS). Under the normal circumstances, the generation of ROS was easily counterbalanced by the efficient endogenous anti-oxidant mechanism. However, in HIBD, the endogenous antioxidant system could not be able to fully consume the overproduced ROS because of diminished anti-oxidant activity. Thus, extra produced ROS in mitochondria causes oxidative damage to lipids, proteins and DNA. The elevated level of malonaldehyde (MDA) produced via lipid peroxidation in plasma has been correlates well with the intensity of HIBD and deemed as specific biomarker for linked oxidative stress. Moreover, various studies have also indicated the significance of endogenous antioxidant enzymes, such as, superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) against the ROS mediated injury. In the present study, as shown in fig. 7A, the level of brain MDA has been found elevated in the HI group in comparison to sham treated group ($p < 0.01$). Whereas, upon treatment with 5k, the level has been decreased considerably ($p < 0.01$). Moreover, in the case of endogenous anti-

oxidant enzymes (fig. 7B, 7C, 7D and 7E), the groups treated with test compound 5k showed elevated level of SOD, GSH-Px, CAT and T-AOC in dose-dependent manner. Thus, it has been suggested that, compound 5k might improve the HIBD in neonatal rats together by reduction in lipid peroxidation and boosting antioxidant enzymatic activity.

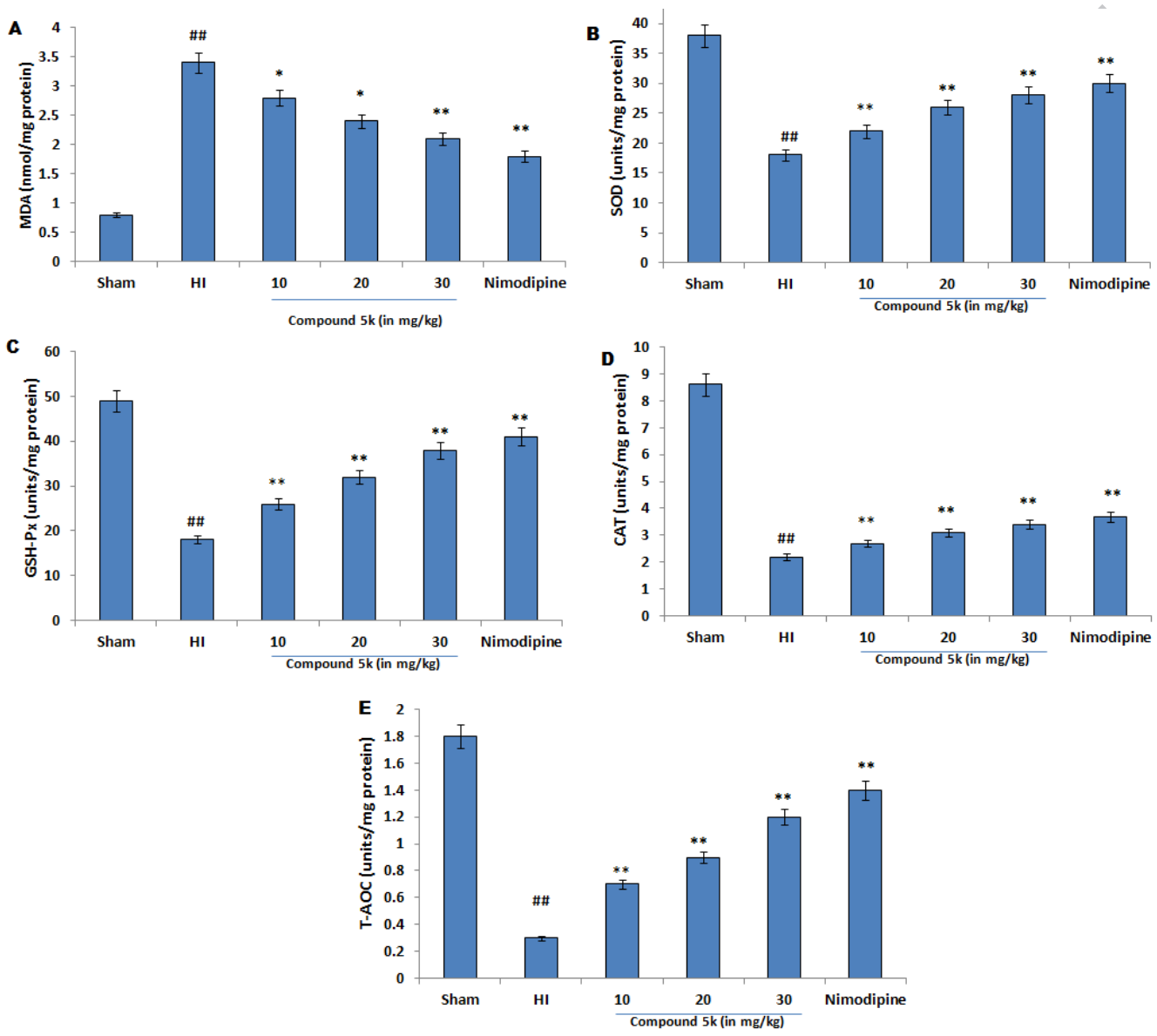


Figure 7. Effect of compound 5k on oxidative stress after hypoxic-ischemic in neonatal rats. (A) Effect on the MDA activity at 48 h after HI. (B–E) Effect of 5k on the endogenous level of SOD, GSH-Px, CAT, T-AOC at 48 h after HI. The data are expressed as mean ± SEM (n = 6). $^{\#\#}p < 0.01$ versus sham group; **p < 0.01 versus HI group.

As a concluding remark, the present study successfully developed a series of substituted nicotine hydrazide analogues as selective caspase-3 inhibitor. The most promising inhibitor 5k was efficiently inhibited caspase-3 in western blot analysis. It also showed to interact with the active site of caspases-3 via formation of vital bonds with His121 and critical enzyme thiols, i.e., Cys163. The results are further supplemented with in vivo pharmacological experiments which suggest that, compound 5k considerably

improves the brain injury and exert neuroprotective effects in HIBD. It also improves the condition of oxidative stress in the brains of neonatal rats.

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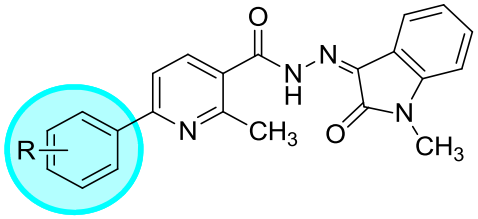

Order of Group

F > Cl > Br > NO_2 > CH_3 > H > OCH_3 > 3,4-OH_3 > 3,4,5-OCH_3

Position of group

4 > 3 > 2