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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.

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

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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]

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.

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

Compound	Structural 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 ± 0.04 nM	60.4 ± 3.3 nM

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

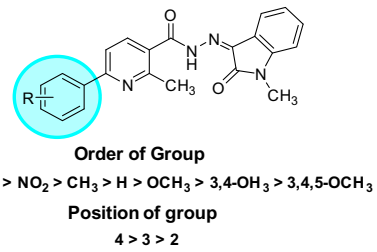
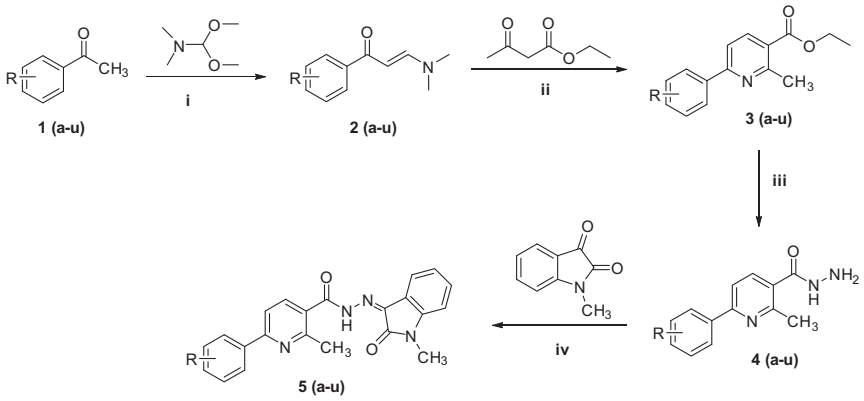


Figure 1. Structure activity relationship of target analogues.

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



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.