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Mechanism underlying the inhibitory effect of nitrative stress on the sulfation of dopamine and its methylated product by human SK-N-MC neuroblastoma cells

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Mechanism Underlying the Inhibitory Effect of Nitrative Stress on the Sulfation of Dopamine and its Methylated Product by Human SK-N-MC Neuroblastoma Cells

by

Prince Tuffour Ampem

Submitted to the Graduate Faculty as a partial fulfillment of the requirement for the Master of Science in Pharmacology and Toxicology

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An Abstract of

Mechanism Underlying the Inhibitory Effect of Nitrative Stress on the Sulfation of Dopamine and its Methylated Product by Human SK-N-MC Neuroblastoma Cells

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Dopamine has a wide range of important physiological roles in humans, but elevated levels due to disruption in its metabolic pathway has been implicated in the etiology of several neurodegenerative disorders. Excessive production of nitric oxide (NO) resulting in "nitrative stress" may lead to the generation of the highly reactive free radical, peroxynitrite, which is also known to be a risk factor for certain inflammatory conditions and neurodegenerative abnormalities. Sulfation, as mediated by the sulfotransferase enzymes, has been reported to play a key role in the regulation of dopamine and its metabolite, 3-methyldopamine, which are predominantly excreted in the sulfated form. Thus, the present study was aimed at finding out whether nitrative stress, as simulated by two NO donors, may affect the homeostasis of dopamine and its metabolite, 3-methyldopamine; and to elucidate possible mechanisms underlying this phenomenon. The sulfotransferase enzyme, SULT1A3, was identified as the main cytosolic sulfotransferase responsible for catalyzing the sulfation of dopamine and 3-

methyldopamine. Kinetic studies using [^{35}S]sulfate-labeled SK-N-MC cells revealed that the inhibitory effect of two NO donors, 3-morpholinosydnonimine (SIN-1) and diethylenetriamine NONOate (DETA NONOate), on the generation and release of dopamine 3-O-[^{35}S]sulfate and 3-methyldopamine 4-*O*-[^{35}S]sulfate occurred in a time-dependent manner. Metabolic labeling experiments using increasing concentrations of SIN-1 and DETA NONOate showed a dramatic decrease in the generation and release of dopamine 3-O-[^{35}S]sulfate and 3-methyldopamine 4-*O*-[^{35}S]sulfate by [^{35}S]sulfate-labeled SK-N-MC cells. Furthermore, cell lysates prepared from SK-N-MC cells treated with increasing concentrations of DETA NONOate showed significant decrease in dopamine- and 3-methyldopamine-sulfating activities, compared to cell lysates prepared from untreated SK-N-MC cells. Cell viability assay using SK-N-MC cells treated with increasing concentrations of SIN-1 or DETA NONOate revealed no significant cytotoxic effect of the NO donors compared to untreated cells.

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1. Introduction

1.1 Dopamine: Biochemistry and Functional Roles

Dopamine is a catecholamine which functions as a neurotransmitter in the central nervous system (CNS), and performs other multifunctional roles in the periphery. Though dopamine was first discovered in 1910, its physiological role as a neurotransmitter was recognized in 1958 by Arvid Carlsson when he found dopamine in the brains of reserpinized animals treated with L-DOPA, using spectrophotofluorimetry (Marsden, 2006; Fahn, 2006). This novel discovery identified dopamine as not only a biosynthetic precursor of norepinephrine and epinephrine, but also a catecholamine neurotransmitter possessing a signaling function of its own (Fahn, 2006; Benes, 2001). Neurotransmitters are chemical compounds that are released from neurons, and transmit chemical messages to other neurons or other target cells resulting in a net change in electrical activity (Campeau *et al*., 2007). An imbalance in the biosynthesis or metabolism of neurotransmitters could result in serious neurological problems or diseases (Campeau *et al*., 2007; Valenstein *et al*., 2005).

As a catecholamine, dopamine possesses a catechol moiety and an ethyl amine side chain (Sanders-Bush and Hazelwood, 2011) (Fig 1.1).

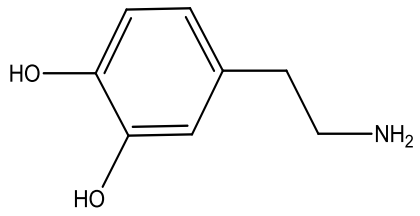


Fig 1-1: Chemical structure of dopamine

Catecholamines are organic compounds that contain a catechol nucleus (a benzene ring with two adjacent hydroxyl substituents) and an amine side chain (Flatmark, 2000). Catecholamines are involved in the regulation of various physiological processes, and are implicated in the pathogenesis of neurological, psychiatric, endocrine, and cardiovascular diseases (Eisenhofer *et al*., 2004). Dopamine, noradrenaline and adrenaline, which are all derivatives of 3, 4- dihydroxyphenylethylamine, are the three catecholamines *in vivo*. Although the three major catecholamines possess similar structures, their distributions and physiological functions in the central and peripheral nervous systems and the endocrine system are quite variable. Studies have shown that the three catecholamines are localized to specific parts of the brain, and maps of the course of distribution of all the catecholamines for various animal species have been shown (first identified by Vogt in 1954 for noradrenaline and adrenaline) (Nagatsu, 2006). In most mammalian organisms, about half (50%) of the total catecholamine content in the CNS is dopamine (Flatmark, 2000). The brain has the highest amount of dopamine, but detectable amounts are also present in the adrenal medulla and in the plexuses of the GI tract and enteric nervous system. Dopamine, unlike its biosynthetic precursor, L-DOPA, is a polar molecule that does not readily cross the blood-brain barrier (Sanders-Bush and Hazelwood, 2011). In addition to their major role as neurotransmitters in the central and peripheral nervous

systems, catecholamines have also been found to play an important role in the endocrine system where they function as hormones in the adrenal medulla (Nagatsu, 2006).

1.1.1 Physiological Role of Dopamine

Dopamine receptors were identified in the CNS in 1972 from studies that showed the ability of dopamine to act as a stimulus for adenylyl cyclase (Marin-Valencia *et al*., 2008). Dopamine receptors are usually categorized into two main groups: two D1-like receptor subtypes (D1 and D5) couple to the G protein, Gs and result in activation of adenylyl cyclase; while the D2-like subfamily (D2, D3, and D4) which are prototypical of G protein-coupled receptors exert their physiological function by inhibition of adenylyl cyclase and activation of potassium channels (Missale C. *et al*., 1998).

Physiologically, dopamine produces a wide range of effects such as control of voluntary locomotor activity, cognition, neuroendocrine secretion (prolactin), food intake and control of motivated behaviors including emotion, affect, and reward mechanisms (Missale *et al*., 1998; Kurian *et al*., 2011). As a classical neurotransmitter in the CNS, dopamine plays a key role in stress response and adaptive responses to stress (both physical and psychological) via coregulation of additional chemical transmitters (Stanwood, 2007). In peripheral tissues, dopamine acts to modulate renal hemodynamics, cardiovascular function, vascular tone and gastrointestinal motility (Marin-Valencia *et al*., 2008; Missale *et al*., 1998). Dopamine's role as a neurohormone in the hypothalamo-hypophysial axis, where it acts as the primary inhibitor of prolactin secretion in pituitary lactotrophs, has been well studied. It has been shown that abnormal regulation of

hypothalamic dopamine results in hyperprolactinemia as well as reproductive disturbances (Ben-Jonathan *et al*., 2001).

1.1.2 Metabolism and Fate of Dopamine

The biosynthetic pathway of dopamine starts with the amino acids, phenylalanine and tyrosine. Exogenous or dietary phenylalanine is converted *in vivo* by mammals to tyrosine using the enzyme phenylalanine hydroxylase. The highly lipid-soluble tyrosine easily traverses the blood brain barrier into the brain and is converted to L-DOPA (3, 4-dihydroxyphenylalanine) in dopamine neurons by the enzyme tyrosine hydroxylase, which is the rate-limiting step in dopamine synthesis (Sanders-Bush and Hazelwood, 2011; Bressan *et al*., 2005). L-aromatic amino acid decarboxylase (AADC) quickly converts L-DOPA to DA, and due to the rapid and avid action of this enzyme (AADC) in the CNS and peripheral system, levels of L-DOPA in the brain cannot be readily measured under normal conditions. Biosynthesized dopamine is transported by the vesicular monoamine transporter (VMAT2) from the cytoplasm of the neuronal cell, and is stored in secretory vesicles or in granular bodies within adrenal chromaffin cells, being concentrated to a level of about 0.1M (Sanders-Bush and Hazelwood, 2011; Bressan *et al*., 2005). Under physiological conditions, storage of dopamine in these specialized vesicles or granules prevents biodegradation of the monoamine in dopaminergic neurons and ensures release of dopamine in definite amounts when needed. On the other hand, dopamine synthesized in adrenergic or noradrenergic neurons is not stored in the manner described above, but rather it is converted to noradrenaline by dopamine beta-

hydroxylase and subsequent conversion to adrenaline by phenylethanolamine N-methyltransferase in adrenergic cells (Sanders-Bush and Hazelwood, 2011; Bressan *et al*., 2005). When an action potential reaches a dopaminergic neuron, "neuronal firing" triggers the release of dopamine into the synaptic cleft by an exocytotic mechanism which leads to activation of presynaptic and postsynaptic dopamine receptors. Presynaptic autoreceptors ensure regulation of neurotransmission by feed-back mechanisms to control synthesis, storage, and release of dopamine (Bressan *et al*., 2005). Dopamine released into the synapse can be degraded or cleared postsynaptically by organic cation transporter 3 (OCT3), but the predominant mechanism of dopamine synaptic clearance and termination of dopamine action is reuptake into presynaptic neurons by the dopamine transporter (DAT) which is non-selective for dopamine. The DAT quickly uptakes most of the released DA into the pre-synaptic neuron which is either stored in the granular bodies or metabolized to 3, 4-dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (MAO). However, in brain areas with low DAT density (such as the prefrontal cortex (PFC)), metabolism of dopamine by catechol-*O*-methyltransferase (COMT) and uptake by the norepinephrine transporter (NET) predominate as homoestatic mechanisms of dopaminergic transmission (Sanders-Bush and Hazelwood, 2011; Mikko *et al*., 2010). Dopamine reuptake by the DAT ensures adequate recycling and vesicular storage of dopamine, and the DAT provides a mechanism for dopamine reuptake to be regulated via phosphorylation (Bressan *et al*., 2005).

Dopamine is inactivated by degradative and/or conjugative pathways. The major inactivating enzyme in the degradative pathway of cytosolic dopamine is monoamine

oxidase (MAO). Oxidative deamination of dopamine by cellular MAO enzymes leads to production of a physiologically inactive, but potentially toxic aldehyde derivative (3, 4-dihydroxyphenylacetaldehyde (DOPAL)), which is further metabolized by aldehyde dehydrogenase to form 3, 4-dihydroxyphenylacetic acid (DOPAC) (Sanders-Bush and Hazelwood, 2011).

Methylation and sulfation of dopamine and its degradative products, catalyzed by COMT and sulfotransferase enzyme respectively, are important for inactivation and subsequent excretion (Marchitti *et al*., 2007; Buu *et al*., 1985). COMT is capable of converting DOPAC to form homovanillic acid (HVA). HVA represents the predominant end product of dopamine degradation and is excreted in urine at up to twice the rate of VMA (vanillylmandelic acid), the metabolic end product of adrenaline and noradrenaline (Eisenhofer *et al*., 2004). Hence, HVA is used as a reliable index of dopamine turnover in the brain. An alternative pathway leading to generation of HVA is by direct O-methylation of dopamine by COMT resulting in production of 3-methoxytyramine, which is further converted to HVA by MAO (Sanders-Bush and Hazelwood, 2011; Birtwistle and Baldwin, 1999).

Sulfate conjugation in the metabolism and disposition of catecholamines was first identified in 1940 by Richter, who conclusively stated that the major pathway for degradation and excretion of adrenaline was sulfation (Eisenhofer *et al*., 1999). Subsequent studies which showed that deamination and methylation were rather the main inactivation pathways refuted Richter's claim, thus the significance of sulfation in catecholamine metabolism was almost neglected (Eisenhofer *et al*., 1999). Notwithstanding, several studies have shown that sulfate conjugation represents a

significant pathway that prevents binding of dopamine to its receptors, and thus this metabolic pathway is highly important in the inactivation and excretion of dopamine. Sulfate conjugation by sulfotransferase enzyme, specifically SULT1A3, represents up to 15% of total dopamine inactivation while methylation by COMT contributes less than 5% of total dopamine inactivation (Eisenhofer *et al*., 1999; Keinzl *et al*., 1990; Werle, 1988). Other studies in humans have shown that in circulation, dopamine produced endogenously as well as from dietary sources exists predominantly (at least 95%) as dopamine sulfate (Goldstein *et al*., 1999). Sulfate conjugation of dopamine results in the production of two sulfate esters: dopamine-3-O-sulfate (DA-3S) and its regioisomer, dopamine-4-O-sulfate (DA-4S), with the former being the predominant metabolite (about 10-fold higher). The extremely high level of DA-3S relative to DA-4S level in plasma is due to the regiospecificity of SULT1A3 enzyme for the 3-hydroxyl group of dopamine (Itaaho *et al*., 2007; Keinzl *et al*., 1990). Sulfate conjugation of the dopamine degradative products, DOPAC and HVA, occurs predominantly in mesenteric organs and are excreted in urine basically as sulfoconjugates (Marchitti *et al*., 2007; Eisenhofer *et al*., 1999).

1.1.3 Disorders of Dopamine metabolism

A dysfunction in the metabolic pathway of dopamine and other biogenic amines results in significant neurodegeneration of central and peripheral neurons by producing certain neurotoxins endogenously (Eisenhofer *et al*., 2004). This homoestatic imbalance has been shown to result in neuropathological conditions such as Parkinson's disease, Segawa disease, schizophrenia, Tourette's syndrome, Alzheimer's disease, bipolar

disorders, anxiety and depression, Huntington's chorea, autism, addictions, suicide, behavioral problems, attention deficit hyperactivity disorder (ADHD) syndrome in children, and hyperprolactinemia (Marin-Valencia *et al*., 2008; Nieoullon, 2002).

In the etiology of Parkinson's disease, which affects mainly locomotor activity, there is degeneration and extensive loss of dopamine-producing neurons in the substantia nigra and mesostriatal pathway (Eisenhofer *et al*., 2004; Birtwistle and Baldwin, 1999). During its metabolic pathway, dopamine is deaminated to form a potentially toxic aldehyde derivative (3, 4-dihydroxyphenylacetaldehyde (DOPAL)), which is further metabolized and detoxified by aldehyde dehydrogenase to form 3, 4-dihydroxyphenylacetic acid (DOPAC). Thus, any defect or dysregulation in the function of aldehyde dehydrogenase (ALDH), which is exclusively responsible for oxidative metabolism of biogenic amines, could result in neurotoxicity associated with Parkinson's disease and Alzheimer's disease. It has also been shown that the formation of DOPAL by MAO gives rise to hydrogen peroxide (H_2O_2), which can generate other ROS and free radicals ultimately leading to cytotoxictiy and neurodenegeration (Marchitti *et al*., 2007; Li *et al*., 2001).

Schizophrenia, which is literally defined as "splitting of the mind", has certain core clinical manifestations including abnormal thoughts and perceptions, motor abnormalities and cognitive defects (Birtwistle and Baldwin, 1999). Contrary to the classical dopamine hypothesis, the revised hypothesis postulates that there is actually a co-existence of subcortical dopamine excess and cortical dopamine deficit in a typical schizophrenic brain (Abi-Dargham *et al*., 2004). It has been shown that negative and cognitive symptoms in schizophrenia are the result of decreased dopamine in the cortex

which leads to hypostimulation and hypoactivity of D1 receptors, while positive symptoms basically results from subcortical hyperstimulation of D2 receptors by dopamine. Positive symptoms of schizophrenia include hallucinations, delusions, inappropriate affect (emotions) and thought disorder, while negative symptoms include social withdrawal, poor thought and speech, impaired volition, and blunted affect (Abi-Dargham *et al*., 2004; Birtwistle and Baldwin, 1999).

1.2 Conjugation Reactions in Biotransformation of Endobiotics and Xenobiotics

Biotransformation of xenobiotics consists of two phases. Phase I (functionalization) reactions occur by addition of certain functional groups (-OH, COOH, -SH, -O- or NH_2) to a parent compound, while Phase II (conjugation) reactions involve the conjugation of Phase I products, which can then be readily excreted from the body (Gonzalez and Tukey, 2011). Endogenous compounds and certain xenobiotics that already possess a conjugatable functional group proceed directly through Phase II reaction (Mulder, 1990). In addition to their importance in xenobiotic metabolism, conjugation (Phase II) reactions, including, methylation, sulfation, and glucuronidation, have been recognized to play an important role in the metabolism of essential endogenous compounds such as catecholamines and steroid hormones (Gibson and Skett, 2001). The enzymes responsible for metabolism of xenobiotic compounds are also capable of catalyzing the metabolism of endogenous compounds, and as a large family, Phase II enzymes comprise individual groups of enzymes which have distinguishable but

overlapping substrate specificities. Conjugation in biotransformation reactions is generally known to result in the production of inactive hydrophilic metabolites which can be excreted more readily from the body (Hayes, 2008; Gonzalez and Tukey, 2011).

1.2.1 Methylation

Methylation is known to be a major pathway involved in the metabolism and disposition of some endogenous compounds (neurotransmitters, amino acids, proteins, carbohydrates, etc.) drugs and xenobiotics. Methylation is defined as the transfer of a methyl group ($-CH_3$) from a methyl donor to an acceptor substrate, leading to the generation of a methyl conjugated product (Mulder, 1990; Axelrod *et al*., 1966; Axelrod and Tomchick, 1958). The universal methyl donor in methylation reactions is S-adenosyl methionine (SAM). The main methylation reactions identified are O-, S-, and N-methylation, and their corresponding methylating enzymes are: catechol-O-methyltransferase (COMT), hydroxyindole-O-methyltransferase and proteincarboxy-O-methyltransferase (Hayes, 2008; Mulder, 1990). In mammals, COMT is widely distributed in various regions of the body, predominantly in the brain, and peripheral organs such as kidney, liver and gastrointestinal tract (Männistö and Kaakkola, 1999). There are two isoforms of the COMT enzyme: cytosolic or soluble (S-COMT) and membrane-bound (MB-COMT) forms, which are encoded by a single gene (Hayes, 2008; Männistö and Kaakkola, 1999). The COMT enzyme catalyzes the magnesium-dependent transfer O-methylation of catechol oxygen atoms in catecholamines (dopamine, norepinephrine, and epinephrine), their hydroxylated metabolites, catecholestrogens,

ascorbic acid, and dihydroxyindolic intermediates of melanin, and other catechol drugs. Methylation of catechols is not only essential for their inactivation, but also as a pathway that prevents them from producing ROS and reactive semi-quinone/quinone metabolites (Hayes, 2008). For both S-COMT and MB-COMT, methylation occurs preferentially at the 3'-hydroxyl instead of the 4'-hydroxyl, which is due to the fact that they are capable of being oriented differently in the catalytic site of COMT.

1.2.2 Sulfation

Although the existence of sulfate-conjugated compounds was discovered far back in 1876, it was about 80 years later when the mechanism of action of sulfoconjugation became clear (Baumann, 1876; Robbins and Lipmann, 1956). It was shown that an active sulfate donor, 3'-phosphoadenosine 5'-phosphosulfate (PAPS) was involved in the process of sulfation, and that the reaction was catalyzed by enzymes called the sulfotransferases (Robbins and Lipmann, 1956). Sulfotransferases are a large family of enzymes catalyzing the transfer of a sulfonate group (SO_3^{-1}) from PAPS to a variety of amine, sulfuryl and hydroxyl functional groups present in xenobiotics and some endogenous compounds (depicted as R-OH in Fig. 1-2), resulting in the generation of a sulfate conjugated product ($R\text{-}OSO_3^{-1}$) (Wong *et al*., 2004).

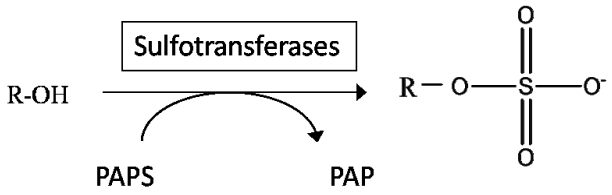


Fig 1-2: Formation of sulfonate ester and its hydrolysis.

Generally, the sulfotransferases are classified as either cytosolic sulfotransferases (SULTs) or membrane-bound sulfotransferases. SULTs catalyze the sulfation of a larger number of exogenous and endogenous biomolecules which have relatively small size and includes steroid hormones, thyroid hormones, catecholamines and several xenobiotics (Hayes, 2008; Wong *et al*., 2004). Membrane-bound sulfotransferases are located in the Golgi-apparatus and have not been implicated in xenobiotic metabolism, but rather have been shown to play an important role in sulfate conjugation of large endogenous biomolecular compounds such as glycosaminoglycans; carbohydrates; and tyrosine in proteins and peptides (Hayes, 2008; Wong *et al*., 2004). There are basically three classes of sulfation reactions based on the functional group present in the substrate: O-sulfation (ester), N-sulfation (amide), and S-sulfation (thioester). Among these, O-sulfation is the most prevalent sulfation reaction involving compounds like catecholamines, steroids, vitamins, proteoglycans and glycosaminoglycans (Wong *et al*., 2004; Strott, 2002). From a general perspective, sulfate conjugation of a xenobiotic or endobiotic result in a biologically inactive and less toxic sulfated product which is readily excreted because of increased hydrophilicity. It is however noteworthy that some exceptions to this general notion exist whereby sulfation reactions rather produce bioactive compounds with increased toxicity and usually possessing mutagenic and carcinogenic properties (Falany, 1997; Hayes, 2008).

As mentioned previously, PAPS is the cofactor which donates its sulfonate group to an acceptor substrate during the process of sulfate conjugation catalyzed by sulfotransferases. PAPS is synthesized in a process involving two successive steps: first step being the production of adenosine-5'-phosphosulfate (APS) from inorganic sulfate

and ATP as mediated by ATP-sulfurylase; with subsequent phosphorylation of APS by APS kinase produces PAPS (Strott, 2002; Cho *et al*., 2004). Studies have shown that enzymes involved in the formation of PAPS (ATP sulfurylase and adenosine 5'-phosphosulfate kinase (APS kinase)) are highly conserved among all organisms and lack of sulfate supply for PAPS synthesis can lead to certain abnormalities, but this rarely occurs because plasma levels of sulfate are relatively stable (Strott, 2002; Hayes, 2008; Wong *et al*., 2004).

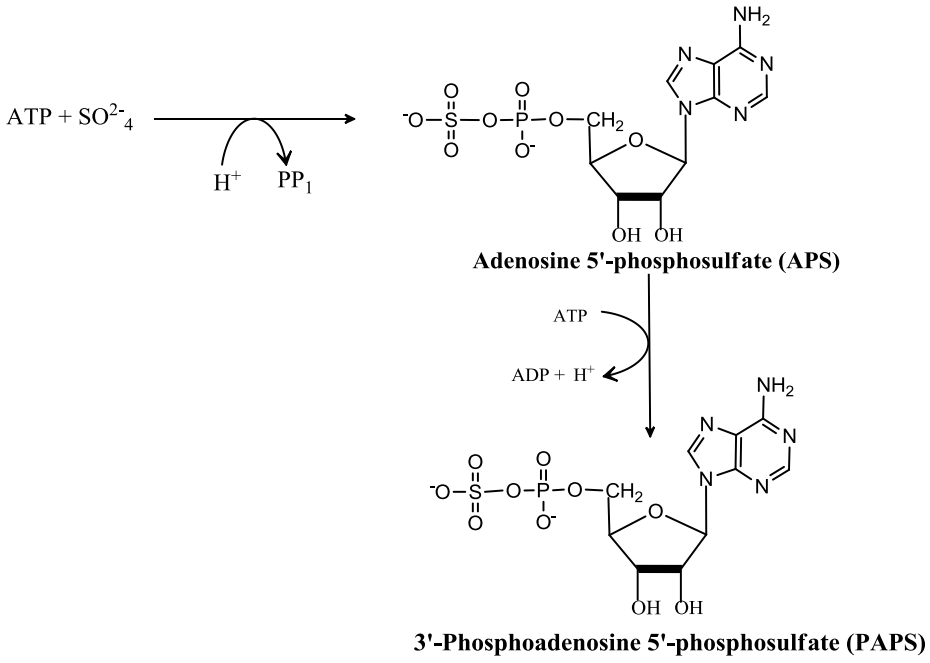


Fig 1-3: Two-step catalytic reaction in the formation of PAPS.

1.2.2.1 Cytosolic Sulfotransferases (SULTs)

Over 50 SULT enzymes have been reported in both mammalian and avian species. Molecular cloning and amino acid sequencing of the different SULT families on a genetic level has revealed up to 40% homology in terms of amino acid sequence. For members within each subfamily, more than 60% amino acid sequence homology is found (Nagata and Yamazoe, 2000; Nimmagadda *et al*., 2006). Notwithstanding the high extent of homologous amino acid sequence, different SULT enzymes seem to exhibit diversity in their functional roles (Allali-Hassani, 2007). Regarding substrate specificity, the membrane-associated sulfotransferases seem to be more specific in their choice of substrate, while the SULTs are less selective in their reactivity towards various substrates (Wong *et al*., 2004). In addition to the apparent overlapping substrate reactivities of the SULT enzymes, the ubiquitous nature of the SULTs in human tissues makes it possible for a wide range of endogenous and exogenous compounds to be sulfated in the human system under physiological conditions (Falany, 1997).

Of the five main SULT families in mammals, SULT1 and SULT2 are the prominent ones which have received considerable attention (Strott, 2002). It has been shown that members in the SULT1 family have a higher affinity and reactivity towards phenolic compounds such as catecholamines, while SULT2 SULTs are more specific for hydroxysteroids (Wong *et al*., 2004). Eleven human SULTs have been identified and well characterized (Sakakibara *et al*., 1998a, b, 2002; Suiko *et al*., 2000; Pai *et al*., 2002).

Table 1.1: Nomenclature and Substrates of human cytosolic sulfotransferases (Modified from Wong *et al.,* 2004)

Name	Substrate	Representative substrates	Amino acid sequence identity *
SULT1A1	phenols	p-Nitrophenol, 2-napthol, T3, Dopamine, Tyramine, 3,3'-T2	100
SULT1A2	phenols (low affinity)	PNP, dopamine	95
SULT1A3	catecholamines	PNP, tyramine, dopamine	93
SULT1B2	thyroid hormones	PNP T4 ,T3 r,T3	54
SULT1C#1	aryl hydroxylamines	PNP, dopamine, N -hydroxy-2- acetylaminofluorene	37
SULT1C#2	aryl hydroxylamines	PNP, dopamine, N -hydroxy-2- acetylaminofluorene	41
SULT1E1	estrogens	Estradiol, DHEA Androstenediol, PNP	51
SULT2A1	hydroxysteroids	Estradiol, DHEA Androstenediol	37
SULT2B1a	hydroxysteroids	DHEA, pregnenolone, 17α-hydroxypregnenolone	41
SULT2B1b	hydroxysteroids	DHEA, Pregnenolone 17α-hydroxypregnenolone	39
SULT4A1	Unknown	Unknown	32

*Relative to SULT1A1

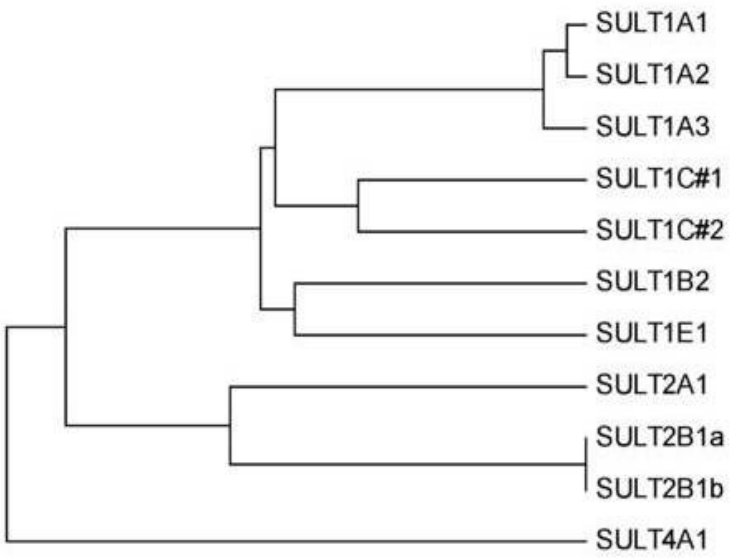


Fig 1-4: Classification of human SULTs based on homology in amino acid sequence

1.2.2.2 Phenol Sulfotransferases: Overview, Biochemical Properties and Regional Localization

At least seven members of the SULT1 subfamily (1A1, 1A2, 1A3, 1B1, 1C1, 1C2, and 1E1) have been expressed in humans, and members of this SULT1 subfamily are referred to as phenol sulfotransferases because of their ability to sulfate phenolic drugs and catecholamines (Strott, 2002). As far as metabolism of xenobiotics and endobiotics are concerned, the phenol sulfotransferases (PSTs) have been found to play a vital role which can be partly attributed to their abundance, wide tissue distribution and high activity towards a broad range of substrates (Strott, 2002; Falany, 1997). There are two major forms of PSTs: P-form of phenol sulfotransferase (SULT1A1) and M-form of phenol sulfotransferase (SULT1A3) which were originally identified and characterized in platelet cytosol (Falany, 1997). Their nomenclature derives from studies which showed

that M-PST distinctively catalyzes the sulfation of monoamine neurotransmitters (dopamine, norepinephrine and epinephrine), whereas P-PST is involved in the sulfate conjugation of neutral phenols such as p-nitrophenol and α-naphthol (Falany, 1997). Depending on their sensitivities to thermal inactivation *in vitro* at temperatures above 37^0C, P-PST and M-PST have also been classified as the thermostable and thermolabile isoforms of PST respectively (Falany, 1997). Although these two isoenzymes have significant difference in their substrate reactivity, the amino acid sequence of M-PST is 92.5% homologous to that of P-PST, and their cDNA sequences encode proteins with molecular weights of 34,134 and 34,097 Da, respectively (Strott, 2002). Among the SULT1 isoenzymes, SULT1A3 previously called thermolabile form or M-form of phenol sulfotransferase, shows significant selectivity and reactivity toward catecholamines. SULT1A3 catalyzes sulfate conjugation reaction of catecholamines usually at carbon-3 of the phenyl ring, and the enzyme has highest affinity for dopamine (Strott, 2002; Falany, 1997; Itaaho *et al*., 2007). Molecular cloning of SULT1A3 reveals that it is highly expressed and localized in the upper gastrointestinal tract which is the major site for dopamine sulfate production (Eisenhofer *et al*., 1999; Goldstein *et al*., 1999; Strott, 2002). Other studies on regional localization and tissue distribution of SULT1A3 show its presence in other tissues in such as brain, platelets and other peripheral tissues (Strott, 2002). With a resolution of 2.4-Å, Bidwell *et al* showed the crystal structure of the SULT1A3 isoenzyme as having a sulfate bound at its active site (Bidwell *et al*., 1999).

1.2.2.3 Concerted action of SULT1A3 and COMT in dopamine metabolism

As mentioned previously, sulfation and methylation by SULT1A3 and COMT respectively, play key role in the metabolism and disposition, hence homeostasis of dopamine (Falany, 1997; Strott, 2002). Several studies have reported the detection of sulfated and methylated dopamine in cerebrospinal fluids, blood and urine samples (Palkovits and Brownstein, 1983; Oeltmann *et al*., 2004; Buu *et al*., 1985). Other studies have also shown that SULT1A3 and COMT (both soluble and membrane) are expressed and localized in glial cells (Yu and Walz, 1985; Hong *et al*., 1998). Yasuda *et al* reports a study which shows that conjugation reaction of dopamine occurs in two different pathways; methylation by COMT and subsequent sulfation by SULT1A3; and that it is the 3-methyldopamine-O-sulfate which is the predominant end product (Yasuda *et al*., 2009). It is interesting to note that both SULT1A3 and COMT enzymes have preferential reactivity at the 3-hydroxyl group of dopamine (Buu *et al*., 1985). Recent studies have however clarified that COMT specifically catalyzes the 3-O-methylation of dopamine (Lotta *et al*., 1995; Mannisto and Kaakkola, 1999), while SULT1A3 is capable of sulfating not only the 3-hydroxyl group but also the 4-hydroxyl group (although with a relatively lower efficiency) of dopamine (Itaaho *et al*., 2007). This double conjugation reaction is known to irreversibly inactivate dopamine, therefore contributing to excretion of excess dopamine which, if not efficiently regulated, could lead to abnormalities in neurotransmission.

1.3 Nitrative Stress: Role of Reactive Nitrogen Species in Toxicology

The free radical nitric oxide (NO) was discovered by Louis Ignarro 1986 who referred to it as 'endothelium-derived relaxing factor (EDRF)' (Ignarro *et al*., 1986; Kroncke, 2003). NO has been found to be an ubiquitous biosignalling molecule present in the cells of almost all vertebrates and other organisms, and unlike ROS, which is usually produced by non-enzymatic reaction, NO is synthesized by enzymes called nitric oxide synthases (NOS) in the body (Parcher *et al*., 2007; Kroncke, 2003). NO is an inevitable and highly essential messenger molecule which plays multifunctional roles including neurotransmission, vasodilation, fertilization, differentiation, inflammation and apoptosis in humans (Chandru *et al*., 2010; Kroncke, 2003). As a key modulator of several transcription factors, NO has been found to be involved in the regulation of gene expression (Chandru *et al*., 2010). Basically, the NO pathway involves changes in the normal physiological redox status and this is accomplished by covalent binding of NO to certain metal- and thiol-containing proteins, specifically cysteines, and other amino acid residues such as tyrosine (Hausladen, 1996; Dietrich-Muszalska *et al*., 2009). This process, referred to as nitrosylation, when occurring in a dysregulated manner can overwhelm cellular defense mechanisms resulting in injury or death of cells. This kind of imbalance in cellular physiology, initiated by "nitrosants" (reactive nitrogen species, RNS), is called nitrative or nitrosative stress, a term which was first coined by Jonathan Stamler (Hausladen, 1999; Kroncke, 2003).

1.3.1 Generation of Peroxynitrite and Physiological Consequences

It is worth noting that the detrimental and cytotoxic effects of NO is rather an "indirect mechanism" which is mediated by the "more toxic" and strong oxidizing agent, peroxynitrite ($ONOO^-$), generated in vivo from a reaction between NO and superoxide anion ($O^{\cdot}{}_2^-$) (Fig. 1.5) (Parcher *et al*., 2007; Beckman *et al*., 1996). Being a very potent scavenger of $O^{2-\bullet}$, NO has been found to be the only biomolecule which has a higher affinity for $O^{2-\bullet}$ than SOD (superoxide dismutase) and thus NO can itself act as an efficient antioxidant (Lubos *et al*., 2008). Notwithstanding the involvement of peroxynitrite ($ONOO^-$) in NO toxicity, other studies have shown that NO and $ONOO^-$ have different mechanisms, and that NO can be potentially toxic independent of $ONOO^-$ formation (Meij *et al*., 2004).

$$\mathbf{NO + O^{\cdot}{}_2^- \longrightarrow ONOO^-}$$

Fig. 1-5: Formation of peroxynitrite from nitric oxide.

The physiological consequences of nitrative stress, mediated by peroxynitrite are diverse, and includes adduct formation of peroxynitrite with lipids, DNA and proteins as well as direct cytotoxic effects, ultimately resulting in cell necrosis or apoptosis via certain inflammatory pathways. Another potential biochemical effect of peroxynitrite formation is the oxidation and inactivation of enzymes which are involved in metabolism of key endogenous compounds and xenobiotics (Fig 1.6) (Hausladen, 1996; Parcher *et al*., 2007; E. Lubos *et al*., 2008).

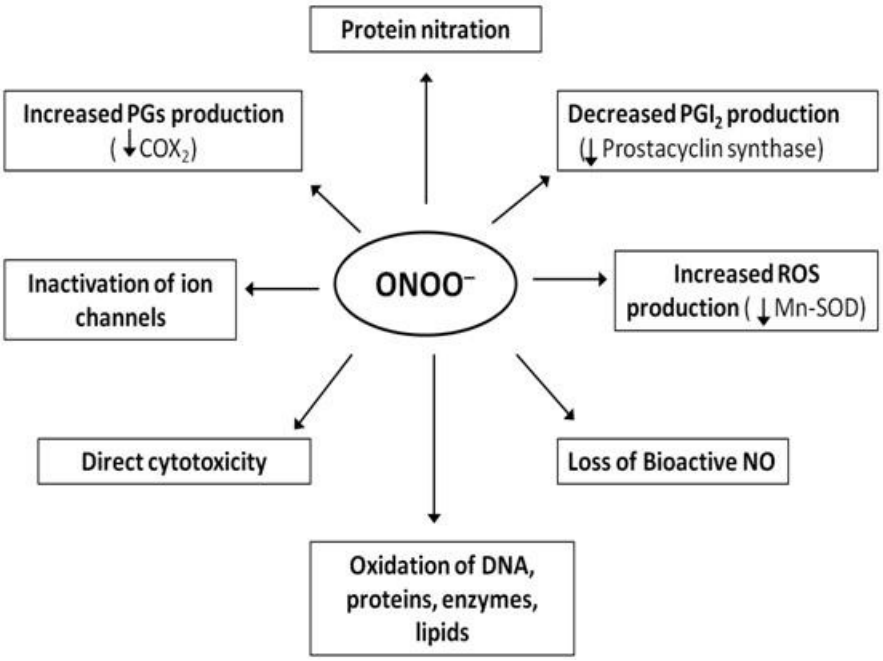


Fig 1-6: Reactive Pathways and Biological Consequences of Peroxynitrite (Modified from Lubos *et al*., 2008)

Several studies have investigated the effects of nitrative stress on the activity of sulfotransferase enzymes. One such study found that upon exposure to NO, both in vivo and *in vitro*, the sulfating activity of SULT2B1a in rats was suppressed; while another experiment reported the down-regulation of SULT2A1 in human Hep G2 cells (Chandru *et al*., 2010; Kohjitani *et al*., 2008). Liu *et al*. also reports of a significant decrease in the sulfating activity of the human cytosolic sulfotransferase SULT1E1 under nitrative stress conditions (Liu *et al*., 2010).

Though the pathway involved remains vague, nitrative stress mediated by peroxynitrite, has been reported to affect dopamine metabolism and hence plays a role in the pathogenesis of Parkinson's disease, schizophrenia and other neurodegenerative

disorders (Dietrich-Muszalska *et al*., 2009; Chen *et al*., 2004; Parcher *et al*., 2007). There is evidence of colocalization of nitric oxide and superoxide anion in tissues of the brain; and elevated levels of 3-nitrotyrosine, a biomarker for reactive nitrogen species (RNS), has been identified in patients with neurodegenerative disorders (Kerry and Rice-Evans, 1999). Peroxynitrite is known to cause nitration and consequent inactivation of tyrosine hydroxylase and MAO-B, which are key enzymes in the dopaminergic pathway, hence leading to disrupted dopamine metabolism (Parcher *et al*., 2007). Aldehyde dehydrogenase (ALDH), another enzyme in the metabolic pathway of dopamine is known to be highly sensitive to ROS and RNS. In its degradative pathway, dopamine is deaminated to form a toxic aldehyde derivative, DOPAL, which is further metabolized and detoxified by ALDH to form DOPAC. Thus, an inhibition of ALDH by RNS leads to elevated levels of DOPAL, which is highly toxic to dopaminergic neurons, and has been implicated in the pathophysiology of PD and other neurodegenerative diseases (Roberts *et al*., 2009; Florang *et al*., 2007; Jinsmaa *et al*., 2009).

1.3.2 NO Donors: Overview, Properties and Mechanism of Action

Because of the versatile role of NO as a biosignalling molecule, the use of NO donors in biochemical and pharmacological research has greatly increased. Generally, due to their ability to release nitric oxide (NO), NO donors simulate endogenous NO-related effects (Feelisch, 1998). There have been numerous studies and reports on the various classes of NO donors which are primarily distinguished based on the factors outlined: whether NO donor decomposition resulting in NO liberation is via enzymatic

pathway or not; rate of NO formation; redox state in which NO is generated (NO+, NO• or NO–); as well as other physicochemical factors affecting NO donor decomposition in biological systems (Feelisch, 1998; Klink and Sulowska, 2007; Wang *et al*., 2002). Because of the extremely short half-life of NO, NO donors have proven useful over the years because they serve a readily available source of continuous release of NO for research purposes (Thompson *et al*., 2009).

1.3.2.1 SIN-1 (3-Morpholinosydnonimine)

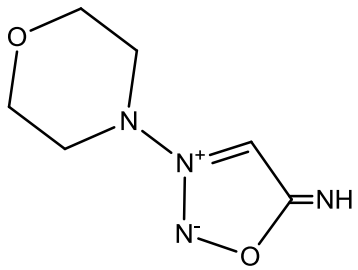


Fig 1-7: Structure of SIN-1 (3-Morpholinosydnonimine).

SIN-1 **(3-Morpholinosydnonimine)** is a well known NO donor, and it is the active metabolite of molisdomine, a prodrug which is commonly used for its vasodilatory effect. Based on its NO production, the half-life of SIN-1 has been found to be 230 minutes (approximately 4 hours) (Coert *et al*., 2002), and it undergoes a catalytic reaction in aqueous solution to produce nitric oxide (NO) and superoxide anion ($O^{\cdot}{}_2^-$) which ultimately leads to the production of the potent oxidant, peroxynitrite ($ONOO^-$) (Trackey *et al*., 2001). The generation of peroxynitrite ($ONOO^-$) from SIN-1 at physiological pH

results from a three-step chemical reaction, and starts with a non-enzymatic hydrolytic reaction of SIN-1 to form an open ring isomer SIN-1A, which, in the presence of molecular oxygen can further react by oxidative cleavage to produce an intermediate SIN-1 cation radical and $O^{\cdot}{}_2^-$. This SIN-1 intermediate can then undergo subsequent reaction by losing protons to produce SIN-1C (N-morpholino-aminoacetonitrile) with the release of NO (Fig 1.8) (Feelisch, 1998; Singh *et al*., 1999). Because of the considerable amounts of protons produced as a result of SIN-1 decomposition, a suitably buffered solution is necessary when using this sydnonimine during *in vitro* studies and for other research purposes (Feelisch, 1998; Lomonosova *et al*., 1998).

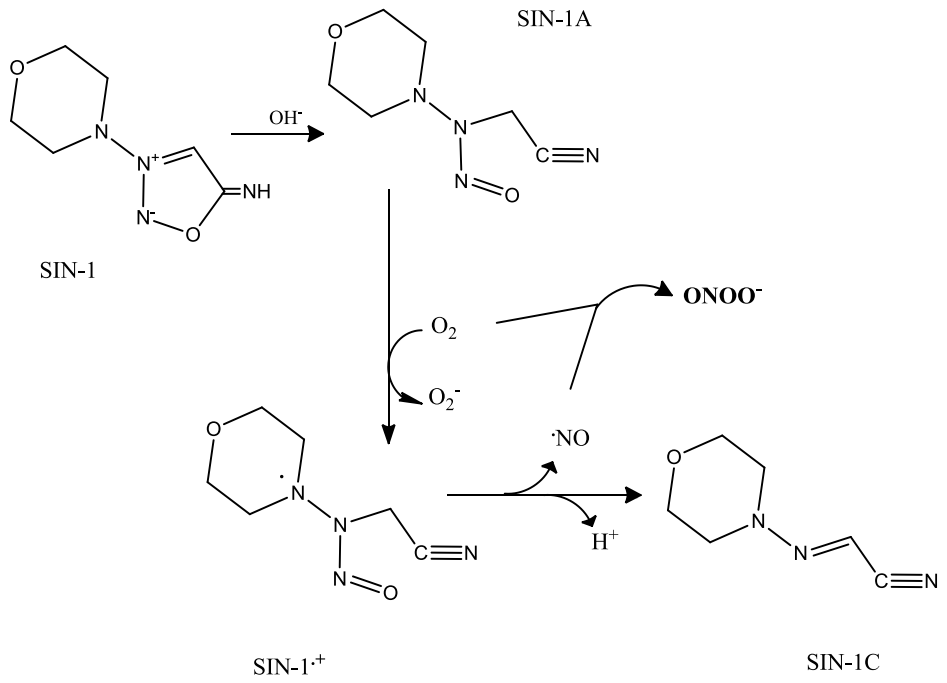


Fig 1-8: Oxidative decomposition of SIN-1 yielding NO and SIN-1C.

There have been several reports on the detrimental and toxic effects (lipid peroxidation, enzyme inactivation, protein nitration, etc) of peroxynitrite produced from SIN-1 (Feesisch, 1998; Hogg *et al*., 1992; Hausladen, 1999; Parcher *et al*., 2007; E. Lubos *et al*., 2008). Though peroxynitrite generation is the predominant pathway of SIN-1 decomposition, some studies suggests that it is just one of many reactive pathways (Feesisch, 1998).

1.3.2.2 Diethylenetriamine (DETA NONOate)

NONOates are a class of NO donors which are basically made up of a NO dimer which is attached to a nucleophilic moiety to form the general chemical structure, X-[N(O)NO]$^{-}$ (Feelisch, 1998). One such compound which is widely used in enzymology and general pharmacological research as an NO donor is diethylenetriamine NONOate (DETA NONOate), (Keefer *et al*., 1996) which has proven very useful for its NO liberation properties due to its relatively long half-life (about 20 hours at pH 7.4 and 37°C), hence sustained release of NO over a longer period; and also due to the fact that its decomposition yields only NO and DETA which is known to be non-toxic (Thompson *et al*., 2009). In addition, diethylenetriamine NONOate has the advantage of withstanding effect of light, metal and thiol compounds (Thompson *et al*., 2009; Feelisch, 1998; Wang *et al*., 2002).

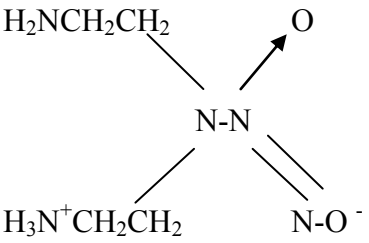


Fig 1-9: Structure of Diethylenetriamine NONOate.

Unlike SIN-1, diethylenetriamine NONOate decomposition does not produce peroxynitrite ($ONOO^-$), but rather it undergoes a catalytic reaction promoted by acid and/enzymes to liberate two moles of NO per one mole of diethylenetriamine NONOate (Feelisch, 1998; Trackey *et al*., 2001).

1.4 Human SK-N-MC Neuroblastoma cell line

A neuroblastoma is a solid tumor which usually affects the sympathetic nervous system but may also occur in nerve tissues of the neck, abdomen, pelvis or chest. Studies have shown that elevated levels of catecholamines (dopamine, noradrenaline and adrenaline) and their metabolites are found in neuroblastoma cells (Brodeur, 2003; Haase *et al*., 1999).

SK-N-MC, a neuroblastoma cell line, was established by Biedler in 1971 and has since then been used as an important *in vitro* model in biomedical research. Using histochemical fluorescence method of detection, Helson and Biedler showed the presence of neurotransmitter catecholamines in SK-N-MC cells, (Helson and Biedler, 1973) but it

was discovered later that SK-N-MC cell showed no presence of dopamine-3-hydroxylase (Biedler *et al*., 1973). Other studies which identified the presence of D1 dopamine receptor have also confirmed existence of dopamine in SK-N-MC neuroblastoma cells (Biedler *et al*., 1978; Sidhu and Fishman, 1990; Sidhu, 1997). The expression and biochemical function of SULT1A3 and COMT in SK-N-MC neuroblastoma cells have also been investigated in recent studies. Results of one study showed the ability of SK-N-MC cells to produce sulfated dopamine and sulfated 3-methyldopamine (Yasuda *et al*., 2009).

1.5 Objectives and goals

As mentioned previously, regulation of the metabolic pathway of dopamine is critical for normal functioning of the nervous system, and disruption in the homeostasis of dopamine has been implicated in the pathogenesis of several neurological disorders including Parkinson's disease and schizophrenia (Marin-Valencia *et al*., 2008; Nieoullon, 2002; Marchitti *et al*., 2007; Li *et al*., 2001). Nitrative stress is known to affect dopamine metabolism, and has been suggested by several reports as an etiologic factor for neurodegenerative diseases (Dietrich-Muszalska *et al*., 2009; Chen *et al*., 2004; Parcher *et al*., 2007).. Considering the key role of sulfation in the inactivation and homeostatic regulation of dopamine, we hypothesized that nitrative stress could suppress the sulfation and subsequent disposal of dopamine and its methylated product, hence resulting in their accumulation, which has been implicated in the pathogenesis of neurodegenerative diseases (Strott, 2002; Marchitti *et al*., 2007; Eisenhofer *et al*., 1999). Thus, the current study aims to test this hypothesis and elucidate the underlying mechanism, using two NO

donors, SIN-1 and DETA, to simulate nitrative stress conditions. As part of the overall aim, the objectives of the study were: to first identify the sulfotransferase enzyme responsible for catalyzing the sulfation of dopamine and 3-methyldopamine; to study the time-dependent effect of nitrative stress on the sulfation of dopamine and 3-methyldopamine; and to study the concentration-dependent effect of nitrative stress on sulfation of dopamine and 3-methyldopamine in metabolic labeling experiments and enzymatic assays.

2. Materials and methods

2.1 Materials

Dopamine, 3-methoxytyramine hydrochloride (3-methyldopamine hydrochloride), adenosine 5'-triphosphate (ATP), 3'-phosphoadenosine-5'-phosphosulfate (PAPS), trizma base, 3-(N-morpholino) propanesulfonic acid (Mops), inorganic pyrophosphatase, dithiothreitol (DTT), dimethyl sulfoxide (DMSO), tropolone (2-hydroxy- 2,4,6-cycloheptatrien-1-one), Nonidet P-40, silica gel thin-layer chromatography (TLC) plate and minimum essential medium (MEM) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetal bovine serum was from Biomeda (Foster City, CA). 3-Morpholinosydnonimine (SIN-1) was from Tocris Bioscience (Ellisville, MO) and diethylenetriamine NONOate (DETA) was purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.). SK-N-MC human neuroblastoma cell line (ATCC HTB-10) was obtained from American Type Culture Collection (Manassas, VA). PAP[^{35}S] was synthesized from ATP and carrier-free [^{35}S] sulfate using the bifunctional human ATP sulfurylase/adenosine 5'-phosphosulfate kinase and its purity determined as previously described (Yanagisawa *et al*., 1998). Carrier-free sodium [^{35}S] sulfate was from ICN Biomedicals Inc. (Irvine, CA, U.S.A.). HyClone trypan blue dye solution was from Thermo Scientific (Waltham, MA, U.S.A.). Ecolume scintillation cocktail and carrier free sodium [^{35}S] sulfate were products of MP Biomedical (Solon, OH). All other chemicals were of the highest grade commercially available.

2.2 Preparation of Purified Human Cytosolic SULTs and Sulfotransferase Assay

Recombinant human P-form (SULT1A1 and SULT1A2) and M-form (SULT1A3) phenol SULTs, thyroid hormone SULT (SULT1B2), SULT1C#1, SULT1C #2, estrogen SULT (SULT1E1), dehydroepiandrosterone (DHEA) SULT (SULT2A1), SULT2B1a, SULT2B1b and a neuronal SULT (SULT4A1), expressed using pGEX-2TK or pET23c prokaryotic expression system, were prepared as previously described (Sakakibara *et al*., 1998a,b, 2002; Suiko *et al*., 2000; Pai *et al*., 2002).

The sulfating activity of the recombinant human cytosolic SULTs was assayed using PAP[^{35}S] as the sulfate group donor. The standard assay mixture, in a final volume of 20 ml, contained 50 mM of Mops buffer at pH 7.0, 1mM DTT, and 14 mM PAP[^{35}S]. Stock solutions of the substrates, dopamine or 3-methyldopamine, was dissolved in DMSO at 20 times the final concentration (5 μM) in the reaction mixture, and the required volume was added subsequent to Mops buffer, DTT and PAP[^{35}S]. A control with water instead of enzyme was also prepared. The reaction was started by the addition of the SULT enzyme (2 μg), allowed to proceed in a water bath for 10 minutes at 37°C, and the reaction terminated by placing the thin-walled tube containing the assay mixture on a heating block, pre-heated to 100°C, for 2 minutes. The precipitates were cleared by centrifugation at 13,000 rpm for 3 minutes, and the supernatant was subjected to analysis of [^{35}S]sulfated product using the TLC procedure as previously described in Section 2.4.

2.3 Treatment of SK-N-MC Cells with SIN-1 or DETA in a Time-dependent Metabolic Labeling Study

In a humidified atmosphere of 95% air and 5% carbon dioxide (CO_2) and a temperature of 37°C, SK-N-MC cells were routinely maintained in MEM supplemented with 10% fetal bovine serum (FBS), penicillin G (30 μg/ml), and streptomycin sulfate (50 μg/ml). For metabolic labeling, confluent SK-N-MC cells grown in individual wells of a 24-well culture plate were pre-incubated for 4 hours in sulfate-free MEM, prepared by omitting streptomycin sulfate and using magnesium chloride instead of magnesium sulfate. After pre-incubation, cells were labeled with 0.7 ml aliquots of the same medium containing [^{35}S]sulfate (0.3 mCi/ml), and was first incubated for 3 hours to allow PAP [^{35}S] to be generated prior to the addition of 50 μM dopamine and SIN-1 (2.5 mM) or DETA (0.5 mM). A control without the addition of NO donor was also included, and triplicates were prepared for each of the control and test samples. Twenty microliter aliquots of the labeling media were collected hourly from the wells containing untreated or NO donor-treated SK-N-MC cells for 24 hours. Collected labeling medium samples were spin-filtered, and the supernatants were subjected to TLC as described in Section 2.4.

In parallel experiments, cells grown in individual wells were pre-incubated for 4 h in sulfate-free MEM and, afterwards, labeled with the same medium containing [^{35}S]sulfate. After an hour and a half, 250 μM of tropolone (a COMT inhibitor) was added to respective wells. This was followed by addition of dopamine (50 μM) and SIN-1 (2.5 mM) or DETA (0.5 mM) to respective wells after 1hour and 30 minutes. A control

with neither NO donor nor tropolone, and another control with the addition of tropolone but not NO donor were also included. Triplicates were prepared for each of the four experimental conditions. Twenty microliter aliquots of the labeling media were collected hourly from individual wells containing untreated or NO donor-treated SK-N-MC cells for 24 hours. Collected labeling medium samples were spin-filtered, and the supernatants were subjected to TLC as described in Section 2.4.

2.4 Treatment of SK-N-MC Cells with SIN-1 or DETA in a Concentration-dependent Metabolic Labeling Study

Confluent SK-N-MC cells grown in individual wells of a 24-well culture plate were pre-incubated for 4 hours in sulfate-free MEM. After pre-incubation, cells were labeled with 0.25 ml aliquots of the same medium containing [^{35}S]sulfate (0.3 mCi/ml), 50 μM dopamine and different concentrations of SIN-1 (0, 0.5, 1.0, 1.5, 2.0, 2.5 mM) or DETA (0, 0.1, 0.2, 0.3, 0.4, 0.5 mM). After an 18-hour incubation, the labeling media were collected from individual wells and spin-filtered. The filtrates were subjected to TLC analysis of [^{35}S]sulfated product(s) by spotting 1 μl of each sample on silica gel TLC plates and subsequently developed using a solvent system containing n-butanol and acetonitrile in a ratio of 2:1 (by volume). Upon completion of TLC, the silica gel plate was air-dried, and an autoradiograph was taken from the plate to reveal radioactive spots corresponding to [^{35}S]-sulfated dopamine and/or its metabolite(s). The radioactive spots on the TLC plate corresponding to the [^{35}S]sulfated products were located carefully, cut out and eluted by shaking in 0.5 ml water in a glass vial using an orbital shaker.

Afterwards, four and a half milliliters of Ecolume were added to each vial, mixed thoroughly, and [^{35}S]-radioactivity therein was counted using a liquid scintillation counter.

2.5 Preparation of cell lysates and sulfotransferase assay

SK-N-MC cells grown in individual wells of a 24-well culture plate were incubated in MEM containing the same concentrations of SIN-1 or DETA as used in the concentration-dependent metabolic labeling study described above. After a 15-hour incubation, media were discarded and cells in individual wells were rinsed with 1 ml aliquots of ice-cold phosphate-buffered saline (PBS), and subsequently incubated at room temperature for 10 minutes in 50 µl of a lysis buffer (containing 1% (w/w) Nonidet P-40 (NP-40); 0.02 M potassium phosphate, pH 7.5; 0.15 M NaCl; 5 mM EDTA; 50 mM sodium fluoride, and a protease inhibitor cocktail). Cell lysates thus prepared were cleared by centrifugation at 14,000 rpm for 10 minutes at 4°C and the supernatants collected were used immediately in enzyme activity assays because preliminary experiments showed instability of the cell lysates upon storage.

Using radioactive PAP[^{35}S] as the sulfate group donor, dopamine- and 3-methyldopamine-sulfating activities in the cell lysate samples were tested. For all enzymatic assays, the final concentration of PAP[^{35}S] in the reaction mixture was 14 µM. The total volume of the standard assay mixture was 20 µl, and contained 50 mM of Mops buffer at pH 7.0, 5 mM DTT, 50 mM NaF, and dopamine or 3-methyldopamine as substrate (5 µM final concentrations). Substrate, dopamine or 3-methyldopamine, was

dissolved in DMSO at 20 times the final concentration in the reaction mixture, and the required volume was added subsequent to Mops buffer, NaF and DTT. Control with DMSO instead of the substrate was also prepared. As the source of the enzyme, 5 µl of each cell lysate sample were added to the assay mixture and pre-incubated at room temperature for 3 minutes, which allowed NaF to inhibit any phosphatase activity in the cell lysate prior to the addition of PAP[^{35}S]. A positive control using recombinant SULT1A3 (0.1 µg) instead of cell lysate was also included. The reaction was started by the addition of PAP[^{35}S], allowed to continue for 30 minutes at 37°C, and terminated by placing the assay mixture-containing thin-wall tube on a heating block, pre-heated to 100°C, for 3 minutes. Precipitates in the heat-treated reaction mixture were cleared by centrifugation for 3 minutes, and the supernatant was subjected to TLC analysis for [^{35}S]sulfated products using same conditions as previously described in Section 2.4. Resulting cpm counts were used to calculate specific activities of SK-N-MC cell lysates as expressed in picomoles of sulfated product formed/minute/milligram protein.

2.6 Trypan Blue Exclusion Cell Viability Assay

SK-N-MC cells grown in individual wells of a 24-well culture plate were incubated in MEM containing varying concentrations of SIN-1 (0, 0.5, 1.0, 1.5, 2.0, 2.5 mM) or DETA (0, 0.1, 0.2, 0.3, 0.4, 0.5 mM). Upon an 18-hour incubation, the media were discarded and the cells in individual wells were rinsed three times with PBS and incubated in 0.2% trypan blue in PBS. After a 3-minute incubation at room temperature,

the cells were rinsed three times with PBS and observed for trypan blue penetration using an inverted microscope.

2.7 Miscellaneous methods

Determination of protein concentration in cell lysates was based on the method of Bradford with bovine serum albumin (BSA) as the standard (Bradford, 1976). Cell lysates were diluted in the ratio 1:400 with de-ionized water to a final volume of 800 µL. Exactly 200 µL of the Bradford reagent was added to the diluted sample and de-ionized water was used as the blank. The absorbance of the mixture was read at 595 nm, and the protein concentration was determined by comparing absorbance of the sample at 595 nm to a standard curve prepared using BSA [Bradford, 1976].

2.8 Statistical analysis

Statistical analysis of all the data obtained was performed using Statistical Package for the Social Sciences (SPSS) software. In time-course studies, a two-way ANOVA followed by one-way ANOVA and Dunnett's post hoc test, was used to calculate the statistical significance of the difference between the control means and SIN-1- or DETA-treatment means. For concentration-dependent studies and trypan-blue exclusion assay, a one-way ANOVA was used to determine whether the difference between control and SIN-1- or DETA-treated samples were statistically significant. In all cases, $p<0.05$ and $p<0.001$ were considered significant.

3. Results

3.1 Identification of the human SULT(s) responsible for the sulfation of dopamine and 3-methyldopamine

As a preliminary step, a sulfotransferase assay was performed to confirm previous studies which identified SULT1A3 as the sulfotransferase enzyme responsible for catalyzing the sulfation of dopamine and 3-methyldopamine (Yasuda *et al*., 2009). All eleven human SULTs: SULT1A1, SULT1A2, SULT1A3, SULT1B2, SULT1C#1, SULT1C#2, SULT1E1, SULT2A1, SULT2B1a, SULT2B1b and SULT4A1, previously cloned, expressed and purified, (Sakakibara *et al*., 1998a,b, 2002; Suiko *et al*., 2000; Pai *et al*., 2002) were analyzed for sulfating activity towards dopamine and 3-methyldopamine. Based on the results obtained, nine (SULT1A2, SULT1B2, SULT1C#1, SULT1C#2, SULT1E1, SULT2A1, SULT2B1a, SULT2B1b, and SULT4A1) of the eleven SULTs showed no detectable activity toward dopamine or 3-methyldopamine. Specific activity values obtained for the other two SULTs are shown in Table 3.1. SULT1A3 displayed stronger sulfating activity toward dopamine and 3-methyldopamine, while SULT1A1 showed relatively weaker, yet significant, sulfating activity toward dopamine and 3-methyldopamine.

Table 3.1 Specific activities of human SULT1A1 and SULT1A3 with dopamine and 3-methyldopamine as substrates

Substrate	Specific activity (nmol/min/mg) [a)]	
	SULT1A1	SULT1A3
3-Methyldopamine	0.42 ± 0.01	10.83 ± 0.09
Dopamine	1.37 ± 0.01	17.15 ± 0.10

a) Data shown represents mean ± SD derived from three independent experiments.

3-2: Kinetics of the generation and release of dopamine 3-O-[^{35}S]sulfate and 3-methyldopamine 4-*O*-[^{35}S]sulfate by [^{35}S]sulfate-labeled SK-N-MC cells treated with SIN-1or DETA

The primary aim of this part of the study was to gain insight into the kinetics of the generation and release of dopamine 3-*O*-[^{35}S]sulfate and 3-methyldopamine 4-*O*-[^{35}S]sulfate by [^{35}S]sulfate-labeled SK-N-MC cells, and to concurrently investigate the effect of nitrative stress on the production and release of these two sulfated species using two nitric oxide (NO) donors: 3-morpholinosydnonimine (SIN-1) and diethylenetriamine NONOate (DETA) to simulate the nitrative stress conditions. In time-dependent metabolic labeling experiments, as described in the **Materials and Methods section**, 20 µl of labeling medium was collected every hour from wells of untreated or NO donor-treated SK-N-MC cells, and were analysed using thin-layer chromatograpy. As shown in Figures 3.1A and B, a time-dependent production of dopamine 3-*O*-[^{35}S]sulfate and 3-methyldopamine 4-*O*-[^{35}S]sulfate was observed in control, SIN-1- and DETA- treated media samples collected during the 24-hr labeling period. A 3 x 25 (Treatment group by Time) two-way ANOVA performed on the amount of 3-methyldopamine 4-*O*-

[^{35}S]sulfate produced by control and SIN-1- and DETA- treated SK-N-MC cells, followed by Dunnett's *t*-test (two-sided), showed a significant main effect of Time [F(1, 24) = 29022.39, p<0.001] and a significant Time x Treatment group interaction effect [F(2, 48) = 234.65, p<0.001]. One-way ANOVA analyses for individual time points revealed significant difference [F (2, 8) > 23.0, p<0.001] in amounts of 3-methyldopamine 4-*O*-[^{35}S]sulfate between control and SIN-1- and DETA-treated samples starting at 4 hour through 24 hour time point. Dunnett's (two sided) multiple comparisons test analyses revealed significant decrease in the amount of 3-methyldopamine 4-*O*-[^{35}S]sulfate starting at 3 hr through 24 hr time point for DETA-treated samples. A significant decrease in the amount of methyldopamine 4-*O*-[^{35}S]sulfate in SIN-1-treated samples compared to control was also observed starting at 6hr which was inconsistent through 18 hr time point, but a significant and sustained decrease was observed from 19 hr through 24 hour time point (Fig 3.1A; p<0.001).

Similarly, a 3 x 25 (Treatment group by Time) two-way ANOVA performed on the amount of dopamine 3-*O*-[^{35}S]sulfate produced by control, SIN-1- and DETA- treated SK-N-MC cells, followed by Dunnett's *t*-test (two-sided), showed a significant main effect of Time [F(1, 24) = 4075.54, p<0.001] and a significant Time x Treatment group interaction effect [F(2, 48) = 747.15, p<0.001]. One-way ANOVA analyses for each time point also revealed significant difference [F (2, 8) > 14.45, p<0.001] in amounts of dopamine 3-*O*-[^{35}S]sulfate between control and SIN-1- and DETA-treated samples starting at 4 hr through 24 hr time point. Dunnett's (two sided) multiple comparisons test analyses revealed a significant decrease in the amount of dopamine 3-*O*-[^{35}S]sulfate starting at 3 hour which was inconsistent through 17 hr time point, but a significant and

sustained decrease was observed from 18 hr through 24 hr time point for DETA-treated samples (Figs 3.2; $p<0.001$). Furthermore, a significant and sustained decrease in the amount of dopamine 3-*O*-[^{35}S]sulfate in SIN-1-treated samples compared to control was observed starting at 4 hr through 24 hour time point (Fig 3.2B; $p<0.001$). Overall, a significant suppression of the generation of 3-methyldopamine 4-*O*-[^{35}S]sulfate, by the two NO donors (SIN-1 and DETA) was observed in a time-dependent manner, with DETA showing a higher degree of inhibition than SIN-1 (Table 3.2A; Fig 3.1A). In contrast, the time-dependent generation of dopamine 3-*O*-[^{35}S] sulfate was strongly suppressed by SIN-1 treatment, whereas DETA moderately suppressed the sulfation of dopamine (Table 3.2A; Fig 3.1B).

In parallel experiments, the effect of tropolone (a COMT inhibitor) on the generation of dopamine 3-*O*-[^{35}S]sulfate and 3-methyldopamine 4-*O*-[^{35}S]sulfate in the presence of NO donor was studied. Figure 3.2A shows the amount of 3-methyldopamine 4-*O*-[^{35}S]sulfate produced by untreated or NO-treated SK-N-MC cells, in the presence or absence of 250 μM of tropolone, over a 24 hr period. A 4 x 25 (Treatment group by Time) two-way ANOVA performed on the amount of 3-methyldopamine 4-*O*-[^{35}S]sulfate produced by control, control+Tropolone, and SIN-1- and DETA-treated (in the presence of Tropolone) SK-N-MC cells, followed by Dunnett's *t*-test (two-sided), showed a significant main effect of Time [$F(1, 24) = 9058.86$, $p<0.001$] and a significant Time x Treatment group interaction effect [$F(3, 72) = 3513.65$, $p<0.001$]. One-way ANOVA analyses for individual time points revealed significant difference [$F(3, 11) > 1.30$, $p<0.001$] in amounts of 3-methyldopamine 4-*O*-[^{35}S]sulfate between control, control+tropolone and SIN-1- and DETA-treated samples starting at 1 hr through 24 hr

time point. Dunnett's (two sided) multiple comparisons test analyses revealed a significant and sustained decrease in the amount of 3-methyldopamine 4-*O*-[^{35}S]sulfate starting at 1 hr through 24 hr time point for tropolone-treated samples (control+tropolone; and SIN-1- and DETA-treated samples in presence of tropolone) compared with control (no tropolone and no NO donor) (Fig 3.3A; $p<0.001$). In a similar manner, a 4 x 25 (Treatment group by Time) two-way ANOVA performed on the amount of dopamine 3-*O*-[^{35}S]sulfate produced by control, control+tropolone, and SIN-1- and DETA-treated SK-N-MC cells, followed by Dunnett's *t*-test (two-sided), showed a significant main effect of Time [$F(1, 24) = 3168.01$, $p<0.001$] and a significant Time x Treatment group interaction effect [$F(3, 72) = 545.51$, $p<0.001$]. One-way ANOVA analyses for each time point revealed significant difference [$F(3, 11) > 4.64$, $p<0.001$] in amounts of dopamine 3-*O*-[^{35}S]sulfate between control, control+tropolone, and SIN-1- and DETA-treated samples starting at 1 hr through 24 hr time point (Fig 3.2B). As a summary for this particular experiment, a marked inhibition of 3-methyldopamine 4-O-[^{35}S]sulfate production by tropolone-treated samples (control+tropolone, and SIN-1- and DETA-treated samples in presence of tropolone) was observed compared with control (Fig 3.2 A, C, D). Moreover, it was observed that compared to control+tropolone samples, sulfation of dopamine was suppressed by SIN-1 treatment in the presence of tropolone (Fig 3.2B), whereas for DETA-treated samples, the presence of 250 μM of tropolone caused approximately a four-fold increase in the amount of [^{35}S]sulfated dopamine produced (Fig 3.2B).

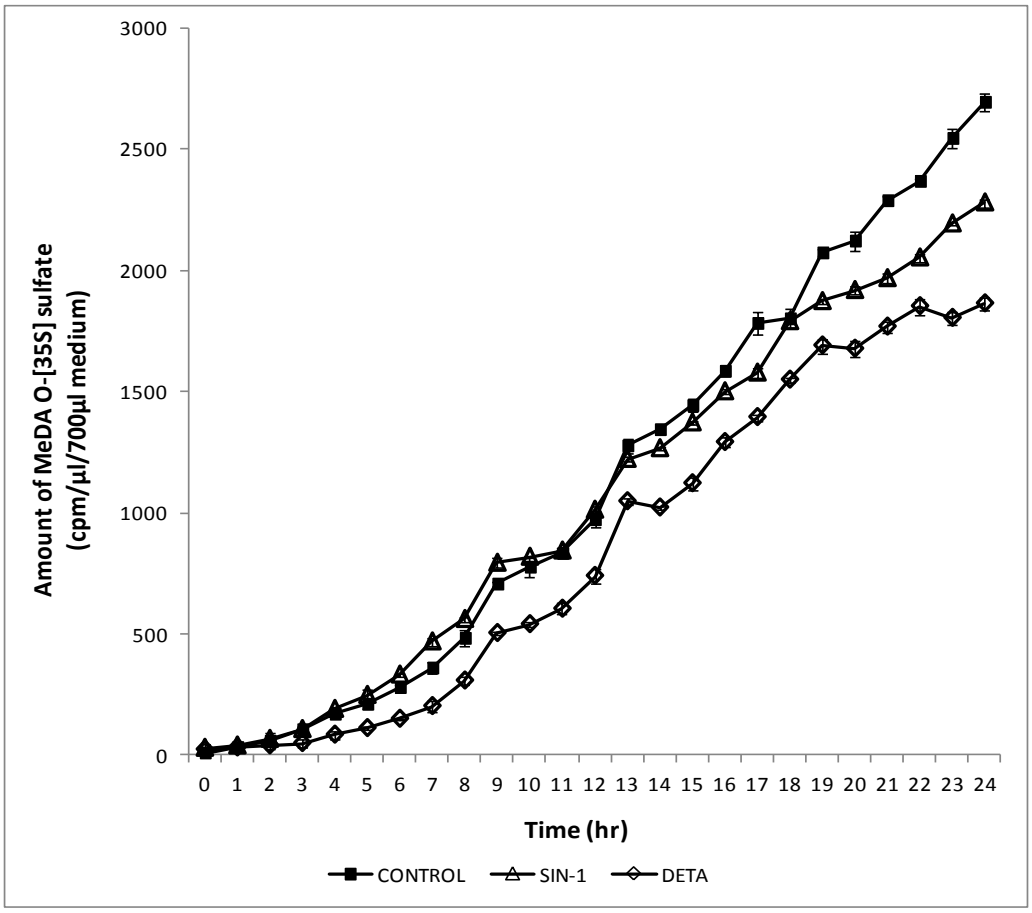


Fig 3-1 (A): Time-dependent generation and release of 3-methyldopamine 4-*O*-[^{35}S]sulfate (measured in cpm) in media samples* of NO donor-treated or untreated (control) SK-N-MC cells. Graph was plotted from mean ± S.D. data (not shown) derived from triplicate experiments (n=3). One-way ANOVA analyses for individual time points revealed significant difference [$F(2, 8) = 74.56$, $p<0.001$] in amounts of 3-methyldopamine 4-*O*-[^{35}S]sulfate between control and SIN-1- and DETA-treated samples starting at 4 hour through 24 hour time point.

*700µl of labeling medium was used for each of untreated or treated set and 20 µl of the medium was collected each hour. A volume correction factor (medium volume remaining/700 µl) was used accordingly at the various time points.

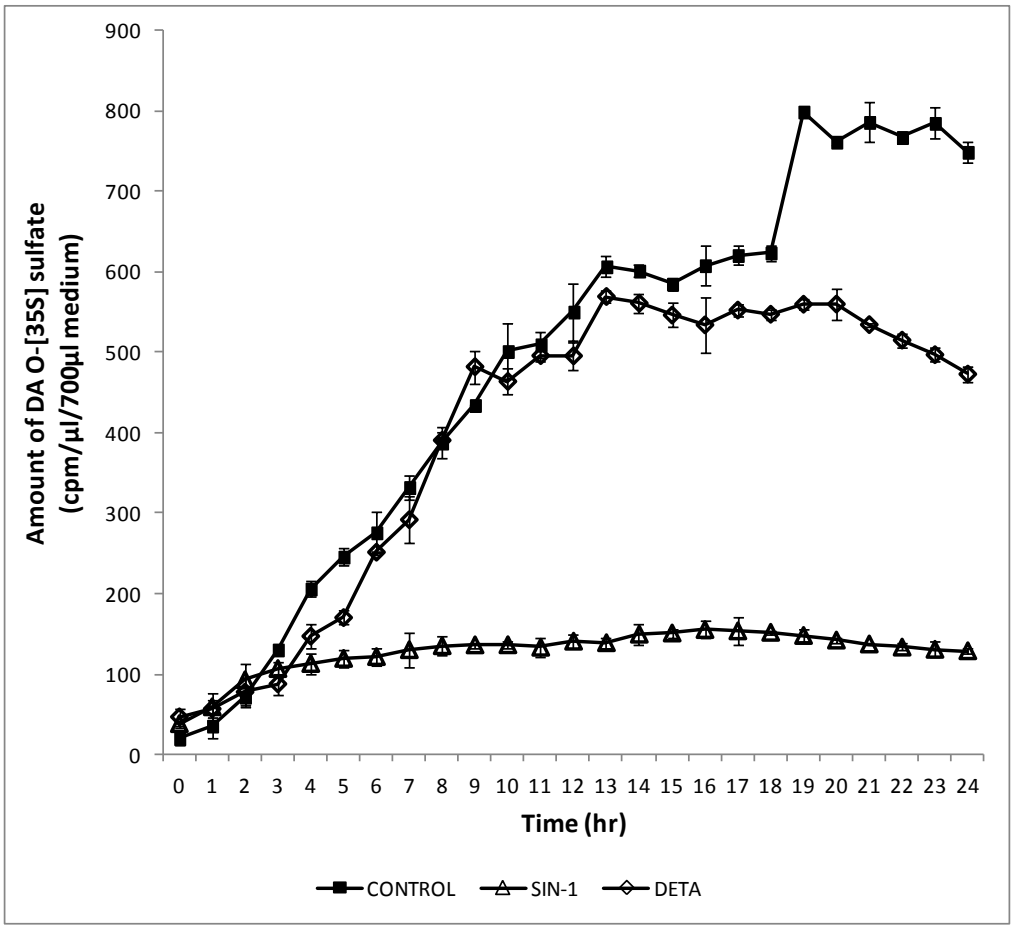


Fig 3-1(B): Time-dependent generation and release of dopamine 3-*O*-[^{35}S]sulfate (measured in cpm) in media samples* of untreated (control) or NO donor-treated SK-N-MC cells. Graph was plotted from mean ± S.D. data (not shown) derived from triplicate experiments (n=3). One-way ANOVA analyses for individual time points revealed significant difference [$F(2, 8) = 40.68$, $p<0.001$] in amounts of dopamine 3-*O*-[^{35}S]sulfate between control and SIN-1- and DETA-treated samples starting at 4 hr through 24 hr time point.

*700µl of labeling medium was used for each of untreated or treated set and 20 µl of the medium was collected each hour. A volume correction factor (medium volume remaining/700 µl) was used accordingly at the various time points.

Table 3.2A: Ratio (in percentage)* of dopamine 3-*O*-[35S]sulfate (DAS) or 3-methyldopamine 4-*O*-[35S]sulfate (MDAS) produced in medium of NO donor-treated SK-N-MC cells, calculated based on data (not shown) used for Fig 3.1 (A) and (B).

Time (hrs)	SIN-1		DETA	
	(MDAS)	(DAS)	(MDAS)	(DAS)
0	317.4%	191.2%	243.9%	233.1%
1	123.3%	165.9%	98.0%	158.9%
2	114.9%	129.7%	68.6%	108.4%
3	101.4%	81.9%	46.5%	67.3%
4	112.2%	54.9%	49.5%	71.4%
5	117.8%	48.6%	52.7%	69.2%
6	118.8%	44.1%	53.4%	91.1%
7	130.2%	39.2%	56.0%	87.8%
8	116.3%	34.9%	63.7%	100.8%
9	112.3%	31.4%	71.2%	111.0%
10	105.4%	27.2%	69.5%	92.5%
11	101.3%	26.2%	72.5%	97.3%
12	104.1%	25.6%	76.0%	90.0%
13	95.7%	22.9%	82.0%	93.9%
14	94.2%	24.9%	76.0%	93.3%
15	95.1%	25.8%	77.7%	93.4%
16	94.7%	25.5%	81.5%	87.8%
17	88.6%	24.8%	78.3%	89.0%
18	99.4%	24.4%	86.0%	87.7%
19	90.4%	18.5%	81.5%	70.1%
20	90.4%	18.7%	79.0%	73.5%
21	86.0%	17.5%	77.3%	68.0%
22	86.7%	17.4%	78.2%	67.1%
23	86.2%	16.7%	70.9%	63.3%
24	84.6%	17.2%	69.2%	63.1%

*Amount of [^{35}S]sulfated product in control sample was set to 100% and the amount of [^{35}S]sulfated product generated in NO donor-treated samples were calculated relative to control.

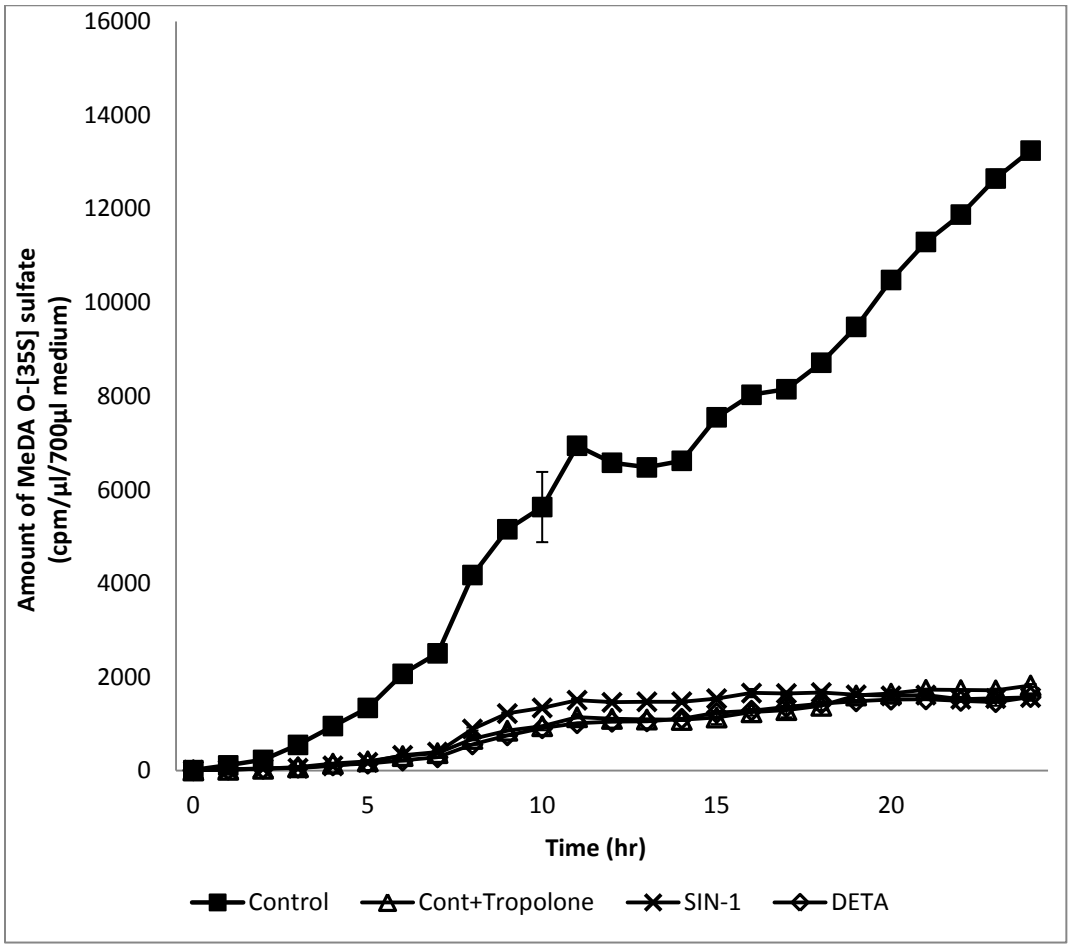


Fig 3-2 (A): Time-dependent generation and release of 3-methyldopamine 4-*O*-[^{35}S]sulfate (measured in cpm) in media samples* of untreated or NO donor-treated (control) SK-N-MC cells in the absence or presence of 250 µM of tropolone. Graph was plotted from mean ± S.D. data (not shown) derived from triplicate experiments (n=3). One-way ANOVA analyses for individual time points revealed significant difference [$F(3, 11) > 1.30$, $p<0.001$] in amounts of 3-methyldopamine 4-*O*-[^{35}S]sulfate between control, control+tropolone and SIN-1- and DETA-treated samples starting at 1 hr through 24 hr time point.

*700µl of labeling medium was used for each of untreated or treated set and 20 µl of the medium was collected each hour. A volume correction factor (medium volume remaining/700 µl) was used accordingly at the various time points.

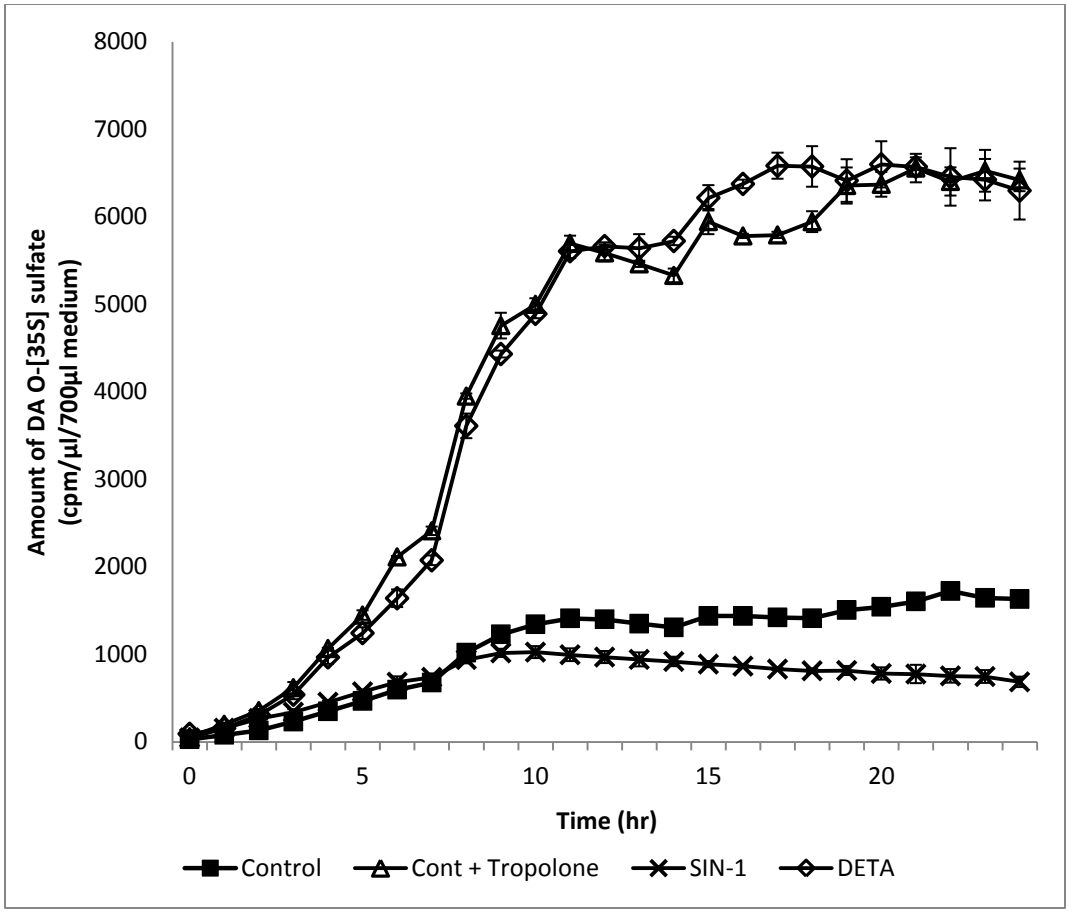
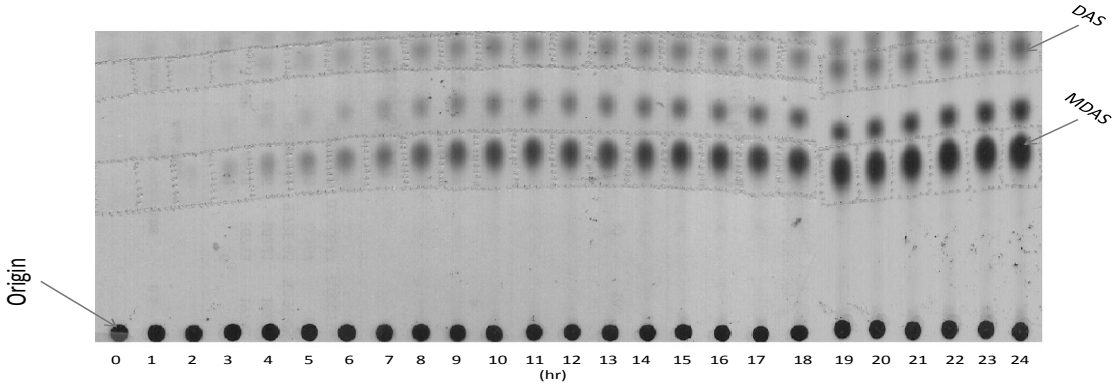


Fig 3-2 (B): Time-dependent generation and release of dopamine 3-*O*-[^{35}S]sulfate (measured in cpm) in media samples* of untreated (control) or NO donor-treated SK-N-MC cells in the absence or presence of 250 µM of tropolone. Graph was plotted from mean ± S.D. data (not shown) derived from triplicate experiments (n=3). One-way ANOVA analyses for each time point revealed significant difference [$F(3, 11) > 4.64$, $p<0.001$] in amounts of dopamine 3-*O*-[^{35}S]sulfate between control, control+tropolone, and SIN-1- and DETA-treated samples starting at 1 hr through 24 hr time point (Fig 3.2B).

*700µl of labeling medium was used for each of untreated or treated set and 20 µl of the medium was collected each hour. A volume correction factor (medium volume remaining/700 µl) was used accordingly at the various time points.

(C)



(D)

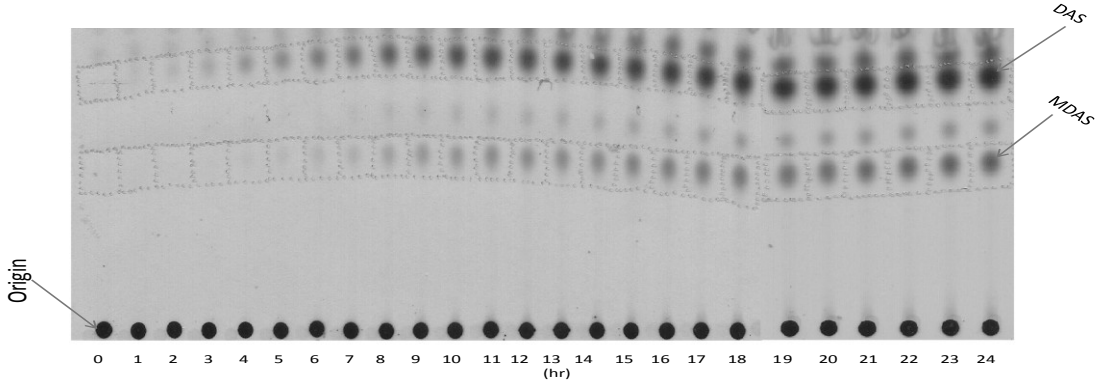


Fig 3-2: Autoradiograph from the thin-layer chromatography analysis of time-dependent generation and release of dopamine 3-*O*-[^{35}S]sulfate (DAS) and 3-methyldopamine 4-*O*-[^{35}S]sulfate (MDAS) in media samples of untreated (control) or NO donor-treated SK-N-MC cells in the absence (C) or presence (D) of 250 μM of tropolone.

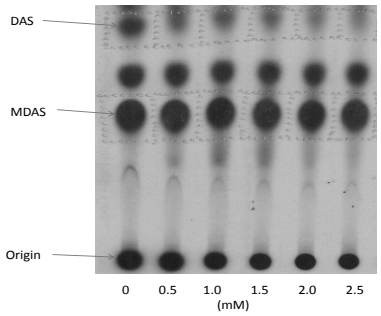
3.3 Concentration-dependent effect of SIN-1 or DETA on generation and release of dopamine 3-*O*-[^{35}S]sulfate and 3-methyldopamine 4-*O*-[^{35}S]sulfate by [^{35}S]sulfate-labeled SK-N-MC cells

This part of the study was aimed at investigating the effect of nitrative stress on the sulfation of dopamine and its metabolite, 3-methyldopamine; using two nitric oxide (NO) donors: 3-morpholinosydnonimine (SIN-1) and diethylenetriamine NONOate (DETA), in a concentration-dependent manner, to simulate the nitrative stress conditions. In the presence of 50 μM dopamine, confluent SK-N-MC cells were metabolically labeled with [^{35}S]sulfate and different concentrations of SIN-1 (ranging from 0 - 2.5 mM) or DETA (ranging from 0 - 0.5mM) were added accordingly. The selected concentrations of the two NO donors were found to be optimum based on preliminary experiments which were performed to determine the limit beyond which cytotoxicity occurred (medium turned yellow and microscopic examination revealed changes in cell morphology and dead cells). After an 18 hr incubation, the labeling media were collected and analyzed using thin-layer chromatography as described in the **Materials and Methods** section.

As shown in Figures 3.3A and C, 3-methyldopamine 4-*O*-[^{35}S]sulfate was the predominant sulfated product compared with dopamine 3-*O*-[^{35}S]sulfate, in both untreated and NO donor-treated cells. Figure 3.3B is a graphical representation showing the amount of 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-*O*-[^{35}S]sulfate produced by SK-N-MC cells metabolically labeled with [^{35}S]sulfate in medium containing 50 μM dopamine and increasing concentrations of SIN-1. One-way ANOVA

analyses revealed a significant main effect between all groups [$F(5, 23) = 459.26$, $p<0.001$]; [$F(5, 23) = 216.40$, $p<0.001$] for 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-*O*-[^{35}S]sulfate respectively. Post hoc Dunnett's multiple comparison test analyses showed significant decrease in the amounts of 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-*O*-[^{35}S]sulfate produced with increasing concentration (0.5 mM through 2.5 mM) of SIN-1 (Figure 3.3B; $p<0.001$). Figure 3.3D shows a graphical representation of the amount of 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-*O*-[^{35}S]sulfate produced by SK-N-MC cells metabolically labeled with [^{35}S]sulfate in medium containing 50 μM dopamine and increasing concentrations of DETA. Using one-way ANOVA analyses, a significant main effect between all groups was observed [$F(5, 23) = 1580.30$, $p<0.001$]; [$F(5, 23) = 65.07$, $p<0.001$] for 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-*O*-[^{35}S]sulfate respectively. Post hoc Dunnett's multiple comparison test analyses revealed significant decrease in the amounts of 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-*O*-[^{35}S]sulfate produced with increasing concentration (0.1 mM through 0.5 mM) of DETA (Figure 3.3D; $p<0.001$). It was worth noting that with SIN-1-treated cells, the suppression of dopamine 3-*O*-[^{35}S]sulfate generation with increasing concentration of SIN-1 was more dramatic (81.6% at highest SIN-1 concentration) than that of 3-methyldopamine 4-*O*-[^{35}S]sulfate (27.7% at the highest SIN-1 concentration). In contrast, the suppression of [^{35}S]sulfated dopamine (32.8%) was more gradual than that of [^{35}S]sulfated 3-methyldopamine (54%) in DETA-treated cells.

(A)



(B)

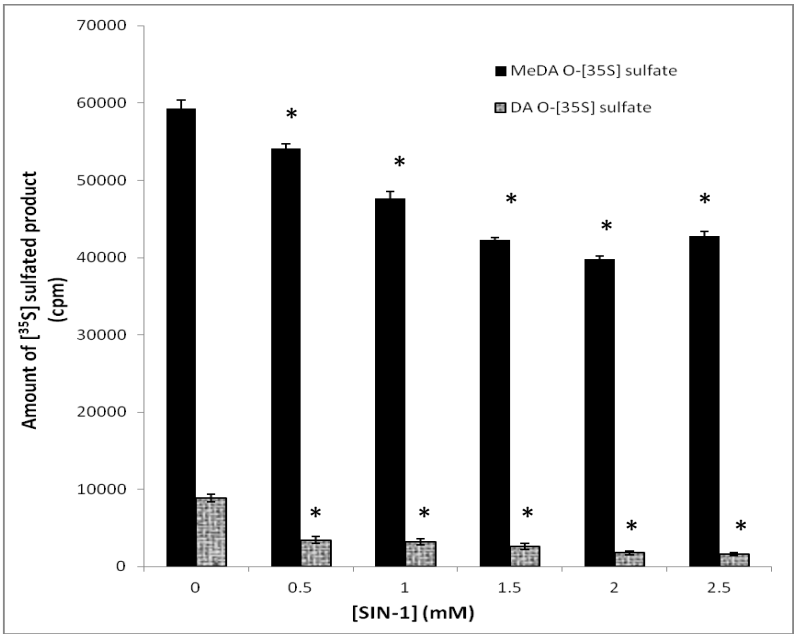
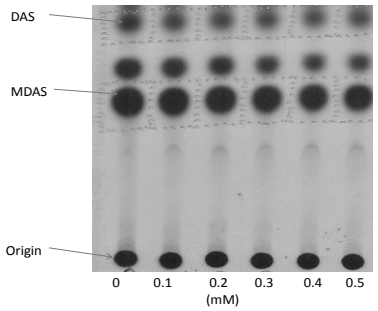


Fig. 3-3: Autoradiograph (A) and graphical representation (B) showing amount of 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-O-[^{35}S]sulfate produced by human SK-N-MC neuroblastoma cells metabolically labeled with [^{35}S]sulfate in medium containing 50 μM dopamine and increasing concentration of SIN-1. Graph was plotted from mean ± S.D. data (not shown) derived from four experiments (n=4). Compared to control, a significant decrease in the amounts of 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-O-[^{35}S]sulfate was observed with increasing concentration of SIN-1 (*$p<0.001$).

(C)



(D)

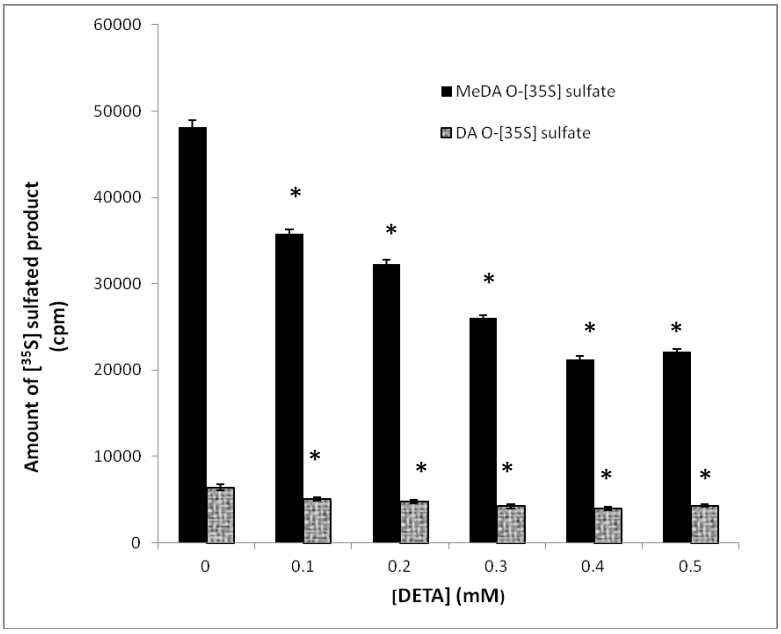


Fig. 3-3: Graphical representation (C) and autoradiograph (D) showing amount of 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-*O*-[^{35}S]sulfate produced by human SK-N-MC neuroblastoma cells metabolically labeled with [^{35}S]sulfate in medium containing 50 µM dopamine and increasing concentrations of DETA. Graph was plotted from mean ± S.D. data (not shown) derived from four experiments (n=4). Compared to control, a significant decrease in the amounts of 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-O-[^{35}S]sulfate was observed with increasing concentration of SIN-1 (*$p<0.001$).

3.4 Decreased Dopamine- and 3-Methyldopamine-sulfating activity in Cell Lysates of SK-N-MC Cells Treated with Different Concentrations of SIN-1 or DETA

With a 30-minute reaction time, the sulfotransferase assay was performed using cell lysates prepared from SK-N-MC cells treated with different concentrations of SIN-1 (ranging from 0-2.5 mM) or DETA (ranging from 0-0.5 mM) and final substrate (dopamine and 3-methyldopamine) concentration of 5 μM, as described in the **Materials and Methods** section. Both dopamine- and 3-methyldopamine-sulfating activities were detected in all cell lysates from untreated and SIN-1- or DETA-treated cells.

Figure 3.4A shows a graphical representation of the specific activity (pmol/min/mg protein) for cell lysates prepared from untreated and SIN-1-treated cells. One-way ANOVA analyses revealed a significant main effect between all groups [$F(5, 17) = 18.69$, $p<0.001$]; [$F(5, 23) = 22.36$, $p<0.001$] for 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-*O*-[^{35}S]sulfate respectively. Post hoc Dunnett's multiple comparison test analyses showed significant decrease in the amounts of 3-methyldopamine 4-*O*-[^{35}S]sulfate starting at 1.5 mM through 2.5 mM SIN-1, with $p<0.05$ (Figure 3.4A). Similar post hoc analyses revealed significant decrease in the amounts of dopamine 3-*O*-[^{35}S]sulfate at 0.5 mM, 2 mM and 2.5 mM SIN-1, with $p<0.05$ (Fig 3.4A).

The specific activity (pmol/min/mg protein) of cell lysates prepared from untreated and DETA-treated cells is shown as a graph in Fig 3.4B. One-way ANOVA analyses showed a significant main effect between all groups [$F(5, 17) = 444.59$, $p<0.001$]; [$F(5, 17) = 87.45$, $p<0.001$] for 3-methyldopamine 4-*O*-[^{35}S]sulfate and

dopamine 3-*O*-[^{35}S]sulfate respectively. Post hoc Dunnett's multiple comparison test analyses showed significant decrease in the amounts of 3-methyldopamine 4-*O*-[^{35}S]sulfate starting at 0.2 mM through 0.5 mM DETA, with $p<0.001$ (Figure 3.4B). Using similar post hoc analyses, a significant decrease in the amounts of dopamine 3-*O*-[^{35}S]sulfate was observed starting at 0.1 mM through 0.5 mM DETA with $p<0.001$ (Fig 3.4B). Furthermore, it was noted that SIN-1 showed a much less inhibition of dopamine 3-*O*-[^{35}S]sulfate production (12.8% inhibition at highest SIN-1 concentration used) compared with DETA which showed a significant and much higher suppression (73.6% inhibition at highest DETA concentration used) of dopamine sulfating activity (Figs. 3.4A, B). With regards to 3-methyldopamine 4-*O*-[^{35}S]sulfate, the same trend was observed, with 8.1% inhibition at highest concentration of SIN-1, and 44.2% suppression at highest concentration of DETA (Figs. 3.4A, B).

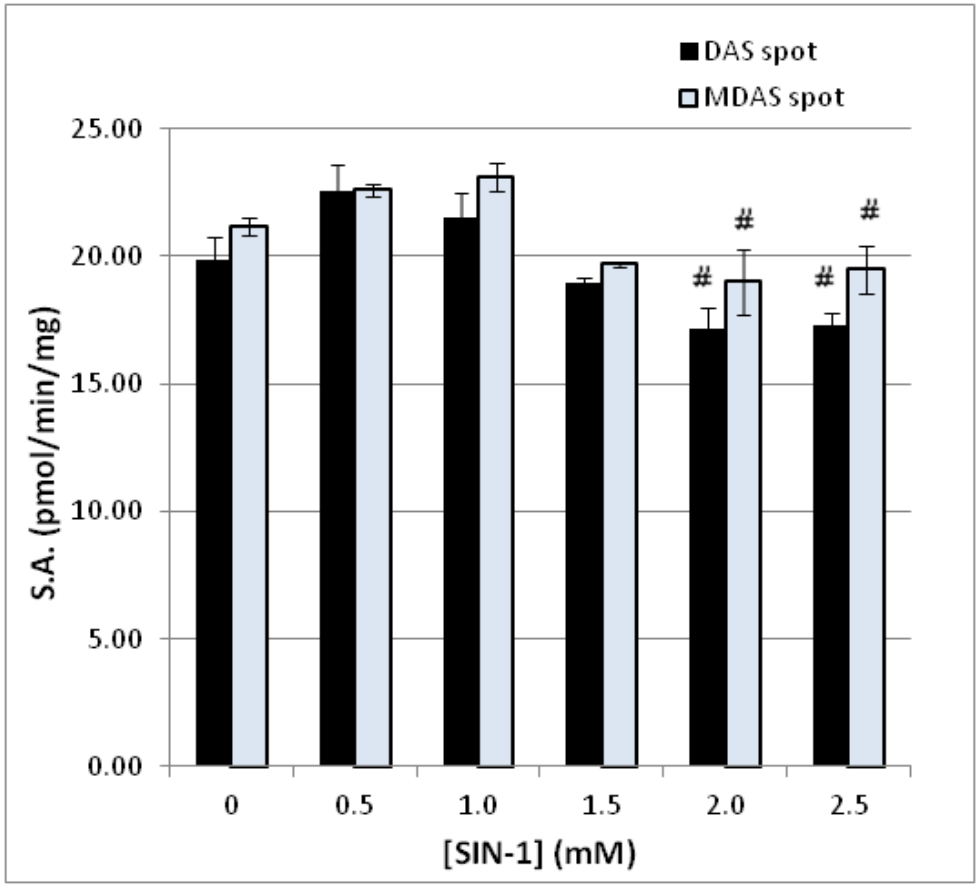


Fig. 3-4 (A): Graphical representation of specific activity (pmol/min/mg protein) of cell lysates prepared from SK-N-MC cells treated with increasing concentration of SIN-1 using dopamine and 3-methyldopamine (5 μM concentration) as substrates. Graph was plotted from mean ± S.D. data (not shown) derived from triplicate experiments (n=3). One-way ANOVA analyses revealed a significant main effect between all groups [$F(5, 17) = 18.69$, $p<0.001$]; [$F(5, 23) = 22.36$, $p<0.001$] for 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-*O*-[^{35}S]sulfate respectively. Post hoc Dunnett's multiple comparison test analyses was performed (# $p<0.05$).

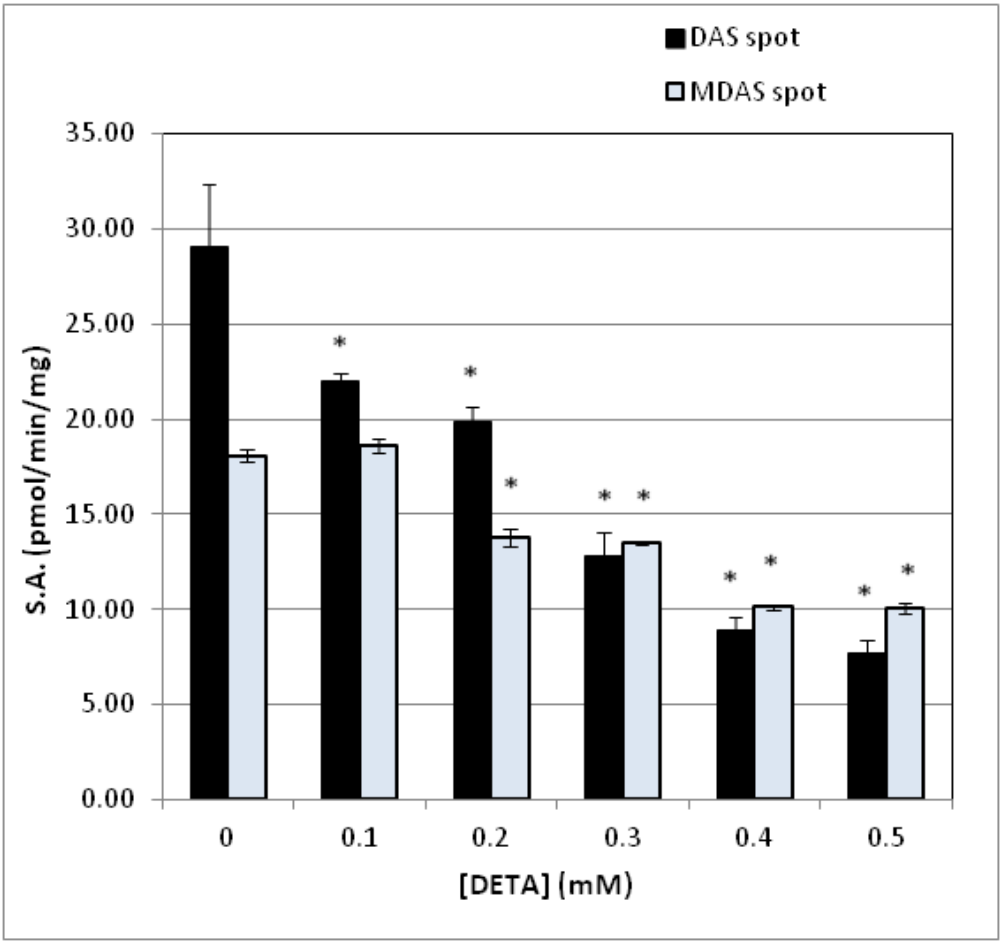


Fig. 3-4 (B): Graphical representation of specific activity (pmol/min/mg protein) of cell lysates prepared from SK-N-MC cells treated with increasing concentration of DETA using dopamine and 3-methyldopamine (5 μM concentration) as substrates. Graph was plotted from mean ± S.D. data (not shown) derived from triplicate experiments (n=3). One-way ANOVA analyses showed a significant main effect between all groups [$F(5, 17) = 444.59$, $p<0.001$]; [$F(5, 17) = 87.45$, $p<0.001$] for 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-*O*-[^{35}S]sulfate respectively. Post hoc Dunnett's multiple comparison test analyses was performed (*$p<0.001$).

3.5 Cell Viability of SK-N-MC cells upon Treatment with Different Concentrations of SIN-1 or DETA

To verify whether, under the experimental conditions adopted, SIN-1 or DETA may exert cell toxicity thereby resulting in cell death and consequently the decreased level of dopamine- or 3-methyldopamine-sulfating SULT(s), further studies were performed in which untreated (control) or SIN-1- or DETA-treated SK-N-MC cells were subjected to trypan blue exclusion test as described in the **Methods and Materials** section.

Average counts of trypan blue-stained cells in untreated or NO donor-treated SK-N-MC cells (not shown) were used to plot a graph as shown in Fig 3.5A and B. One-way ANOVA analyses showed no significant main effect between all groups [$F(5, 23) = 0.979$, $p=0.48$]; [$F(5, 23) = 1.32$, $p=0.302$] for SIN-1- and DETA-treated samples respectively. Moreover, post hoc Dunnett's multiple comparison test analyses showed no significant difference in the average number of trypan blue-stained cells in control samples and SIN-1- or DETA-treated samples (Figs 3.5A-F; 3.6; 3.7A-F; and 3.8; in all cases $p>0.05$).

.

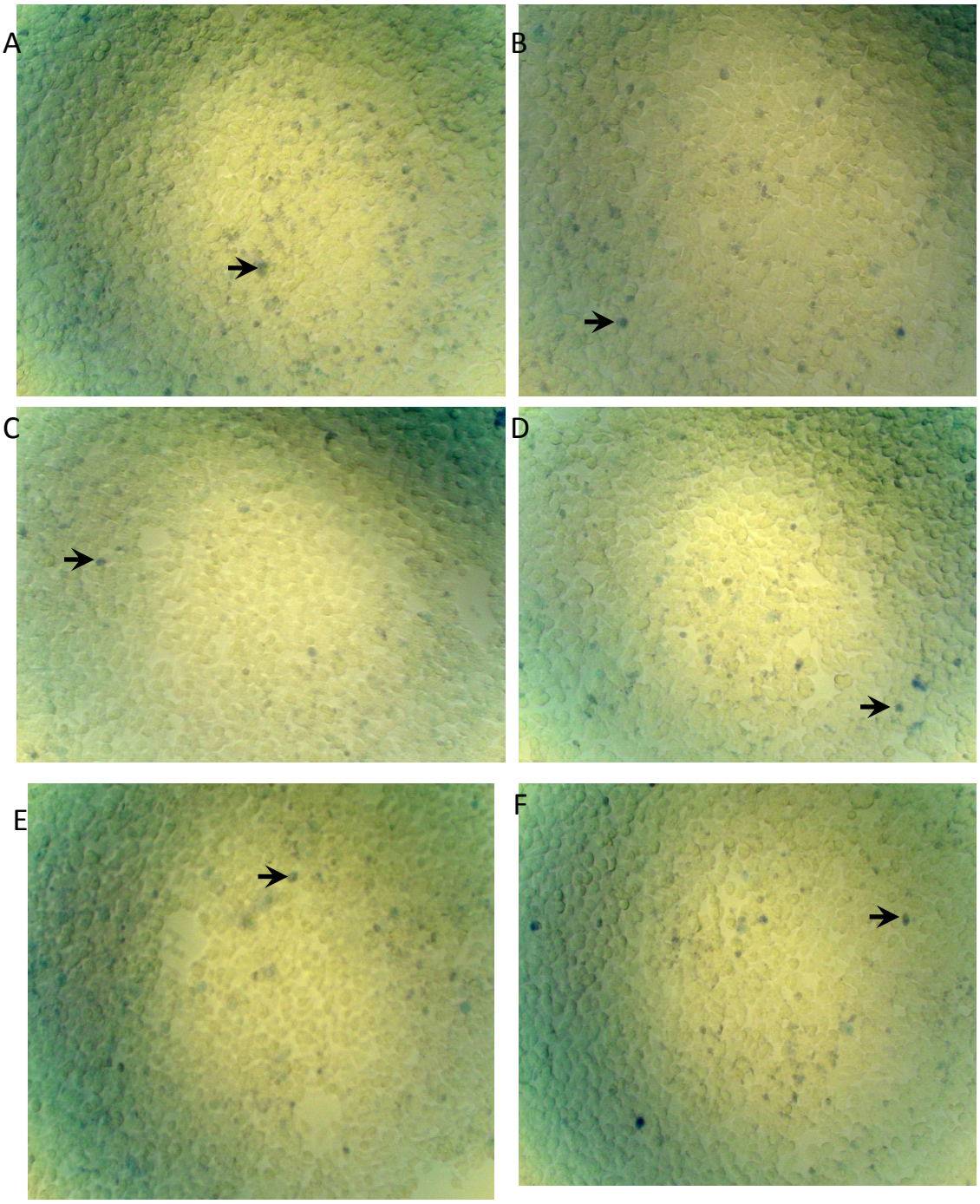


Fig. 3-5. SK-N-MC cells untreated (A) or treated (B, C, D, E, F) with 0.5, 1.0, 1.5, 2.0, 2.5 mM, respectively, of SIN-1 were assayed for cell viability using trypan blue exclusion test. The figure at ×20 original magnification is a representative microscopic image of four independent experiments. Arrows represents SK-N-MC cells that stained with trypan blue.

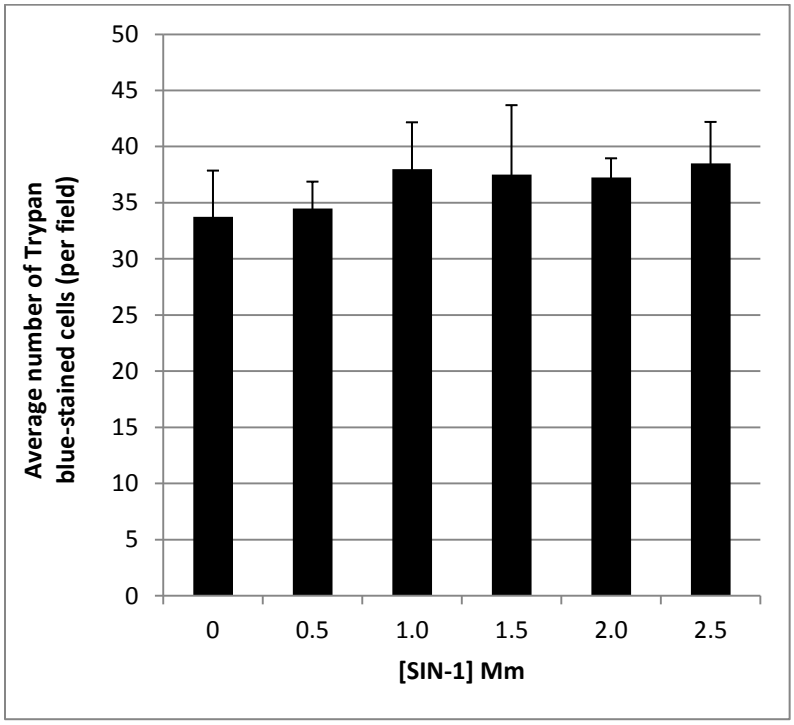


Fig 3-6. Graphical representation of average number of trypan blue-stained cells per microscopic field in untreated (0 mM) or SIN-1-treated SK-N-MC cells. Graph was plotted from mean ± S.D. counts from four independent experiments (n=4). Post hoc Dunnett's multiple comparison test analyses showed no significant difference in the average number of trypan blue-stained cells in control samples and SIN-1-treated samples ($p>0.05$).

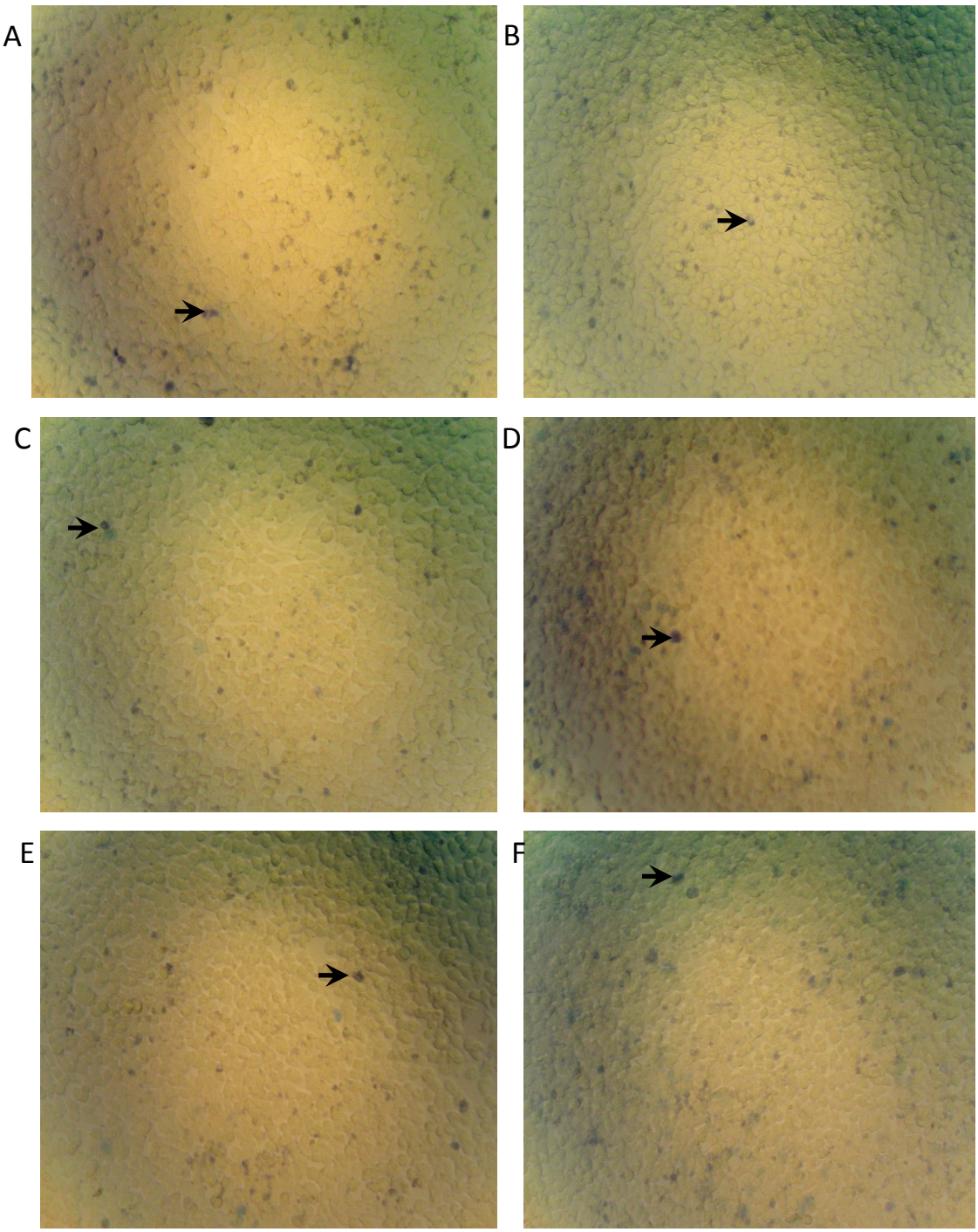


Fig. 3-7. SK-N-MC cells untreated (A) or treated (B, C, D, E, F) with 0.1, 0.2, 0.3, 0.4, 0.5 mM, respectively, of DETA were assayed for cell viability using trypan blue exclusion test. The figure at ×20 original magnification is a representative microscopic image of four independent experiments. Arrows represents SK-N-MC cells that stained with trypan blue.

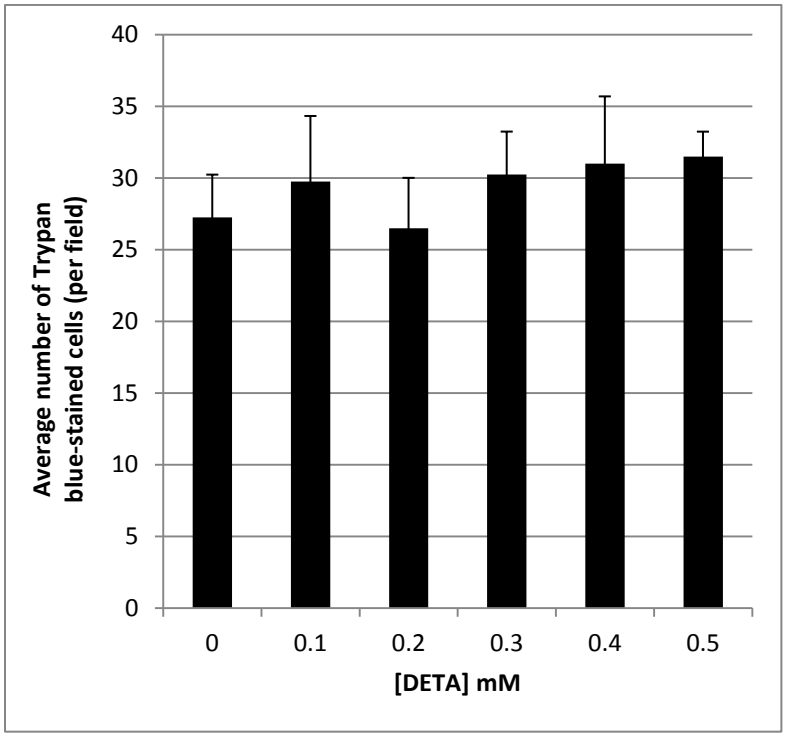


Fig 3-8: Graphical representation of average number of trypan blue-stained cells per microscopic field in untreated (0 mM) or DETA-treated SK-N-MC cells. Graph was plotted from mean ± S.D. counts from four independent experiments (n=4). Post hoc Dunnett's multiple comparison test analyses showed no significant difference in the average number of trypan blue-stained cells in control samples and DETA-treated samples ($p>0.05$).

4. Discussion

The present study was designed to investigate the effect of nitrative stress on the sulfation of dopamine and its metabolite, 3-methyldopamine, in human SK-N-MC neuroblastoma cells. In humans, dopamine is involved in a wide range of physiological effects, particularly its role as a classical neurotransmitter in the brain (Fahn, 2006; Benes, 2001). Hence, its homeostasis is critical for normal functioning of the nervous system, and abnormal regulation of dopamine has been implicated in the pathogenesis of several neurological disorders including Parkinson's disease and schizophrenia (Marin-Valencia *et al*., 2008; Nieoullon, 2002; Marchitti *et al*., 2007; Li *et al*., 2001). Sulfation as mediated by the SULTs has been reported to play a key role in the regulation of dopamine, which is predominantly excreted as sulfoconjugates (Strott, 2002; Marchitti *et al*., 2007; Eisenhofer *et al*., 1999). In circulation, up to 95% of dopamine exists in the sulfated form (Strott, 2002). Nitric oxide (NO) is ubiquitous in the body and possesses pleiotropic functional roles. Although NO is part of the normal physiology, its excessive production may lead to the generation of the highly reactive free radical, peroxynitrite, which is often associated with inflammatory conditions and neurodegenerative abnormalities. One of the consequences of reactive nitrogen species (RNS) is the oxidation and inactivation of enzymes and other proteins by modulating gene expression and binding of NO or RNS to certain metal- and thiol-containing proteins, on their cysteine residues and/or other amino acid residues such as tyrosine (Hausladen, 1996; Dietrich-Muszalska *et al*., 2009).

The main focus of the current study was to find out whether under nitrative stress conditions - as simulated by the use of two NO donors, the sulfation of dopamine and 3-methyldopamine may be affected; and to elucidate possible mechanisms underlying this phenomenon. As a preliminary step, a sulfotransferase assay was performed to confirm previous studies which identified SULT1A3 as the sulfotransferase enzyme responsible for catalyzing the sulfation of dopamine and 3-methyldopamine (Yasuda *et al*., 2009). All eleven known human cytosolic SULTs were examined for sulfating activity towards the two substrates, dopamine and 3-methyldopamine. Results obtained from sulfotransferase assay (Table 3.1) corroborated the fact that though its isoform, SULT1A1, shows significant activity, SULT1A3 is the main enzyme responsible for sulfate conjugation and subsequent excretion of dopamine and 3-methyldopamine.

To gain insight into the kinetics of generation and release of dopamine 3-O-[^{35}S]sulfate and 3-methyldopamine 4-*O*-[^{35}S]sulfate, a time-course study was performed using [^{35}S]sulfate-labeled SK-N-MC cells without (control) or with treatment with each of the two NO donors, SIN-1 and DETA. Results obtained indicated that sulfate conjugation of dopamine and 3-methyldopamine occurs in a time-dependent manner, and that the effects of the two NO donors, SIN-1 and DETA on the generation and release of sulfated products were also time-dependent. For SIN-1- and DETA-treated samples, higher amounts of 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-O-[^{35}S]sulfate were observed at initial time points, which suggests an initial stimulatory effect of NO donor in the cell milieu prior to the onset of NO-donor effect. Compared to control, DETA-treated samples started to show significant suppression in the amounts of 3-methyldopamine 4-*O*-[^{35}S]sulfate from the 3rd hour through the 24 hour time-point (Fig

3.2.A). It was however surprising to note that compared to control and DETA-treated samples, SIN-1 caused a marked suppression in the production of dopamine 3-*O*-[^{35}S]sulfate starting at 4 hour time point and sustained throughout the time course study, whereas DETA-treated samples showed a milder suppression of dopamine 3-*O*-[^{35}S]sulfate production from the 3 hour through the 17 hour time-point, and then a very significant decrease from 18 hour through the 24 hour time-point (Fig 3.2.B). This difference might have been due to the possible difference in the pathways of NO generation by SIN-1 and DETA. As mentioned previously, SIN-1 undergoes a three-step reaction to generate peroxynitrite ($ONOO^-$), whereas DETA only reacts to liberate two moles of NO per one mole of compound. Moreover, during its oxidative decomposition, SIN-1 generates high amounts of protons and superoxide anion ($O^{\cdot}{}_2^-$) (Feelisch, 1998; Trackey *et al*., 2001). All these could be potential factors causing the differential effects of SIN-1 and DETA on the generation and release of dopamine 3-*O*-[^{35}S]sulfate. In an attempt to clarify whether or not the decreased production of 3-methyldopamine 4-*O*-[^{35}S]sulfate caused by DETA or SIN-1 was due to an inhibitory effect on COMT enzyme, the effect of tropolone (a COMT inhibitor) treatment on untreated and NO-treated SK-N-MC cells was studied. Results showed no significant inhibition of the COMT enzyme since the amounts of sulfated product in control and NO-treated samples did not vary significantly, except for dopamine 3-*O*-[^{35}S]sulfate in SIN-1-treated samples which showed a sustained suppression as previously mentioned. Future studies will be needed in order to clarify this finding.

The effect of increasing concentrations of DETA or SIN-1 was examined in metabolic labeling experiments using an incubation period of 18 hours. This time

duration was adopted based on the consideration of the half-lives of DETA and SIN-1, as well as the results from the kinetic studies of 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-O-[^{35}S]sulfate production. Consistent with the results from the kinetic studies, 3-methyldopamine 4-*O*-[^{35}S]sulfate was the predominant product compared with dopamine 3-O-[^{35}S]sulfate. In the presence of increasing concentrations of SIN-1 or DETA, there was a significant and dramatic decrease in the generation and release of both 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-O-[^{35}S]sulfate by SK-N-MC cells (Figs 3.3B and D). As mentioned previously, sulfation of dopamine may render it unable to bind to its receptor, and thus this metabolic pathway is critical for the inactivation and excretion of dopamine. Sulfation is known to be responsible for 15% of total dopamine inactivation while methylation by COMT contributes less than 5% of total dopamine inactivation (Eisenhofer *et al*., 1999; Keinzl *et al*., 1990; Werle, 1988). This suggests that a disruption in the sulfation pathway by nitrative stress may lead to the accumulation of dopamine and its metabolites in the unsulfated form. Excessive dopamine is potentially deleterious to cells due to its enzyme-catalyzed autoxidation to generate reactive quinones which are capable of covalently modifying and damaging cellular macromolecules and culminating in cytotoxicity and neurodenegeration (Stokes *et al*., 1999).

To verify findings from the SIN-1 or DETA concentration-dependent metabolic labeling, and gain supporting evidence for the decrease in sulfating activity under nitrative stress, lysates were prepared from untreated SK-N-MC cells and cells treated with different concentrations of SIN-1- or DETA. Based on the specific activity data compiled (not shown), a significant decrease in dopamine- and 3-methyldopamine-

sulfating activity in lysates of SK-N-MC cells treated with increasing concentrations of DETA (up to 0.5 mM) was observed. It was however surprising that after repeated attempts, lysates of SIN-1-treated cells (up to 2.5 mM) showed a less significant decrease in dopamine- and 3-methyldopamine-sulfating activity compared to results from concentration-dependent metabolic labeling experiments. Considering the fact that SIN-1 caused a very significant decrease in the generation of both 3-methyldopamine 4-*O*-[^{35}S]sulfate and dopamine 3-O-[^{35}S]sulfate in metabolic labeling experiments; the unexpected finding using lysate may suggest that SIN-1 may not directly affect SULT1A3, which is the enzyme responsible for the sulfation of dopamine and 3-methyldopamine, but it could rather affect other steps involved in the overall sulfation pathway. In a biological system, possible targets in the sulfation pathway that could be disrupted by "stress" include: sulfate transport; synthesis of PAPS, as catalyzed by the PAPS synthetase; release of sulfated product from cell; hydrolytic action of sulfatase enzyme(s); chemistry of substrate. Further studies will be needed in order to fully elucidate the mechanistic basis of the unexpected, but interesting finding on the anomaly of dopamine and its methylated metabolite in SIN-1-treated cells.

Additional studies were performed to verify whether the decreased dopamine- and 3-methyldopamine-sulfating activity could be due to any possible cytotoxic effect of SIN-1 or DETA. At high concentrations, SIN-1 and DETA are known to cause cytotoxicity by the generation of the free radical, peroxynitrite, or by direct effect of NO (Feesisch, 1998; Hogg *et al*., 1992; Meij *et al*., 2004). In a cell viability assay, SK-N-MC cells incubated in the presence of increasing concentrations of SIN-1 or DETA were subjected to trypan blue exclusion test. Results, as shown in Figs 3.5A-F, 3.6, 3.7A-F and

and 3.8, revealed that SIN-1- and DETA-treated cells maintained their cell membrane integrity to a level almost similar to untreated cells. This implies that the inhibition of the sulfation of dopamine or 3-methyldopamine in SIN-1- or DETA-treated SK-N-MC cells was likely not due to the cytotoxic effects of SIN-1 or DETA, but rather, might have been associated with the decrease in the activity of SULT1A3 and/or an inhibitory effect along the sulfation pathway. Since there are other possible targets in the sulfation pathway that could be affected, further studies that extend the present study are warranted in order to understand and elucidate the relative contributions of the various pathways as listed above.

In summary, it was demonstrated in the present study that nitrative stress, as simulated by the treatment of SK-N-MC neuroblastoma cells with SIN-1 or DETA NONOate, could suppress the sulfation of dopamine, resulting in accumulation of its unsulfated form, which has been implicated in the pathogenesis of various neurological disorders mainly schizophrenia. It will however be essential to investigate whether the suppression of the sulfation of dopamine and its metabolite, 3-methyldopamine is physiologically relevant and actually exists in neuropathological states. Although the current study showed the decrease of dopamine-sulfating activity of SULT1A3, in the presence of nitrative stress, this may be just one of several possible pathways. Hence, further studies are warranted in order to fully understand and elucidate other potential pathways that could also be involved in the overall mechanism.

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