Dinitrosyl Iron Complexes and their Role in Patho-physiological Conditions

BY

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THESIS

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This thesis is dedicated to my parents (Mr Ravi Kumar Sahni and Mrs Indu Sahni), sister (Shikha Baweja), nephew (Raghav) and Akanksha Arvind, without whom it would never have been accomplished.

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LIST OF ABBREVIATIONS

•NO	Nitric oxide
•OH	hydroxyl radical
ABH	α/β hydrolase
ATCC	American Type Culture Collection
ATF3	Activating transcription factor 3
BA	Blue acetate
cGMP	cyclic-guanylyl monophosphate
CI	Cell index
CIP	Intracellular Labile/Chelatable Iron Pool
CLA	Chemiluminescence analyzer
Ctsc	cathepsin C
DC	Deactivated control
DETA/NO	Diethylenetriamine nonoate
DFO	desferrioxamine
DHR123	Dihyrorhodamine 123
DNDGIC	Dinitrosyl diglutathione iron complex
DNIC	Dinitrosyl iron complexes

LIST OF ABBREVIATIONS (continued)

DP	2,2’-dipyridal
DTPAC	Diethylenetriamine-pentaacetic acids
EMT	epithelial mesenchymal transition
eNOS	Endothelial nitric oxide synthase
EPR	electron paramagnetic resonance
FAC	Ferric ammonium citrate
FBS	Fetal bovine serum
Ferrozine	3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium
GL	Geldanamycin
GSK3β	glycogen synthase kinase 3β
GTN	Nitrogylcerine
GTP	guanylyl triphosphate
HBED	N,N'-Di(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid
HIF-1	Hypoxia inducible factor -1
HMSNL	Hereditary Motor and Sensory Neuropathy- Lom
HRE	HIF response elements

LIST OF ABBREVIATIONS (continued)

IKK	κB kinase
iNOS	Inducible nitric oxide synthase
IREs	iron response elements
IRPs	iron regulatory proteins
LPS	lipopolysaccharide
mRNA	messenger Ribonucleic acid
NDRG1	N-myc downstream–regulated gene-1
N-myc	Neuroblastoma derived myelocytomatosis
nNOS	Neuronal nitric oxide synthase
NO_2	nitrogen dioxide
Nonoate	Diazeliumdienolate
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drugs
NSCLC	non-small cell lung carcinoma
NT	No treatment

LIST OF ABBREVIATIONS (continued)

ODQ	1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
PBS	phosphate buffer saline
PHD	prolyl hydroxylase
PTEN	Phosphate and tensin homolog deleted on chromosome 10
qRT-PCR	quantitative-real time-polymerase chain reaction
ROS	reactive oxygen species
RsbQ	Bacillus subtilis stress-response regulator
sGC	soluble guanylyl cyclase
SGK1	Serum- and glucocorticoid-regulated kinase 1
SNAP	S-nitroso-N-acetylpenicillamine
Sper/NO	Spermine/nonoate
TAMs	tumor associated macrophages
Tf	diferric transferrin
TfR	transferrin receptor
Thtpa	thiamine triphosphatase
UTR	untranslated region
VHL	Von Hippel-Lindau

SUMMARY

Nitric oxide (•NO) is a small free radical known to play an important role in tumor cell metastasis. Inside the cell, one of its primary interactions occurs wi th iron and thiols leading to the formation of dinitrosyl iron complexes (DNIC). Formation of these complexes renders iron inactive towards the various physiological functions it performs.

The physiological-chemical characterization of DNIC formation and degradation has not yet been established in cancer cells. It is important to measure these parameters in order to understand their role in •NO mediated downstream signaling and phenotypic effects. We observed formation of these paramagnetic complexes on exposure of nitric oxide in different cancer cell lines. We also measured levels of chelatable iron pool (CIP), a methodologically defined quantity that can be chelated by cell permeable chemical iron chelators like desferrioxamine. The amount of DNIC formed equaled the CIP on exposure to lower concentrations of •NO-donor whereas higher concentrations leads to the formation of DNIC in much excess over CIP. DNIC were stable for at least 2 hours after removal of the •NO source in different cancer cell lines. Moreover, we also observed formation of these complexes on exposure to endogenous •NO in lipopolysaccharide (LPS) activated macrophage cell line. There was an inverse relationship between amount of DNIC and the oxygen concentrations at which cells were incubated. As the tumor micro-environment is hypoxic in nature, formation of large amount of these complexes under these conditions could lead to important downstream •NO-mediated effects.

Next, we studied the effect of DNIC on cellular signaling related to cancer cell metastasis. Formation of these complexes renders iron inactive towards various physiological functions it performs. Thus •NO, due to its ability to form DNIC, behaves as an intracellular iron sequestering agent. Recently, N-myc downstream regulated gene 1 (NDRG1), a well known metastasis suppressor gene, was shown to be up-regulated by iron chelation and hypoxia inducible factor 1 alpha (HIF-1α) stabilization. We also observed HIF-1α stabilization in cancer cells (HCC 1806, MDA-MB-231 cell lines) on exposure to •NO. Given the similarities between NDRG1 regulation and the actions of •NO in cancer cells, we hypothesized that exposure of nitric oxide to cells results in NDRG1 up-regulation, in an iron dependent manner. We observed time (0-24 hours) and dose (0-1mM) dependent increase in NDRG1 expression at both mRNA and protein level in cancer cells treated with •NO-donor DETA/NO. Although NDRG1 mRNA was not stable after removal of •NO source, NDRG1 protein once expressed was stable for a prolonged period of time (~48hours). As protein is a functional product of the gene, these results show that NDRG1 could be an important clinically significant target for nitric oxide.

Next we studied possible pathways involved in this up-regulation. Nitric oxide is known to exert the majority of its effects through classical sGC-cGMP pathways. We ruled out involvement of this pathway by performing experiments using ODQ (inhibitor of sGC). HIF-1α is an important transcription factor that is known to be stabilized by •NO under normoxic conditions. There are reports showing HIF-1α dependent up-regulation of NDRG1. Therefore, it was important to study the involvement of this pathway.

Using inhibitor and knockdown studies, we observed HIF-1α independent up-regulation of NDRG1 by nitric oxide. We also ruled out pathways involving p53, c-Myc and N-Myc.

We performed iron supplementation studies in order to determine the role of chelatable iron in this up-regulation. We observed •NO-mediated NDRG1 up-regulation was dependent on the amount of chelatable iron. Higher level of CIP leads to decreased up-regulation of NDRG1 by •NO. There was a strong correlation between the amount of DNIC formed and NDRG1 up-regulation. Thus, we concluded that •NO-mediated NDRG1 up-regulation was an iron dependent process, possibly via formation of DNIC.

As NDRG1 is a well known metastatic suppressor gene, we hypothesized that •NO-mediated NDRG1 up-regulation will lead to suppression in metastasis. Using a real-time migration/invasion/cytotoxicity assay, we observed a significant decrease in migration/invasion potential without affecting the viability of HCC 1806 breast cancer cells on exposure to •NO at doses corresponding to NDRG1 up-regulation (DETA/NO, 125-250μM). Next, using NDRG1 knock-down cells we determined the role of NDRG1 in this migration suppression. We observed a significant reduction in migration suppression in NDRG1 knockdown cells, implying that NDRG1 up-regulation is a major contributor to this migration suppression. As NDRG1 up-regulation is controlled by intracellular CIP levels, we studied the effects of iron supplementation on metastasis suppression. In accordance with the hypothesis, we observed no migration suppression in iron supplemented cells on treatment with •NO-donor. In conclusion, these results will aid in understanding and development of nitric oxide based chemotherapy targeting cancer

metastasis, which is an important clinical problem accounting for almost 90% cancer related deaths.

In another set of studies we examined the role of DNIC in iron mediated oxidative stress. The CIP is known to play an important role in hydrogen peroxide mediated oxidative stress via Fenton type reactions. Fenton-type reactions occur between peroxide and transition metals resulting in formation of hydroxyl radicals leading to oxidative damage. We hypothesized that nitric oxide via formation of DNIC can render this iron unavailable for the Fenton reaction thereby decreasing the level of peroxide mediated oxidative stress. We observed that HCC breast cancer cells treated with •NO (spermine nonoates, 0-100μM) were partially protected against ROS mediated cytotoxicity (H_2O_2 exposure) in a concentration dependent manner. Furthermore, pretreatment with •NO markedly decreases the oxidative stress (proportional to •OH generation) induced by peroxide treatment as measured by DHR fluorescence. These results could be replicated by substituting iron chelators (dipyridal) for •NO indicating a role for iron. Nitric oxide concentrations at which we observe cytoprotective effect correlated with concentration-dependent increases in DNIC formation. Next, we carried out iron supplementation studies in order to determine the role of iron in this cyto-protection. We observed iron supplemented cells showed increased oxidative stress and cytotoxicity on peroxide treatment, and higher doses on •NO-donor were required to circumvent this toxicity. These results support the hypothesis that •NO can act as an antioxidant by its ability to sequester cellular iron. This could play a significant role in a variety of diseases involving ROS mediated toxicity like cancer.

In conclusion, these results show a novel mechanism for nitric oxide mediated effects in a biological system. It also signifies the importance of DNIC in the intracellular environment and especially their role in cancer biology.

CHAPTER 1

INTRODUCTION

1.1 Nitric Oxide and Its Chemistry

Nitric oxide (•NO) is a small free radical present in various biological systems[1]. It is a colorless gas[2], with more solubility in organic solvents than water[3]. This lipophilic character makes it freely diffusible between and within the cells[3]. Nitric oxide is a vital effector and messenger molecule that plays role in variety of biological processes including smooth muscle tone, immune response, angiogenesis, apoptosis and synaptic communication[1].

In biological systems, nitric oxide leads to production of a complex array of reactive species[4], but it directly reacts with very small number of targets[5]. These targets are either other free radicals (e.g., dioxygen, superoxide) or transition metals (e.g., Fe) [5]. This reactivity is due to the ability of these targets to stabilize the unpaired electron on •NO[2].

1.1(a) *Nitric oxide and dioxygen*:

Nitric oxide reacts with O_2 to form the brown gas nitrogen dioxide (NO_2). Under aqueous solution, NO_2 reacts further to produce nitrite[6].

$2{\bullet}NO + O_2 \rightleftharpoons 2{\bullet}NO_2$

${\bullet}NO_2 + {\bullet}NO \rightleftharpoons N_2O_3$

$N_2O_3 + 2OH^- \rightleftharpoons 2NO_2^- + H_2O$

Overall: $4{\bullet}NO + O_2 + 2H_2O \rightleftharpoons 4H^+ + 4NO_2^-$

1.1(b) *Nitric oxide and other radicals*:

Nitric oxide reacts very slowly with most biological molecules, as compared to other free radicals[7]. One of the most important interactions occurs with free radical superoxide to form peroxynitrite[7].

$$\bullet NO + O_2\bullet^- \rightleftharpoons ONOO^-$$

Superoxide is most notably produced as a byproduct of mitochondrial respiration as well as during immune response[8]. This reaction can be of importance under these conditions for at least two reasons[3]: First, it means that •NO and superoxide antagonizes each other's biological actions. As •NO is also generated during the immune response, concomitant generation of both radicals will abate their action. Under physiological conditions, more amount of •NO will be required to elicit a biological response in the presence of superoxide[3]. Secondly, generation of peroxynitrite as a result of this reaction can lead to deleterious cellular events e.g., lipid oxidation, DNA strand breaks, deamination of DNA bases, nitration of aromatic amino acids etc[3].

1.1(c) *Nitric oxide and transition metals*:

Nitric oxide is known to react with transition metals in a biological system to form complexes[2]. These interactions are known to be responsible for the vast majority of effects observed upon exposure to •NO. One of the most important biological interactions of nitric oxide occurs with transition metal iron[2].

Iron is present in Heme centers of various proteins e.g., hemoglobin, soluble guanylyl cyclase (sGC). For example nitric oxide reacts with the ferrous iron center in deoxyhemoglobin to form Fe-NO complex[3].

$$HbFe^{2+} + \bullet NO \rightleftharpoons HbFe^{2+}NO$$

Nitric oxide can also react with the oxygenated form of hemoglobin leading to the formation of methemoglobin and nitrate[3].

$$HbFe^{2+}\text{-}O_2 + \bullet NO \rightleftharpoons HbFe^{3+} + NO_3^-$$

The most important biological effect attributed to •NO results from its reaction with the ferro-heme enzyme, soluble guanylyl cyclase (sGC)[9]. This leads to the formation of a nitrosyl complex with Fe^{2+} leading to labilization of a trans axial (proximal) histidine ligand in the protein backbone[9]. This results in a change in the protein conformation, which activates the enzyme for catalytic formation[9]. This enzyme catalyzes the formation of the secondary messenger cyclic-guanylyl monophosphate (cGMP) from guanylyl triphosphate (GTP)[9]. The formation of cGMP leads to smooth muscle relaxation in the blood vessels and lowering of blood pressure[10].

Other important metalloenzymes •NO is known to inhibit are cytochrome P450[11], cytochrome oxidase[12], nitrile hydratase[13].

Apart from its reaction with heme iron nitric oxide is also known to react with the intracellular chelatable iron pool. This leads to the formation of **Dinitrosyl iron complexes (DNIC)** [14]. They are discussed in detail in **section 1.3**.

1.2 Intracellular Iron

Iron is the most abundant and one of the most important heavy metals present inside the cell[15]. It is required for the activity of numerous proteins, which make it a metal of high importance in biological system[15].

1.2(a) *Iron uptake mechanism*[16]:

In the majority of cells, iron uptake occurs via a receptor mediated endocytosis[16]. Iron is present in serum bound to the protein transferrin. Apo-transferrin binds 2 molecules of iron to form diferric transferrin (Tf), which can then bind to membrane bound transferrin receptors (TfR). The Tf-TfR complex is then internalized via receptor mediated endocytosis into an endosome. Iron is released inside the endosome by a decrease in pH followed by transport into the cytoplasm by unknown receptors. At this state iron enters a very poorly defined **intracellular chelatable iron pool**. Apo-Tf bound to TfR is released back into serum via exocytosis, where is can be used again. The complete process of iron uptake by the cell is illustrated in **Figure 1**[16].

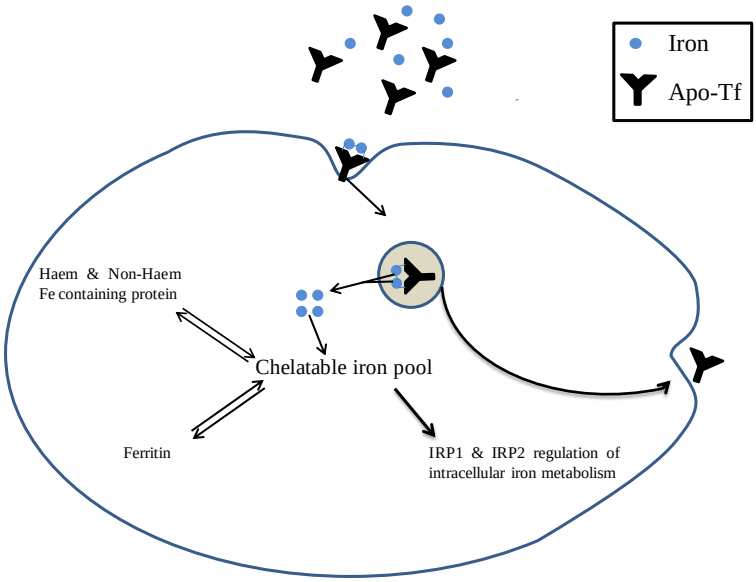


Figure 1: Cellular Iron uptake mechanism[16]

1.2(b) *Intracellular Labile/Chelatable Iron Pool (CIP)* [15,17].

The chelatable iron pool is a methodologically defined quantity which accounts for very small part of total cellular iron[15]. It is named because it can be chelated by chemical iron chelators like desferrioxamine[17]. It comprises both forms of iron (Fe^{2+} or Fe^{3+}) and it is associated with a diverse population of both high and low molecular weight cytosolic ligands such as organic anions (phosphates and carboxylates), polypeptides, and surface component of membranes (e.g., phospholipid head groups)[17]. The CIP is believed to act as a transitory pool between extracellular iron, ferritin bound iron and cellular iron associated with proteins. It represents only a small fraction of total cellular iron (<5%) [17]. Although, CIP is mainly believed to be cytosolic, some studies have shown its presence in other cellular compartments like mitochondria and nucleus[18].

CIP is a dynamic pool, but under normal physiological conditions its concentration is regulated within a narrow range by homeostatic mechanisms (typically involving IRE-IRP pathway)[15]. Only under extreme cases of iron deprivation or overload, it loses its integrity due to loss of homeostatic control[15].

The CIP is a pool of redox active iron complexes. It is known to be involved in reactions involving the formation of reactive oxygen species e.g., the Fenton reaction[19].

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \bullet OH$$

While H_2O_2 reacts slowly with biological molecules, the Fenton reaction results in formation of the highly reactive hydroxyl radical[20]. This highly oxidizing species can react with various biomolecule in a cell leading to lipid peroxidation, base modifications, DNA strand breaks and protein oxidation. These damaging events are considered crucial pathogenic factors for numerous

diseases, like neurodegenerative disorders (Alzheimer's and Parkinson's disease), ischemia-reperfusion injury etc[15].

***1.2(c)** IRE-IRP regulatory mechanism*[16,21]:

CIP homeostasis is maintained by well regulated machinery comprising of iron regulatory proteins (IRPs) and iron response elements (IREs). IRPs sense changing levels of the CIP and modulate iron import and storage machineries via feedback mechanism[15.] IREs are part of mRNA that have stem loop structures and are located in either the 3' (e.g., TfR) or 5' (e.g., ferritin and eALAS) untranslated regions of mRNA's of iron responsive genes. Within the loop structure an unpaired cytidine is found 6 bases 5' of six membered loop (CAGWGH, where W is adenosine or uridine and H is adenosine, cytosine or uridine) [22]. These structural characteristics of stem-loop are important for IRP-IRE binding. Substitution of nucleotides in the loop, disruption of base pairing in the upper part of the stem or replacement of the bulged cytidine decreased the binding affinity of the IRP for the mutant IREs in almost all cases[23].

There are two types of IRPs known as IRP1 and IRP2. Both of these bind to IREs in a sequence specific manner[21]. When iron levels are low in cells, IRPs bind to IREs in mRNA of both ferritin and TfR. These interactions protect TfR mRNA against targeted endonucleocytic degradation, whereas ferritin translation is prevented as IRP binding inhibits association of mRNA with the small ribosomal subunit[21]. Conversely, when iron levels are high, IRP-IRE binding is decreased resulting in degradation of TfR mRNA and translation of ferritin[21]. The detailed illustration of IRE-IRP mechanism is shown in **Figure 2**[21].

In case of IRP1 iron binding is regulated via Fe-S clusters, whereas in IRP2 it is regulated via Fe-mediated degradation by the proteasome[21].

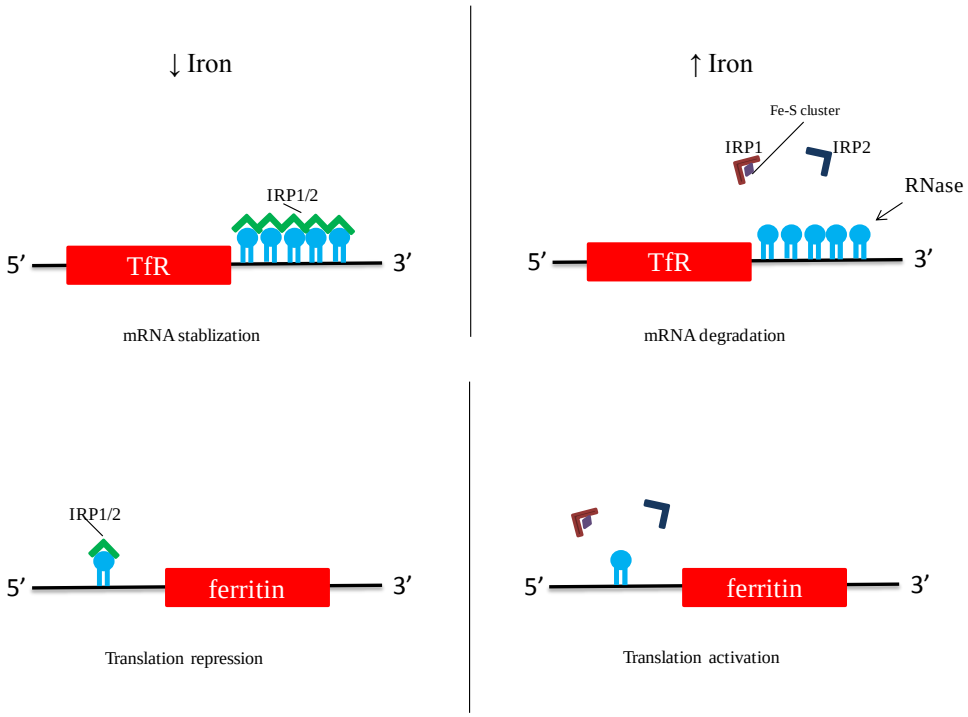


Figure 2: IRE-IRP mechanism[21]

1.2(d) *Role of Iron in Cancer*:

Elevated iron levels have been identified as an important risk factor for the development of cancer[24]. Numerous studies show a positive correlation between body iron stores and an increased risk for the development of cancers (e.g., colorectal, liver, kidney, lung, stomach etc)[25]. There is substantial epidemiological association between dietary iron intake and increased risk of breast cancer[26]. Neoplastic cells are more sensitive to iron depletion than normal cells, probably because of their increased iron requirement for growth and thus it provides an attractive target for cancer chemotherapy[27].

Iron forms the active site of the rate limiting enzyme in DNA synthesis, ribonucleotide reductase[21]. Iron depletion also affects range of cell cycle molecules (p53, GADD45, cyclins D1, D2 and D3, p21 and CDK2), which can be responsible for G1/S arrest or apoptosis[28]. Iron

chelators are also known to play an important role in metastasis[21]. Iron chelators are known to affect expression of a variety of cell surface adhesion molecules like E-selectin, VCAM-1 and ICAM-1[29,30]. Moreover, chelators also decrease the expression of matrix metalloproteinases in general[21]. Recently, Le *et al* have shown up-regulation of metastatic suppressor gene NDRG1 on treatment with iron chelators[31]. For this reason, several iron chelators (e.g., desferrioxamine, DFO) have shown anti-tumor activity[32] and may act as a potential chemotherapeutic agents in the future. Therefore iron metabolism is important to gain an understanding of cancer development.

1.3 Dinitrosyl Iron Complexes (DNIC)

As mentioned above one of the primary interaction of nitric oxide is with the transition metal iron. This can lead to the formation of dinitrosyl iron complexes. These are square planar complexes with iron in the center and two nitrosyl and two anionic ligands at cis-apical position (**Figure 3**)[33]. These complexes are paramagnetic in nature and thus identified by electron paramagnetic resonance (EPR) spectroscopy[5]. The exposure of cells or tissue to •NO results in ubiquitous "g=2.03" axial EPR signal, whose shape is characteristic to DNIC (**Figure4**) [5].

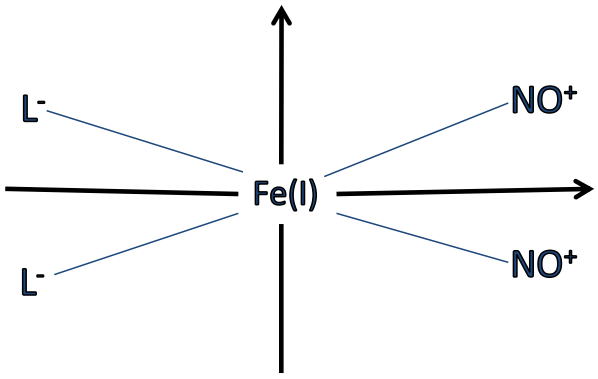


Figure 3: Structure of DNIC[33]

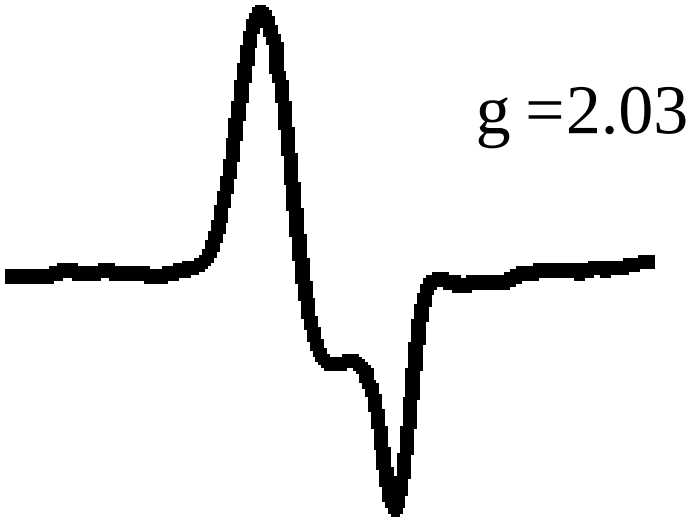


Figure 4: EPR signal of DNIC

1.3(a) *Shape and structure of DNIC*:

The DNIC formed in solution have a square planar coordination. Iron is present in the center of coordination complex with both anionic and nitrosyl ligands at cis-apical position (**Figure 3**) [33]. Iron have d^7 electronic configuration and its valency is described as Fe(I). Nitrosyl ligands are supposed to be present in the form of nitrosonium ion (NO^+) [34]. Anionic ligands (RS^-) can be low molecular weight thiols (e.g., glutathione, cysteine), proteins, phosphates etc [33].

Under acidic conditions or lower thiol:iron ratio dimeric DNIC are formed. The dimer is diamagnetic due to antiferromagnetic coupling between the two constituents. It is proposed by Vanin *et al* that the dimer is composed of two cofacial monomers rotated 180° to each other (**Figure 5**) [33].

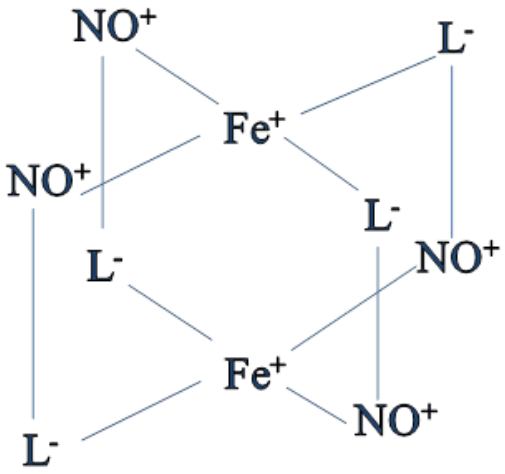
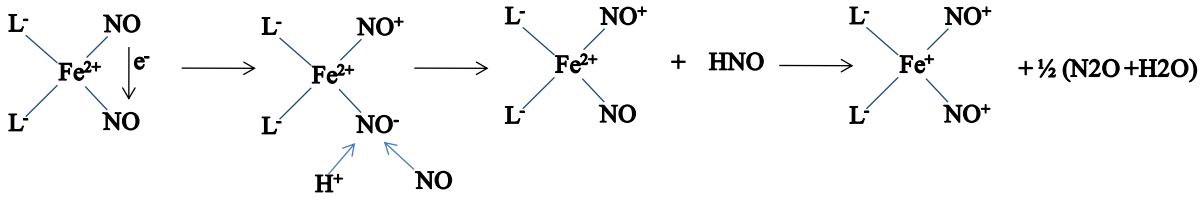


Figure 5: Structure of dimeric DNIC[33]

1.3(b) *Mechanism of formation*:

Vanin *et al* have proposed an evidence based disproportionation mechanism for the formation of DNIC. According to this mechanism, the d-orbitals of iron mediate this disproportionation by coupling unpaired electrons of the nitrosyls. The possible mechanism might proceed as below[35]:



For the formation of every molecule of DNIC, three molecules of •NO are required. The overall reaction can be presented as:

$$H^+ + 2L^- + 3NO + Fe^{2+} \longrightarrow \{(L^-)_2Fe^+(NO^+)_2\}^{+1} + ½\ (N_2O + H_2O)$$

1.3(c) *Iron source*:

The source of iron for the formation of these complexes is not entirely clear. There are studies showing the involvement of the CIP[5]. Others report iron ions localized in Fe-S clusters of iron-sulfur proteins to be involved [36]. Recently, we reported levels of DNIC to be equivalent to CIP levels on exposure to low doses of •NO, but it exceeds the CIP under conditions of high •NO exposure[37]. We concluded that, although CIP is the primary target of •NO for formation of DNIC, it can obtain Fe from other sources as well, like Fe-S proteins[37].

1.3(d) *Stability*:

Low molecular weight synthetic DNIC [e.g., Glutathione (GSH)-DNIC, Cysteine (Cys)-DNIC] are very unstable and undergo rapid oxidation. Cys-DNIC is only stable for 10-15 minutes, whereas GSH-DNIC is stable for 1-1.5 hours in synthetic test tube conditions[33].

There are only a few isolated studies that assessed the stability of these complexes in biological conditions. In hepatocytes, DNIC were shown to be stable, with a half life ranging from 4.5-8 hours[38]. We have recently shown that the half-life of DNIC formed in RAW 264.7 macrophages (either from exogenous or exogenous •NO) is around 80-90 minutes[37].

1.3(e) *Physiological and pathophysiological significance*:

GSH-DNIC are shown to behave like tight binding inhibitors of human glutathione-s-transferase superfamily (GSTA1-a, GSTM2-2, GSTP1-1, and GSTT2-2) [39]. They are also potent inhibitor of glutathione reductase, an important cellular antioxidant[40].

Presence of nitrosonium ion as a ligand in DNIC confers it the ability to nitrosate thiols[41]. Low molecular weight thiols and DNIC form a self regulatory system and are mutually inter-convertible[41]. Bosworth *et al* recently showed that cellular nitrosothiols (RSNO) are formed via

transnitrosation from DNIC[42]. RSNO are implicated in a variety of physiological and pathophysiological processes[43]. Thus, DNIC can play important cellular role by the formation of RSNO. For example, synthetic DNIC can lead to inhibition of caspase-3 via S-nitrosylation of cysteine residues on the protein[41].

Formation of these complexes renders CIP unavailable for other biological processes which may lead to cascade of cellular events.

1.3(e) *Pharmacological actions of low molecular weight DNIC*:

The use of Low molecular weight thiol DNIC (Cys- or Glu– DNIC) as pharmacological agent has been studied. Development of stable powder preparation of these low molecular weight DNIC have further helped in understanding their role as •NO donor and potential therapeutic agent[44].

DNIC are shown to have vasorelaxing activity in rat arotic ring[45]. They also have hypotensive effects, in an experimental animal model, which has biphasic dynamism (i.e. initial short term decrease in arterial pressure followed by prolonged decrease) [46]. Other studies show effects of DNIC in activities like inhibition of platelet aggregation, accelerated wound healing and erectile function[41,47,48].

1.4 Cancer Metastasis

Cancer is the second leading cause of death in United States[49]. Metastasis is the process by which tumor cells spread from the primary tumor to distant locations. The cancer cells have to migrate and invade through the basement membrane, survive in the circulatory system, invade into a secondary site, and start to grow there [50]. As it is difficult to treat and contain metastasis, it is regarded as an important clinical problem. Tumor metastasis is responsible for ≈ 90% of cancer related deaths[51]. Cancer cell metastasis is a complex biological process that involves changes in mRNA, protein, cellular morphology etc.

Figure 6 illustrates the invasion-metastasis cascade of primary tumors to distant sites[52]. Briefly, it involves following stages:

i) *Local invasion*: The cells detach themselves from the primary tumor mass by acquiring invasive phenotype, which results in loss of cell-cell and cell-extracellular matrix adhesion followed by proteolytic degradation of matrix[53]. They also undergo drastic alteration – the epithelial mesenchymal transition (EMT). During EMT cells shed their characteristic morphology and gene expression pattern of an epithelial cell and switch to a transcriptional profile of mesenchymal cell[52]. These EMT modified cells locally invade through the basement membrane.

ii) *Intravastion* of tumor cells through the walls of capillaries and lymphatics into the circulation.

iii) *Transport*: During transport of cancer cells to different sites, they must survive hostile environment in the circulation (e.g., hydrodynamic shear forces, lack of growth factors

for original microenvironment etc). Cells which survive these hostile conditions adhere to solid supporting tissue and form micro-thrombi.

iv) *Extravasation* of cancer cell micro-thrombi into distant tissue, typically lungs, brain, bone or liver.

v) Formation of *micro-metastasis*: After extravasation cells lodge at secondary sites, where they must proliferate and colonize for successful metastasis. This is the most challenging step, primarily because of the foreign tissue has much different microenvironment (i.e. growth and survival factors) compared to the primary tumor.

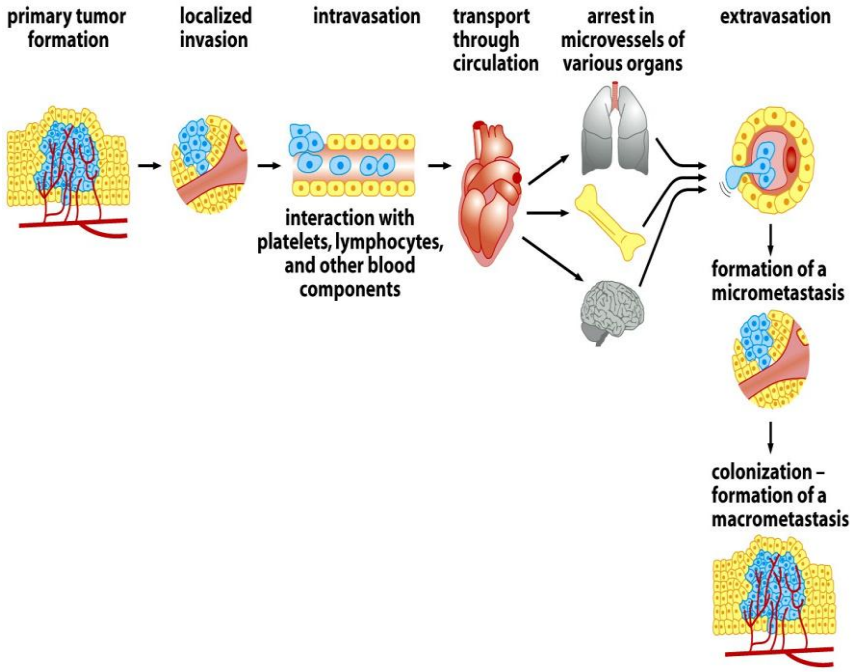


Figure 6: General metastatic Process[54]

These metastatic processes are controlled by number of promoters and suppressors, which orchestrate a complex array of events to achieve successful metastasis[55]. Recently researchers have started targeting these promoters (i.e. inhibition) or suppressors (i.e. up-regulation) of metastasis in order to develop new anti-metastatic therapies.

Various studies have identified a number of metastatic promoter or suppressor genes. The following is a list of metastatic promoter genes[55]: Amf, Hgf/sf, Tgfβ, Mmp, Upa, β-catenin.

1.5 Metastasis Suppressor Genes

The latest discovery of metastasis suppressor genes provides a new insight into cancer treatment. These genes inhibit metastasis at one of the steps involved in metastasis cascade, without affecting growth of primary tumor[50]. Currently, there are 23 well-characterized metastatic suppressors.

TABLE I describes the actions of known metastatic suppressor genes on different steps of metastasis–invasion cascade.

Major Metastasis Steps Inhibited	Metastatic Suppressor gene(s)
Angiogenesis	SSeCKs, TIMPs
EMT	Cadherins
Migration	CD44, DLC1, Gelsolin, MKK4, Nm23, OGR1, RhoGDI2, RKIP, SSeCKs, TIMPs
Invasion	Cadherins , DCC, DLC1, RKIP, RRM1, TIMPs
Intravasation	KAI1
Survival in transport	BRMS1, Caspase-8, KAI1, TIMPs
Motility	DCC, RRM1
Colonization	BRMS1, KISS1, MKK4, Nm23, OGR1, RhoGDI2, TIMPs
Unknown	NDRG1

TABLE I: METASTATIC SUPPRESSOR GENES[50]

Metastatic suppressor genes are shown to have tumor specific effects i.e. they inhibit metastasis in some cancer types but not in others[56]. They can also show their effect by different signaling pathways in different cancers[56]. In the current study, we are interested in the role of NDRG1 metastatic suppressor gene.

1.6 N-myc Downstream–Regulated Gene-1 (NDRG1)

Human NDRG1 is also known as RTP[57], Drg-1[58], Cap43[59] and Rit42[60].

1.6(a) *NDRG family*:

NDRG1 is a member of NDRG family[61]. The NDRG family consist of three other genes, namely NDRG-2,-3 and -4, which have 57-65% sequence identity to each other[61,62]. The NDRG family is divided into two subfamilies, first one consisting of NDRG-1 and -3, and other of NDRG-2 and -4. The NDRG family belongs to α/β hydrolase (ABH) superfamily[61]. The amino acid sequence of member proteins is best conserved in the region spanning ABH domain[63]. All members of family consist of CpG islands in their promoter region, suggesting that they may be controlled by DNA methylation[64,65].

1.6(b) *Molecular structure of NDRG1*:

It has been mapped to chromosome 8q24.3 and encodes a 3kb mRNA that is translated into 43kDa protein[57,58,59,60]. NDRG1 protein consists of 394 amino acid sequence[57,60]. NDRG1 is distinguished from other group members by three tandem repeats of a 10 amino acid sequence, GTRSRSHTSE, at its C-terminal region[62]. The mouse analog, Ndr-1, is highly homologous to human NDRG1, suggesting that it is highly conserved gene with important biological function[66].

The amino acid sequence of NDRG1 indicates the presence of a phosphopantetheine attachment site and a few potential phosphorylation sites (calcium-calmodulin kinase II, protein kinase A and C)[67,68].

Crystal structure of NDRG-2: Recently, crystal structure of NDRG-2 has been obtained[63]. It consists of two domains: a large canonical α/β hydrolase fold domain and a small cap like domain. Similarity search analysis showed similarity between the number of α/β hydrolases and NDRG2, with maximum similarity with Bacillus subtilis stress-response regulator (RsbQ)[63]. As shown in **Figure 7**, the main difference between NDRG2 and α/β hydrolases (e.g., RsbQ) is the absence of the catalytic triad (Ser-His-Asp). Instead, NDRG2 has Gly^{132}-Gly^{279}-Ala^{251}. Another important difference between NDRG2 and RsbQ is the lack of a pocket for substrate binding[63].

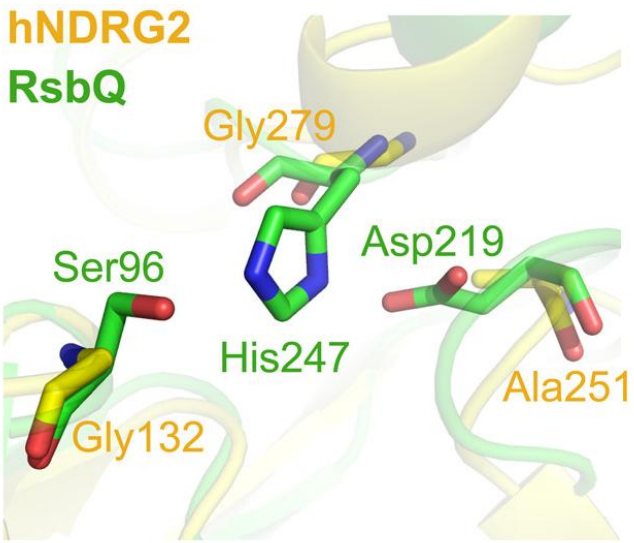
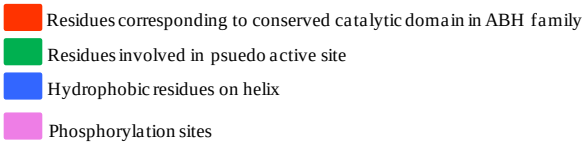


Figure 7: Comparison of catalytic residues of RsbQ and NDRG2[63]

The structure of NDRG2 suggests it to be homologue of ABH superfamily with no catalytic activity. Other members of NDRG family share similar evolutionary characteristics. The Gly-

Gly-Ala triad is conserved in all members, except substitution of Ala to Ser in NDRG1 and -3 (**Figure 8**). Also, all members have His[186], which leads to destruction of the active site (**Figure 8**)[63].

Another important structural difference between RsbQ and NDRG2 lies at helix α6. In RsbQ, like other hydrolases, the helix α6 is involved in formation of the hydrophobic pocket, with charged residues facing outside and hydrophobic residues facing inside of hydrophobic pocket[63]. In NDRG2, unlike other hydrolases, the hydrophobic residues face outside and charged residues face inside[63]. Moreover, α-helix stands out of the rest of the protein and is easily accessible for interaction with its binding target. These features of helix α6 are conserved in all NDRG family members[63].



	57	131	132	133	165	168	169	172	175	183	186	187	207	224	251	253	279	316	318
NDRG1	I	T	G	A	M	A	A	I	W	V	H	L	I	N	S	A	G	T	S
NDRG2a	V	V	G	A	M	A	A	L	L	I	H	L	I	N	A	H	G	T	S
NDRG2b	V	V	G	A	M	A	A	L	L	I	H	L	I	N	A	H	G	T	S
NDRG3	I	V	G	A	I	A	A	L	L	I	H	H	I	N	S	A	G	T	S
NDRG4a	V	V	G	A	I	A	A	L	L	V	H	L	I	N	A	A	G	T	S
NDRG4b	V	V	G	A	I	A	A	L	L	V	H	L	I	N	A	A	G	T	S

Figure 8: Comparison of important residues in NDRG family members[63]

1.6(c) *Cellular and tissue distribution of NDRG1*:

NDRG1 mRNA is ubiquitously expressed in all tissues, with high levels in prostrate, kidney, placenta and intestine[57,58,59,61,62,64,69,70]. NDRG1 protein is mainly found in epithelial cells[69]. On the other hand, NDRG-2 is expressed in spinal cord, NDRG-3 in testis and NDRG-4 in heart and brain[61,62]. This differential expression of all members of NDRG family, suggest different specific function of all these proteins[61].

At the cellular level, NDRG1 protein is predominantly cytoplasmic in nature[69]. But, it can vary between different cell type (e.g., intestinal and breast epithelia has membrane associated protein, whereas prostrate epithelial cells have mostly nuclear localization[69]). In some cells, mitochondrial localization is also observed[69].

1.6(d) *Biological function and regulation of NDRG1*:

The exact function of NDRG1 is not yet known, but is believed to have downstream effects under a variety of biological conditions.

1.6(d.1) *Role in differentiation:* Number of studies show involvement of NDRG1 in cellular differentiation. NDRG1 levels increase during keratinocyte differentiation[71]. Also, ligands of nuclear transcription factors involved in cellular differentiation (e.g., PPARγ) have been shown to induce NDRG1[64,70]. Stable transfection of NDRG1 cDNA in colon cancer cells leads to morphological changes similar to cellular differentiation[64]. Moreover, it also up-regulated various epithelial cell differentiation markers e.g., alkaline phosphatases, E-cadherin[64].

1.6(d.2) *Role in nerve myelination:* Hereditary Motor and Sensory Neuropathy- Lom (HMSNL) is an autosomal recessive disorder caused by demyelination of peripheral nerves. Patients suffering from this disease have a truncating mutation in NDRG1[72].

NDRG1 is shown to be abundantly present in peripheral nerves, especially Schwann cells. It is shown to play an important role in its differentiation and signaling required for its survival[72]. NDRG1 knock-out mice show demyelination in peripheral nerves, suggesting its role in myelin sheath maintenance[73].

1.6(d.3) *Role in Stress response:* Significant increase in NDRG1 mRNA has been observed in homocysteine treated human umbilical vein endothelial cells[57]. It has been proposed that increased homocysteine levels cause cell damage, causing stress response followed by changes in gene expression (e.g., NDRG1). These results imply a possible cytoprotective role of NDRG1 upon exposure to stress causing agents like homocysteine[57].

Further, regulation of NDRG1 by hypoxia inducible factor -1 suggests its role as a stress response gene[74]. In another study, overexpression of NDRG1 in trophoblast led to decrease in p53 expression, suggesting a protective role of NDRG1 against p53 mediated apoptosis[75].

1.6(d.4) Role in Cancer:

NDRG1 has been shown to be down-regulated in breast[76], colon[64] and prostate tumors[77]. Levels of NDRG1 have been found to be inversely related to grade of tumor, with higher grade and poorly differentiated tumors expressing less NDRG1[60,77]. Another study shows higher level of NDRG1 in primary colon cancers compared to their metastasis[64].

During the cell cycle in normal cells, NDRG1 shows a biphasic expression with high expression in G1 and G2/M phases and it is lowest in S phase. In cancer cells NDRG1 levels are consistent

throughout all phases[60]. These results show different roles of NDRG1 in growth and progression of normal and cancer cells.

NDRG1 and tumor suppressors: Phosphate and tensin homolog deleted on chromosome 10 (PTEN) is a phosphatase, known to have tumor suppressive actions. PTEN has been shown to up-regulate NDRG1[78]. Overexpression of PTEN leads to increased NDRG1 levels[78]. Studies have demonstrated time and dose dependent regulation of NDRG1 with PTEN inhibition[78]. Inactivation of PTEN correlates with invasion and metastasis of a wide variety of cancers. PTEN also decreased lymph node metastasis in mice with prostate cancer[78]. It has been suggested that PTEN might act as a tumor suppressor, at least in part, by mediation of NDRG1.

Von Hippel-Lindau (VHL) is another tumor suppressor gene known to regulate NDRG1 expression. VHL is shown to downregulate the expression of NDRG1[79]. VHL leads to degradation of HIF-1α and decreased NDRG1 levels. In renal carcinoma cells, VHL is often mutated, leading to higher levels of both HIF-1α and NDRG1[79].

p53 is a tumor suppressor that has been implicated in NDRG1 expression[75]. Stein *et al* observed NDRG1 up-regulation, at both the protein and mRNA level, following induction of p53 in DLD-p53 colon carcinoma cells but not in H1299-p53 lung carcinoma cells[80]. Another study showed up-regulation of NDRG1 levels upon iron depletion in p53-null H1299 lung cancer cells[81]. These findings point out cell type and conditions specific regulation of NDRG1 by p53.

NDRG1 and MYC: Myc gene expression is deregulated in 15-30% human cancers, resulting in elevated levels of Myc protein[82]. In deregulated conditions, Myc protein acts as an oncoprotein[82]. Both NDRG1 namesake N-myc (Neuroblastoma derived myelocytomatosis) as well as c-myc is known to downregulate NDRG1 expression[83]. In cells with ectopic expression of c- and N-myc, decrease in NDRG1 levels was observed[83].

NDRG1 and primary tumor growth: Kudristani *et al* showed inhibition of primary tumor growth by NDRG1 in a variety of cancer cell lines (breast, prostate and bladder) [60]. In other study, researcher found no significant effect of NDRG1 on primary tumor growth[64,77]. Again, this reiterates the point that NDRG1 can have tissue and cell specific function.

NDRG1 and angiogenesis: Angiogenesis is a process by which tumors induce generation of new blood vessels in order to meet the requirements of oxygen and nutrients for rapid growth. Studies have shown a role of NDRG1 in angiogenesis. Pancreatic cancer cells over-expressing NDRG1 were shown to have diminished matrix metalloproteinase-9 activity, a critical component of the angiogenic process[84]. Similarly, levels of VEGF-1 and IL-8 proteins, which play an important role in angiogenesis, are diminished in cells expressing high levels of NDRG1[84].

A study by Nishio *et al* showed a contradictory role for NDRG1 in angiogenesis in cervical carcinomas. In clinical samples, they observed an increase in tumor angiogenesis, invasion, metastasis and poor survival rate in carcinomas with higher NDRG1 levels [85].

NDRG1 and hypoxia: Hypoxia mediated response in cells occurs via Hypoxia inducible factor -1 (HIF-1) system as described in **Figure 9** [21]. HIF-1 is composed to two functional subunits: HIF-1α and HIF-1β. HIF-1 complex is formed after translocation of HIF-1α from the cytoplasm to nucleus, where it binds HIF-1β which is present in nucleus. HIF-1 complex acts as a transcription factor for genes having HIF response elements (HRE) in their promoter region[21].

Under physiologic oxygen and iron supply, prolyl hydroxylase (PHD) hydroxylate two proline residues on HIF-1α. The hydroxylated HIF-1α then binds to pVHL. This leads to activation of its ubiquitin mediated degradation by proteasome. As a result, expression of HIF targets genes decreases [21].

In hypoxia or iron depleted conditions, PHDs fail to hydroxylate, leading to stabilization of HIF-1α protein. HIF-1α is then translocated into the nucleus where it binds to a second subunit HIF-1β to form the HIF-1 complex[21]. HIF-1 complex interacts with HREs in promoter region of HIF-responsive genes leading to their transcription[21].

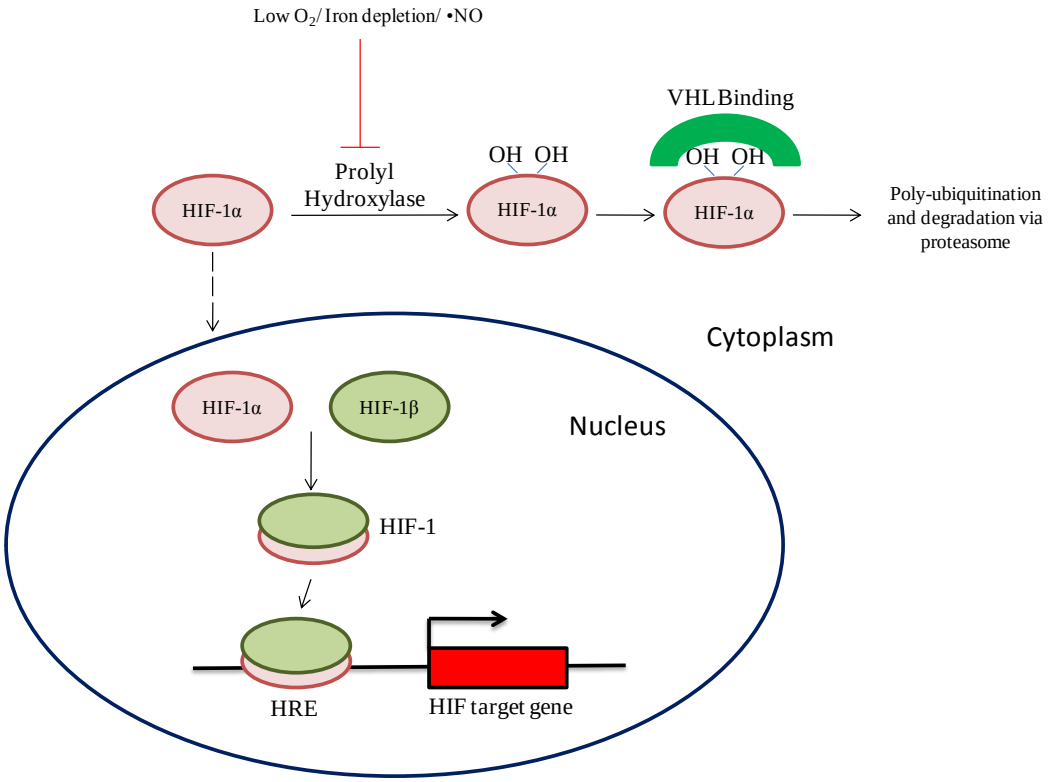


Figure 9: Hypoxia detection system by HIF-1[21]

Le *et al* showed *in-silico* presence of putative HREs upstream of NDRG1 promoter at -1376 bp and -7503 bp [31]. These results points towards possible involvement of HIF-1 in NDRG1 up-regulation. In the same study, they show both HIF-1α dependent as well as independent pathways are involved in up-regulation of NDRG1 on treatment with iron chelators [31]. Other studies have shown up-regulation of NDRG1 under hypoxic conditions[74] or exposure of hypoxia mimetics (e.g., nickel[59]).

NDRG1 and Metastasis: NDRG1 has been shown to have a metastatic suppressor effects in prostate, colon and breast cancers[76,77,86]. Others have shown a decrease in the invasive ability of cells over-expressing NDRG1in colon and prostate cancers [64,77].

Bandopadhyay *et al* showed a significant decrease in invasiveness of MDA-MB-468 cell upon NDRG1 over-expression in both *in-vitro* and *in-vivo* models[76]. MDA-MB-231, an invasive breast cancer cell line, in particular has a 10 fold less NDRG1 mRNA and protein as compared to MCF-7 (non-invasive) cell line[87]. In another study, up-regulation of NDRG1 in metastatic H1299 lung cancer cells leads to inhibition in proliferation, but not in non-metastatic DLD-1 colon cancer cells[80].

In-vivo studies have shown more significant effect in both colon and prostate cancer. There is more than a 50% reduction in liver metastasis in mice injected in SW620 colon cells over-expressing NDRG1[64]. Similar, results were obtained with mice injected with AT6.1 prostate cancer cells over-expressing NDRG1[77].

In human prostate specimens, the primary tumor cells express much higher levels of NDRG1 than lymph-node or bone metastases[77]. Patients with higher levels of NDRG1 in tumor are shown to have a higher survival rate, as indicated by 5 year follow up study in breast and prostate cancer patients[76,77]. Similar results were obtained in another study done on colorectal cancer patients[86]. These observations indicate the usefulness of NDRG1 as a prognostic marker in metastatic disease.

Another study indicated population wise differences in NDRG1 levels in prostate cancer patients. African-Americans are shown to have much lower levels of NDRG1 in tumors as compared to Caucasian patients[88]. Interestingly, African-American patients are known to have a greater risk of prostate cancer, with a more aggressive phenotype as compared to Caucasians[88].

Thus, NDRG1 can possibly be one of the factors attributed to population dependent differences in disease progression.

Downstream targets of NDRG1: The adhesion molecule and metastatic suppressor, E-cadherin was found to be up-regulated by NDRG1 and may be at least one molecular target of NDRG1, explaining its anti-metastatic effects[64]. Increased levels of E-cadherin have been shown to decrease motility of metastatic breast cancer[85].

A microarray study shows NDRG1 dependent up-regulation of thiamine triphosphatase (Thtpa) protein levels in DLD-1 and MIAPaCa-2 cells over-expressing NDRG1[90]. Thtpa, is involved in hydrolyzing thiamine triphosphate (an important energy currency molecule). Thus, increased NDRG1 levels, may lead to a decrease in the energy state of the cell followed by diminished cell growth and metastasis suppression. In the same study, they also showed down-regulation of cathepsin C (Ctsc) mRNA and protein in MIAPaca-2 pancreatic cancer cells overexpressing NDRG1[90]. Ctsc is a cysteine proteinase that plays a role in pancreatic cancer progression. NDRG1 might exert its anti-metastatic effects in pancreatic cancer via Ctsc.

A recent study showed up-regulation of the cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ (or p21) in prostate and lung carcinoma cells over-expressing NDRG1[91]. In the same study, decreasing NDRG1 levels using shRNA led to a decrease in p21 protein levels. Thus, p21 was shown to be closely related to NDRG1 in these cell lines. As p21 is considered as an inhibitor of tumor progression, the up-regulation of this protein by NDRG1 might be in-part responsible for its anti-tumor functions.

Another important downstream target of NDRG1 is activating transcription factor 3 (ATF3)[92]. KAI1, is a metastatic suppressor gene that has a consensus binding motif for ATF3 in its promoter region. The study showed up-regulation of KIA1 on ectopic expression of NDRG1 in

prostate cancer cell lines. Also, silencing of NDRG1 was accompanied with a decrease in KAI1 expression[93].

NDRG1 is also shown to decrease the NF-κB signaling by reducing κB kinase (IKK) β expression and IκBα phosphorylation[94]. Reduced IKKβ expression leads to decrease in nuclear translocation of p65 and p50. Further, binding of p65 and p60 with NF-κB motif also gets reduced[94].

Controversial Role of NDRG1 in Cancer: There are some contradicting reports on the role of NDRG1 in cancer. In some studies, androgen receptor positive prostate cancers show a higher level of NDRG1 compared with normal tissue. They claim the decreased level of NDRG1 observed in other studies is due to loss of hormone-dependence[74].

In a study involving colorectal cancer, NDRG1 expression was found to be higher in more advanced lesions, leading to speculation that NDRG1 is a metastasis promoter gene[95]. This increase might be because of hypoxic conditions present inside tumors, as hypoxia also up-regulates NDRG1[95].

In another clinical study involving 223 prostate cancer specimens, there was no conclusive NDRG1 up-regulation or down-regulation[88]. These expression patterns were considered as differential response to hypoxia and androgens in prostate epithelium of different patients.

In some recent studies, levels of NDRG1 are shown to be up-regulated in a variety of tumors (e.g., liver, cutaneous squamous cell, oral squamous cell, cervical or renal carcinoma)[79,96,97,98,99]. In liver carcinoma, silencing of NDRG1 reduced proliferation, invasion and apoptosis *in vitro* and inhibited tumor growth *in vivo*[96]. These results again show tissue specific effects of NDRG1.

1.6(d.5) *Other regulators of NDRG1:* Recently, NDRG1 has been found to be activated by iron chelators[81]. There are a variety of other inducing agents shown to be involved in expression of NDRG1: vitamin D, retinoic acid, phorbol esters, androgenic and estrogenic hormones, mercaptoethanol, tunicamycin, nickel compounds, okadaic acid, calcium inophores, 5'-aza-2'-deoxycytidine, trichostatin A, mitomycin C etc [57,59,60,64,70,100,101].

Transcription factors which might be involved in regulation of NDRG1 include Erg-1, activator protein 1 (AP-1) and erythroblastosis virus E26 oncogene homologue 1 (ETS1)[102,103,104].

1.6(d.6) Phosphorylation of NDRG1: NDRG1 has been shown to get phosphorylated by a number of kinases, but the specific role of these modifications is still unknown. *In vitro* studies have shown phosphorylation activity by calmodulin kinase-II, PKA and PKC[105].

Serum- and glucocorticoid-regulated kinase 1 (SGK1) is known to phosphorylate the C-terminal region of NDRG1 (Thr^{328}, Ser^{330}, Thr^{346}, Thr^{356} and Thr^{366})[106]. Phosphorylation by SGK1 primes it for further phosphorylation (Ser^{342}, $Ser^{352}$and Ser^{362}) by glycogen synthase kinase 3β (GSK3β)[106].

1.7 Nitric Oxide as an Anti-Tumor Agent

Nitric oxide (•NO) is a small free radical with a variety of functions and activities in biological system[1,6]. Nitric oxide is known to have both anti- and pro- tumor activity in several *in-vitro and in-vivo* models[107]. It is important to note that difference in experimental set-up and the source of •NO in the study can drastically alter the outcome. It is therefore essential to critically analyze the literature, in order to understand the discrepancies in the results. Studies have shown the role of Nitric oxide as an endogenous modulator of tumor progression as well as a pharmacological agent[108].

1.7(a) Role of nitric oxide synthase (NOS) in cancer:

Nitric oxide is produced biologically via enzymatic synthesis by nitric oxide synthase (NOS). NOS exist in three isoforms (eNOS, iNOS and nNOS) and require arginine, oxygen and other cofactors (NADPH, FMN, BH_4 and FAD) for its enzymatic activity[109,110].

Studies have shown both positive and negative effects of •NO on tumor growth and metastasis[111,112,113,114]. Various studies have shown increase in tumor growth, angiogenesis and invasion in cells exposed to endogenous •NO production[115,116,117,118]. Both tumor cells and host stromal cells (tumor associated macrophages (TAMs) or cytokine activated fibroblast) are known to produce •NO, mainly by iNOS activity. Conversely, inhibition or deletion of iNOS in mice has resulted in decreased tumor growth and metastasis[119]. Inhibitors of iNOS or eNOS are also shown to reduce tumor progression[120,121].

On the other hand, increase in •NO signaling via other methods (e.g., iNOS gene delivery or induction of iNOS by cytokines) has beneficial effects by induction of apoptosis, diminished angiogenesis and cell growth and inhibition of metastasis[122,123,124]. Le *et al* demonstrated that different cancer cell lines (PC3, MDA-MB-453, DLD-1, SN12PM6, SKOV-3, HT1080, L3.3, 253J BV) transfected with murine macrophage iNOS showed significantly reduced tumor growth and metastasis compared to cells transfected with control EGFP gene[125].

1.7(b) Role of nitric oxide donating or •NO related drugs in cancer:

Nitric oxide is the active component of several classes of pharmaceuticals that are in various phases of development: pre-clinical models, clinical trials or currently approved to treat patients e.g., organic nitrates, including •NO-donating NSAID's (NO-NSAIDs)[126,127], S-nitrosothiols[127], and diazeniumdiolate[128].

Studies involving different •NO related drugs consider it a potential candidate for anti-cancer therapy. In a study involving PC-3 prostate xenograft mouse model, researchers found significant inhibition in tumor growth on treatment with doxorubicin along with concomitant nitroglycerine patch, as compared to doxorubicin alone[129]. In a similar study, Bonavida *et al* concluded that combination therapy of •NO-donor DETA/NO and cisplatin was effective in inhibiting tumor growth in mice bearing PC-3 tumor xenograft[130].

•NO-Donating Non-steroidal anti-inflammatory drugs (•NO-NSAID) are recent candidates used in the treatment of colon cancer in preclinical models. GT-094, a novel •NO-NSAID, has been shown to reduce the formation of azoxymethane induced aberrant crypt foci in male Fisher rats[131]. Similarly, Ouyang *et al* demonstrated inhibition of angiogenesis by •NO-aspirin in HT-29 colon cancer mouse xenograft model[132].

JS-K is another novel •NO-donating prodrug, which releases •NO on reaction with glutathione. JS-K has been shown to inhibit tumor growth in human multiple myeloma cells xenografted in NIH III mice[133].

Simeone *et al* demonstrated anti-invasion and pro-apoptotic potential of •NO-prodrug JS-K, at doses which were not cytotoxic, in metastatic breast cancer cells. They attributed observed effects to production of TMP and inhibition of p38 on JS-K treatment[134].

S-nitroso-N-acetylpenicillamine (SNAP) is another •NO-donor with anti-metastatic effects in experimental models[133]. Another study showed inhibition of invasion by sodium nitroprusside (SNP) in PC-3M and T24 cancer cells[136].

Baritaki *et al* have recently observed anti-metastatic effects of •NO-donor DETA/NO in PC-3 prostate tumor cell line, in both *in-vitro* and *in-vivo* models[137].

Targeted delivery of •NO to tumor using modified donor compounds has shown great promise in the development of nitric oxide based anti-cancer therapy. Tang *et al* synthesized a •NO-donor PYRRO/NO coupled to a chain of amino acids which is recognized by prostate specific antigen (PSA) [138]. PSA is an important marker of prostate cancer progression. PSA is inactive in blood plasma but highly up-regulated within prostate cancer metastases. Thus, this hybrid molecule gives the possibility of selective release of •NO in metastatic prostate cancer cells[138].

1.7(c) Clinical studies involving •NO containing drugs:

Several studies have been conducted in order to understand the clinical significance of •NO-donor/•NO related molecules.

In a study involving the use of Nitrogylcerine (GTN), Siemens *et al* showed a marked increase in PSA doubling time in patients treated with low dose GTN transdermal patch as compared to vehicle control[139]. Also, no apparent side effects were observed with GTN treatment[139].

In another Phase I study, the NOS inhibitor N-nitro-L-arginine was administered to several cancer patients in order to study its effect on tumor blood volume[140]. They demonstrated that nitric oxide plays an important role in tumor vascularity and inhibition of endogenous •NO production significantly reduces tumor blood volume[140].

Yasuda *et al*, in a phase II trial, showed beneficial effects of the addition of nitroglycerine to vinorelbine and cisplatin regimen in untreated stage IIIB/IV non-small cell lung carcinoma (NSCLC) patients. Addition of GTN increased response rate to chemotherapy (measured as identifiable tumor sizes) by 72% (compared to 42%) and median time of progression to 11 months (compared to 4.2 months) [141].

NCI conducted a phase I clinical trial on •NO-releasing acetylsalicylic acid derivative (NCX4016) in order to assess its multi-dose safety, pharmacokinetics and pharmacodynamics. The results of this trial are still not available.

1.8 Antioxidant properties of Nitric Oxide

Nitric oxide has been shown to have both deleterious as well as cytoprotective effects under biological conditions[142]. The pro- or anti- oxidant effects of •NO depend on its local concentration as well as the presence of other reactive oxygen/nitrogen species in the biological milieu[142]. At high local cellular concentration of •NO, it can react with a number of other reactive nitrogen oxide species and cause deleterious effects to cellular components[142]. In contrast, low physiological doses of •NO are shown to abate the oxidation chemistry of reactive oxygen species (ROS) [142]. In addition, •NO has been shown to protect cell against death caused by oxidizing agents such as hydrogen peroxide, alkylhydroperoxides[143].

Reactive oxygen species are the primary source of oxidative stress. These species (e.g., superoxide and peroxide) are often produced via Fenton-type reactions. Other sources of oxidative stress include reactive nitrogen oxide species (e.g. peroxynitrite, nitrogen dioxide, nitroxyl), and lipid –oxy and –peroxy adducts. •NO has shown to have an anti-oxidant effect against all these sources[142].

Fenton generated ROS: Fenton-type reactions occur between peroxide and transition metals resulting in the formation of hydroxyl radicals, or hypervalent peroxo- or oxo-metal complexes[142].

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \bullet OH,\ Fe^{4+}O,FeO_2$$

These ROS intermediates generated during Fenton reaction can lead to alteration of biomolecules like proteins, lipids, nucleic acids, resulting in cell death[144].

ROS generated during these reactions can be scavenged by •NO at very high rates[145]. But these species react with other biomolecules at similar rates and thus high •NO concentrations are required for •NO to exert its anti-oxidant properties by this mechanism[146]. Moreover, these Fenton products must be produced near the biomolecules in order to cause modifications like DNA strand break, protein oxidation. Diffusion of •NO to these regions would not likely yield a high enough concentration to outcompete deleterious reaction between oxidants and biomolecules[142].

The formation of metal-nitrosyl complexes can also prevent the reaction of peroxides with metals. This can lead to a decrease in the formation of Fenton related oxidants and thus act as a potential antioxidant mechanism of •NO[142].

Moreover, iron is known to be more stable in ferric form *in vivo*. Reduction of ferric to ferrous iron can involve superoxide[147]. Nitric oxide is also known to react with superoxide to form peroxynitrite. In the absence of other reactive species peroxynitrite can rearrange to form nitrate[148]. This conversion of superoxide to nitrate can prevent reduction of ferric to ferrous and stops catalytic formation of ROS[142]. This can be another important mechanism for antioxidant effects of •NO.

Nitric oxide/Superoxide reaction: The formation of peroxynitrite by reaction between •NO and O_2^- is supposed to provide antioxidant effect against Fenton generated ROS, but peroxynitrite can also act as an oxidant itself[149]. Studies have shown that the presence of excess •NO can scavenge reactive intermediates formed during decomposition of peroxynitrite, resulting in

formation of N_2O_3[150]. Thus formation of N_2O_3, a nitrosating agent, may provide an antioxidant effects by reduction in ROS production[142].

Lipid peroxidation: Lipid peroxidation leads to formation of lipid –oxy and –peroxy adduct in the cell membrane[146]. This can lead to compromise in cell membrane. Reaction of •NO with these radicals terminates this free radical chain reaction[148]. •NO can, therefore, act as an anti-oxidant agent by inhibiting deleterious effects of lipid peroxidation[142].

$$LOO\bullet + \bullet NO \longrightarrow LOONO$$

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals and Reagents:

Diethylenetriamine nonoate **(**DETA/NO) and Spermine/nonoate (Sper/NO) were a generous gift from Dr Joseph A. Hrabie. Ferric ammonium citrate (FAC), Diethylenetriamine-pentaacetic acids (DTPAC), phosphate buffer saline (PBS), desferrioxamine mesylate, CelLytic™ M cell lysis reagent, bovine serum albumin, ferrous sulfate, Phenylmethanesulfonyl fluoride (PMSF), vanadium chloride, potassium iodide, 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-*p*,*p′*-disulfonic acid monosodium (Ferrozine™), Neocuproine, ammonium acetate, sodium hydroxide, acetic acid, hydrochloric acid, ascorbic acid, insulin, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), reduced glutathione (GSH), Geldanamycin (GL), and lipopolysacchride (LPS) were purchased from Sigma-Aldrich Corporation. N,N'-Di(2-hydroxybenzyl)ethylenediamine-N,N'-diacetic acid monohydrochloride hydrate (HBED) was purchased from Strem Chemicals. 2,2'-dipyridal (DP) was purchased from Acros Organics. Bio-rad DC™ protein assay reagents, tris-glycine gels (4-20% gradient), laemmeli sample buffer, tris/glycine/sds running buffer were purchased from Bio-rad. Dihyrorhodamine 123 was purchased from Alexis Biochemicals. PVDF gel transfer stacks and alamarBlue® dye was purchased from Invitrogen. Protease inhibitor cocktail set III was purchased from Calbiochem. SuperSignal West Femto Maximal Senstivity Substrate, rainbow protein marker dye, Matrigel™ and Tween 20 were purchased from Fisher Scientific. Dry non-fat milk was purchased from Nestle.

2.2 Cell culture supplies:

All cell culture supplies were purchased from either Invitrogen or Fischer Scientific, unless otherwise specified.

2.3 Cell Culture:

HCC 1806, MDA-MB-231, HT-29, MCF-7, SKOV-3 and SHSY5Y cancer cells were obtained from the American Type Culture Collection (ATCC). Murine macrophage RAW 264.7 was also obtained from ATCC. All cell lines were cultured at 37°C temperature and 5% CO_2 concentration in a tissue culture incubator. The cells were grown to 80-90% confluence in tissue culture plates in growth medium containing 10% fetal bovine serum (FBS). Prior to treatments, growth media was replaced with serum-free growth medium for 16 hours. All experiments were conducted under these culture conditions.

TABLE II illustrates media composition required for growth of different cell lines used.

Iron supplementation: The cells were treated with ferric ammonium citrate (150μg/ml) for indicated time points. Plates were washed with PBS containing DTPAC (100μM) in order to remove excess extracellular iron. Fresh growth medium was added to the plate, followed by desired treatments.

Cell Line	Cell Type	Media Recipe
HCC 1806	Breast cancer	RPMI media + 10% FBS + 1% Pen-strep
MDA-MB-231	Breast cancer	DMEM media + 10% FBS + 1% Pen-strep
HT-29	Colon cancer	RPMI media + 10% FBS + 1% Pen-strep
MCF-7	Breast cancer	DMEM media + 10% FBS + 1% Pen-strep + Insulin
SKOV-3	Ovarian cancer	McCoy's5A modified medium + 10% FBS + 1% Pen-strep
SHSY5Y	Neuroblastoma	RPMI /opti-MEM(1:1) media + 5% FBS + 2% Pen-strep + 2% ABAM
RAW 264.7	Murine macrophages	DMEM media + 10% FBS + 1% Pen-strep

TABLE II: GROWTH MEDIUM RECIPE FOR DIFFERENT CELL LINES

2.4 Western Blot Analysis[151]:

Western blot analysis was performed as previously described[151]. Briefly, cells were lysed using lysis buffer (CelLytic™ M Cell lysis reagent with 1% protease inhibitor cocktail and 1mM PMSF). The samples were centrifuged and the supernatant was collected for further analysis. The protein samples of equal amount were separated on denaturing polyacrylamide gels and then transferred to PVDF membranes by electrophoresis. The membrane was blocked with 5% solution of non-fat dry milk in PBS (containing 0.1% Tween 20), followed by overnight incubation with primary antibodies (**Table III**). Bands were washed and incubated with horseradish peroxidase conjugated secondary antibody. The blots were finally analyzed in a Fluor Chem HD2 imager (Alpha Innotech) using SuperSignal West Femto Maximal Senstivity Substrate. Relevant bands were cropped to size using Microsoft office picture manager. Figures are representative of $n \geq 3$ individual experiments.

2.5 quantitative-Real time-Polymerase chain reaction (qRT-PCR)[152]:

After desired treatments, RNA was isolated using RNAqueous 4PCR kit (Applied Biosystems) following manufacturer's protocol. mRNA was converted to cDNA using Transcriptor First Strand cDNA synthesis kit (Roche) according to manufacturer's protocol. The cDNA was subjected to real time – Polymerase chain reaction using StepOnePlus™ real time PCR system. The reaction was carried out using FastStart Universal Probe Master (Roche) protocol. PCR program includes initial denature at 95^{o}C for 10 min, 40 cycles of denature step at 95^{o}C for 15 s, primer-annealing and extension step at 60^{o}C for 1 min. The oligonucleotide primer sequences (Invitrogen) are described in detail in **TABLE IV**.

Protein	Antibody dilution	Source	Catalogue No.
NDRG-1	1:500	SantaCruz Biotechnology	sc-30040
HIF-1α	1:500	BD Transduction Labs	610959
β-actin	1:2000	Cell Signaling	4970
iNOS	1:500	SantaCruz Biotechnology	sc-651
Anti-rabbit IgG	1:1000	Cell Signaling	7074
Anti-mouse IgG	1:1000	Cell Signaling	7076

TABLE III. LIST OF ANTIBODIES USED FOR DETECTION OF PROTEINS

Gene	Forward Primer	Reverse Primer
NDRG-1	GGGCTGAAAAGCATTATTGG	CTCCACCATCTCAGGGTTGT
HIF-1α	GGTTCACTTTTTCAAGCAGTAGG	GTGGTAATCCACTTTCATCCATT
β-actin	CCAACCGCGAGAAGATGA	CCAGAGGCGTACAGGGATAG
C-myc	TGCTCCATGAGGAGACACC	CCTCATCTTCTTGTTCCTCCA
E-cadherin	GCCGAGAGCTACACGTTCA	GACCGGTGCAATCTTCAAA

TABLE IV. LIST OF OLIGONULEOTIDE PRIMERS SPECIFIC FOR cDNA SEQUENCES

2.6 Cell Migration, Invasion and Viability[153,154]:

xCELLigence® DP system was used for measurement of migration, invasion and cell viability. The system is based on measurement of impedance across the gold electrode arrays on the bottom of wells (E-plates) or on the underside of membrane (CIM-plates).

E-plate: The media was added to individual wells in E-plates and they were then inserted into the DP system. The background impedance of the cell culture media was recorded. The cells were then harvested and added in equal number (1×10^4 cells) to the individual wells on the E-plate. The plates were left for 30min in tissue culture hood at RT, followed by reinsertion on the DP system for measurement of impedance (proportional to cell growth).

CIM plates: CIM plates are trans-well system with a membrane in between two wells. The 10% serum media (160µL) was added to the bottom well as a migratory stimulant. Then top chamber was placed on the bottom chamber and locked. Then 40µL of serum free media was added and left to equilibrate for 1h in incubator followed by measuring a background read on the DP system. The cells were then harvested, counted and diluted in serum free growth media and added in equal number (2×10^4 cells) to each top well. The plates are placed on the DP system for measurement of impedance (proportional to cell migration across micro-porous polyethylene terephthalate membrane).

Cell Invasion: For invasion studies, top chamber of CIM plate was coated with 30µL of 1:40 dilution of Matrigel™ in serum free media and incubated at 37°C for 4 hours. Subsequent steps were performed in the same manner as for cell migration assay.

2.7 Knockdown Cell lines

NDRG1 and HIF-1α knockdown HCC 1806 cells were prepared by transfection with plasmid. Briefly, plasmid vector containing either NDRG1 or HIF-1α specific shRNA were transfected into HCC 1806 cell line using lipofectamine. Individual colonies containing shRNA were grown in puromycin containing media. The cell lines were validated for knockdown by qRT-PCR and western blot analysis.

2.8 siRNA transfection[155]:

The cells were grown until 60-80% confluent in antibiotic free media and were transfected with 6µL of transfection reagent (Santacruz Biotechnology) in transfection medium (Santacruz Biotechnology) along with 8µL of the siRNA purchased from Santacruz Biotechnology. The cells were then cultured in 1ml transfection medium for 6 hours followed by supplementation with media containing 2X serum and antibiotic for 24hours. Then, the media was changed to 10% FBS containing culture medium with antibiotics for another 24hours. The cells were then assayed for various treatments.

2.9 EPR Spectroscopy[37]

Cells were trypsinized and re-suspended in equal volumes of PBS. DNIC signals were measured by EPR at g = 2.03 using a Bruker X-band EMX Plus EPR spectrometer, fitted with a liquid nitrogen dewar (at 77^{o}K) with the following settings: modulation amplitude 10G, 200G scan range, 90 sec scan time, 1 scan. DNIC concentrations were estimated by comparing the signal amplitudes to that of a known standard of GSH-DNIC. For quantitative studies a microwave power saturation profile for EPR signal amplitudes was conducted to ensure we were operating below the saturation. Protein samples were collected from each experiment so that DNIC could be quantitatively compared between individual runs (pmol DNIC/mg protein).

For the CIP quantification, cells were treated with 1mM desferrioxamine (DFO) for 4 h and then harvested for EPR analysis. The concentration of the CIP was calculated by comparing the signal amplitude to the amplitude of known concentrations of a DFO-Fe(III) standard prepared as previously described. EPR settings: g = 4.03, modulation amplitude of 10G, 200 G scan range, 30 sec scan time, 4 scans.

2.10 GSH-DNIC synthesis[156]

1 ml of 0.5 mM $FeSO_4$ (dissolved in degassed water to avoid rapid oxidation to the ferric state) was added to 10 ml (final volume) of 0.1 M of degassed PBS buffer, pH 7.4, containing 20 mM GSH and 2 mM GSNO (25 °C) under hypoxic conditions. After 45 minutes, the reaction was almost 70% complete, and the resulting stock solution of GSH-DNIC was quantified either by UV-Vis spectroscopy (ε_{403}=3000) or EPR spectroscopy.

2.11 Nitrite/Nitrate Chemiluminescence Analysis[157]

Nitrite was measured using ozone based chemiluminescence analyzer (CLA). Nitrite was first reduced to •NO by glacial acetic/KI solution in purge vessel, before final measurement in CLA.

$$2NO_2^- + 2I^- + 4H^+ \rightarrow 2{\bullet}NO + I_2 + 2H_2O$$

$${\bullet}NO + O_3 \rightarrow NO_2^* + O_2$$

$$NO_2^* \rightarrow NO_2 + h\nu$$

Nitrate was measured using a similar method as for nitrites, except acidic VCl_3 at 95 ^oC is used as a reducing agent. It gives combined concentration of both nitrate and nitrite in sample. Nitrate concentration can be obtained by subtracting nitrite concentration from combined (Nitrate+Nitrite) concentration.

$$2NO_3^- + 3V^{3+} + 2H_2O \rightarrow 2{\bullet}NO + 3VO_2^+ + 4H^+$$

2.12 alamarBlue® Assay[158]

Cells (10 x 10^3/well) were plated in 96-well plate 16 hour before the experiment. The cells were treated with Sper/NO for 1hour followed by H_2O_2 for 2hours. They were further incubated in FBS supplemented DMEM media. After 48 hours, media was replaced with media containing 10% alamarBlue® dye for 2-3 hours. Absorbance was recorded at 570nm using 600nm as reference wavelength.

2.13 DHR fluorescence assay[159]

Cells (7.5X10^3/well) were plated in 96-well plate 16 hr before the experiment. The cells were incubated with PBS containing DHR123 (100μM) for 1 hour at 37^{o}C. The cells were washed once with PBS and incubated with the culture medium containing Sper/NO for 1 hour. H_2O_2 were added to the medium and incubated for 1 hour. The fluorescence was measured by Spectra MAX GeminiEM plate reader (excitation: 485nm, emission: 530nm).

2.14 Total iron determination[160]

Cells were lysed using lysis buffer (CelLytic^{TM} M Cell lysis reagent with 1% protease inhibitor cocktail and 1mM PMSF). The samples were centrifuged and the supernatant was collected for further analysis. Aliquots (100μL) of cell lysates were mixed with 100μL of 10mM HCl (the solvent of the iron standard $FeCl_3$), and 100μL of the iron-releasing reagent (a freshly mixed solution of equal volumes of 1.4M HCl and 4.5% (w/v) $KMnO_4$ in H_2O) for 2 hour at 60 °C. After the mixtures were cooled to room temperature, 30 μL of the iron-detection reagent (6.5mM ferrozine, 6.5mM neocuproine, 2.5M ammonium acetate, and 1M ascorbic acid dissolved in

water) was added. After 30 minutes, 250 μL of the solution was transferred into a well of a 96-well plate and the absorbance was measured at 550nm on a microplate reader.

Iron concentrations were estimated by comparing the absorbance of the sample to that of a known standard of $FeCl_3$ (mixture of 100 μL of $FeCl_3$ standards (0–300 μM) in 10mM HCl, 100 μL lysis buffer, 100 μL releasing reagent, and 30 μL detection reagent). The intracellular iron concentrations determined were normalized to the amount of protein in the sample.

2.15 Statistical Analysis

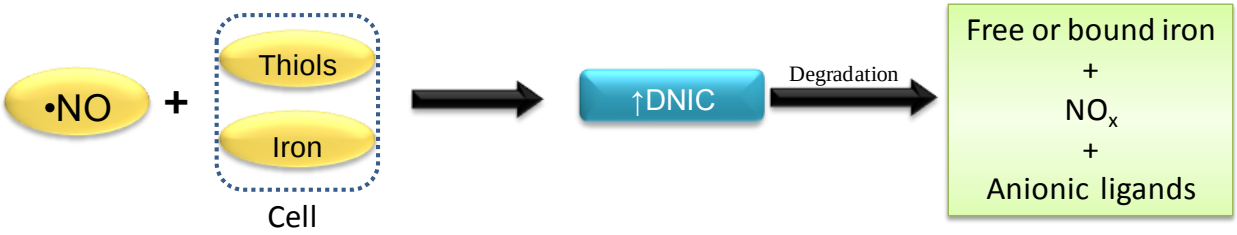
The data were reported as the Mean ± Standard Error of Mean (S.E.M.). One way ANOVA analysis with Fisher's LSD test was done using OriginPro8.1 software, OriginLab.

CHARTER 3

PHYSIO-CHEMICAL CHARACTERIZATION OF DINITROSYL IRON COMPLEXES (DNIC) IN CANCER CELLS

RATIONALE & HYPOTHESIS:

It is well known that DNIC are formed on exposure of •NO to the cells[33]. The concentration of •NO and duration of exposure required for DNIC formation has not yet been established in cancer cells. It is important to measure these parameters. We hypothesize that DNIC formation in cancer cells affect its phenotypic behavior. If this is to be true, DNIC would have to be stable inside the cells to have meaningful biological effects. To test this, we propose to study the DNIC stability inside cancer cells. Also, there are many different views on the source of iron for the formation of DNIC. Although, CIP and iron-sulfur clusters are believed to be the major source of iron, latest paper from *Toledo et al*, claims that DNIC are formed only from CIP in murine macrophages. We want to test the source of iron for DNIC formed in cancer cells, as it can be cell type specific.



Scheme 1 : Schematic representation of hypothesis for Chapter 3

RESULTS:

Total Cellular Iron

We determined total cellular iron in different cell lines (HCC 1806, HT29, MDA-MB-231 and RAW 264.7). Cell lysates were digested with acidic $KMnO_4$ solution to release iron from all intracellular stores. The lysates containing free released iron were then treated with iron detection reagent. Absorbance was measured at 550nm and compared to known iron standard.

We observed varying levels of total iron in different cell lines used. The iron levels varied from 8-20 nmoles/mg protein. (**Figure 10**)

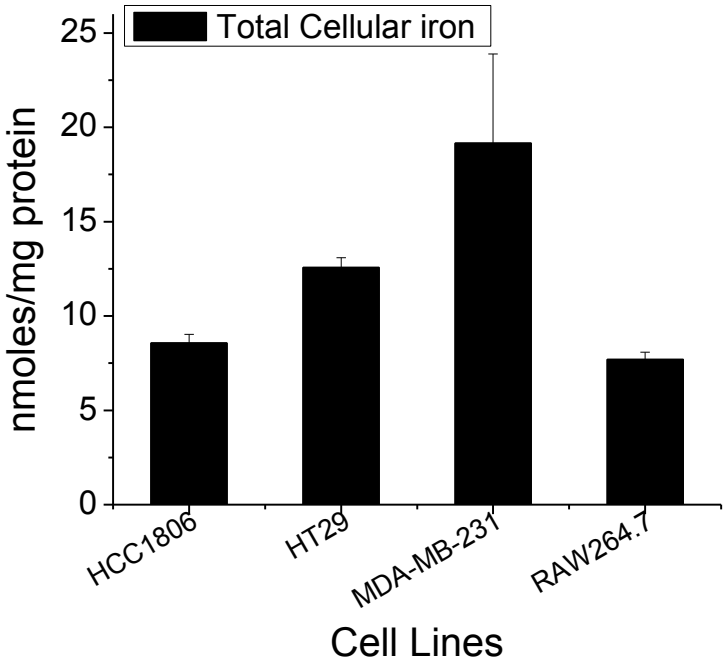


Figure 10 Total Cellular Iron levels: Comparison of total cellular iron levels in different cell lines under study. Data represents mean ± S.E.M (n=3)

Chelatable iron pool (CIP)

The intracellular CIP levels were measured using desferrioxamine (DFO). Iron chelator, DFO, only complex the CIP in the cell and this chelator-iron (DFO-Fe^{3+}) complex give an EPR signal at g=4.3. The amount of CIP was quantified using standard complex of $FeSO_4$ with DFO.

In **Figure 11**, we observed dose dependent linear increase in EPR signal with increasing amount of iron. The standard showed strong coefficient of correlation with r^2=0.9976. This standard curve was used in order to determine the levels of CIP.

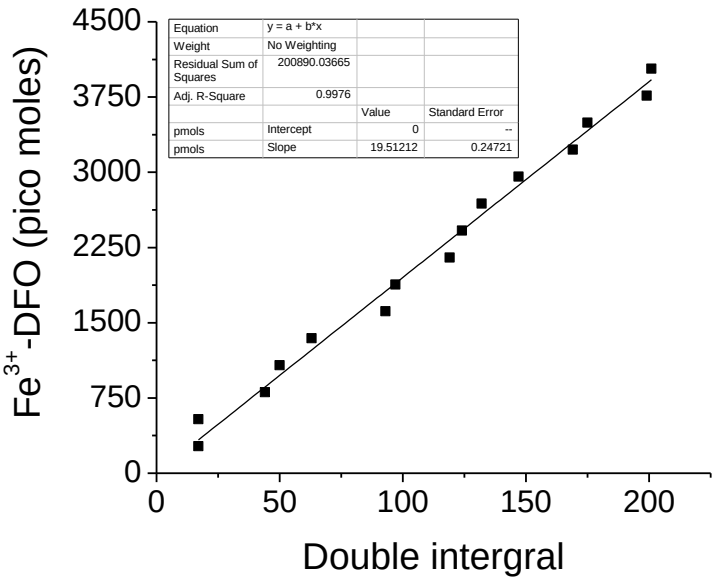


Figure 11 Iron-DFO standard: Plot showing increase in EPR signal (double integral) with increasing amount of Fe^{3+}-DFO (0-4000 pmoles) complex.

We observed varying levels of CIP in different cell lines (100-600 pmoles/mg protein) (**Figure 12**). Then, we calculated the percentage of the CIP compared to total cellular iron. We observed HCC 1806 and MDA-MB-231 cell lines have a similar percentage of the CIP (~1%). RAW 264.7 and HT-29 have much higher percentage of the CIP (~ 5%). (**TABLE V**)

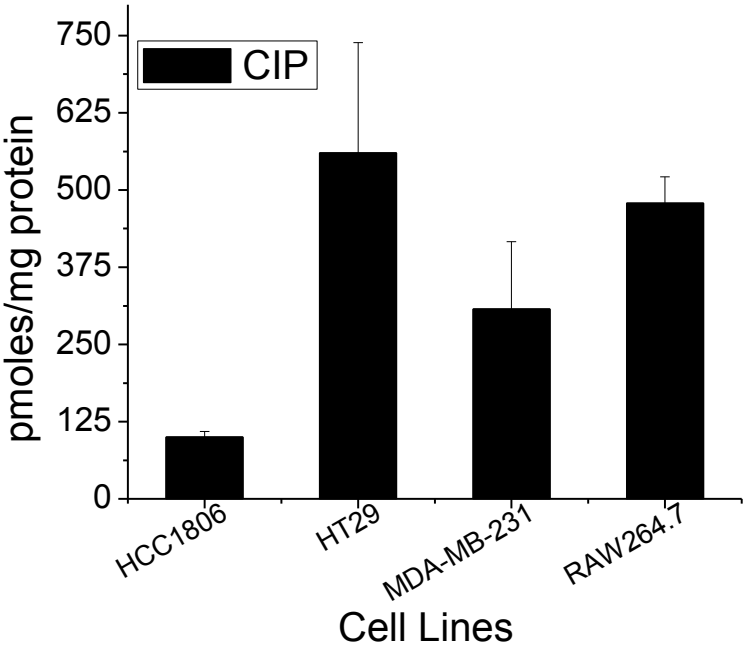


Figure 12 CIP Levels: Comparison of CIP levels in different cell lines under study. Data represents mean ± S.E.M (n=3)

Cell Lines	CIP (pmoles/mg protein)	Total Iron (nmoles/mg protein)	% CIP/iron
HCC 1806	100 ± 9.1	8.6 ± 0.46	1.2
HT 29	559.8 ± 178.6	12.6 ± 0.5	4.4
MDA-MB-231	307.1 ± 109	19.2 ± 4.7	1.6
RAW 264.7	478.5 ± 42.8	7.7 ± 0.37	6.21

TABLE V: COMPARISON OF PERCENTAGE OF CIP/TOTAL IRON IN DIFFERENT CELL LINES UNDER STUDY.

Synthetic Dinitrosyl diglutathione complex (GSH-DNIC) and its standard

In order to quantify DNIC, we synthesized small molecular weight glutathione DNIC (GSH-DNIC). The complex was synthesized under anaerobic conditions to have prolonged stability. We observed 70% yield compared to starting iron concentrations. The complexes give absorbance at λ_{max}=403nm with ε=3000.

In **Figure 13**, we observed dose dependent linear increase in EPR signal with increasing amount of GSH-DNIC. The standard showed strong coefficient of correlation with r^2=0.99245. This standard curve was used to determine the levels of intracellular DNIC.

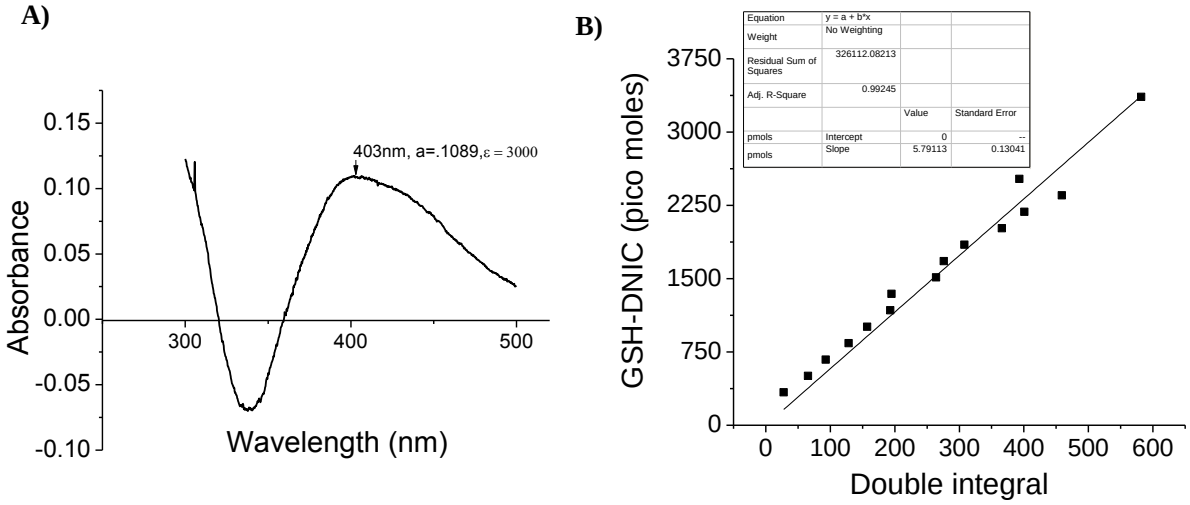


Figure 13 GSH-DNIC: A) UV-Visible absorbance spectra of GSH-DNIC. **B)** Standard plot of amount of GSH-DNIC vs EPR signal (double integral).

Time Course formation of DNIC

DNIC formation was tested in HCC 1806 breast cancer cells. We treated the cells with a high concentration (1mM) of •NO-donor DETA/NO and harvested the cells at various time points (0-12 hours) for EPR analysis.

Significant amount of DNIC were formed within 1 hour of exposure to DETA/NO. There was a slight increase in DNIC formation from 1-4 hour(s), but no increase was observed after that. The amount of DNIC formed were 3-4 folds higher than the observed CIP levels in HCC 1806 cells. (**Figure 14**)

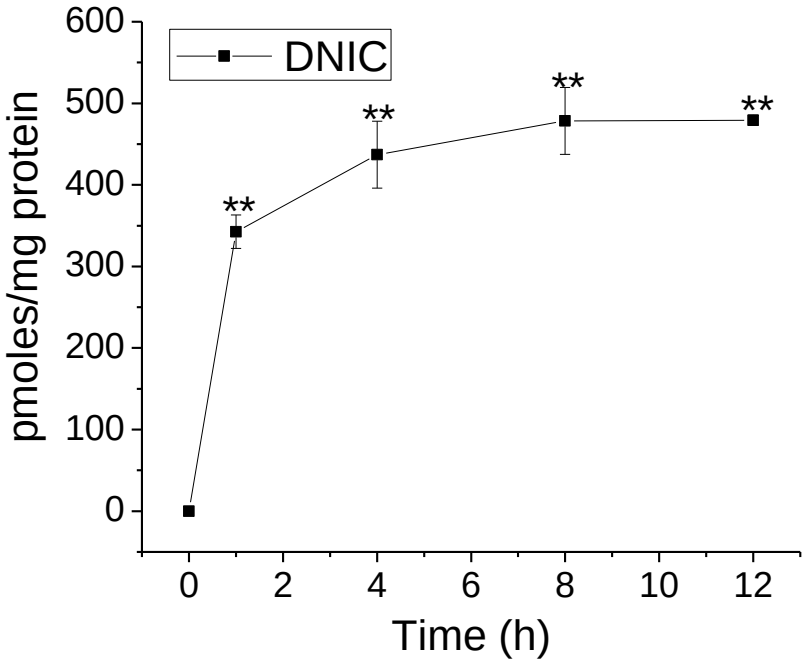


Figure 14 Time Course formation of DNIC. HCC 1806 cells were treated with DETA/NO (1mM) for indicated time points. The cells were harvested and DNIC were measured using EPR analysis. The amount of DNIC is normalized to total amount of protein in the sample. **,$p<0.01$ with respect to untreated control. 100pmoles/mg protein ~ 5-10μM DNIC samples.

Dose Response formation of DNIC

After establishing the time course for DNIC formation, we wanted to determine the dose of nitric oxide required for DNIC formation. We treated HCC 1806 cells with increasing concentration of DETA/NO (0.5-1mM) and harvested the cells for DNIC analysis after 4hours.

DNIC levels start to increase in a concentration dependent manner on treatment with DETA/NO in HCC 1806 cells. Maximal effect was observed at around 1mM DETA/NO, with much higher DNIC compared to the CIP. The DNIC levels were close to the CIP levels on treatment with a lower dose of DETA/NO (500μM). (**Figure 15**)

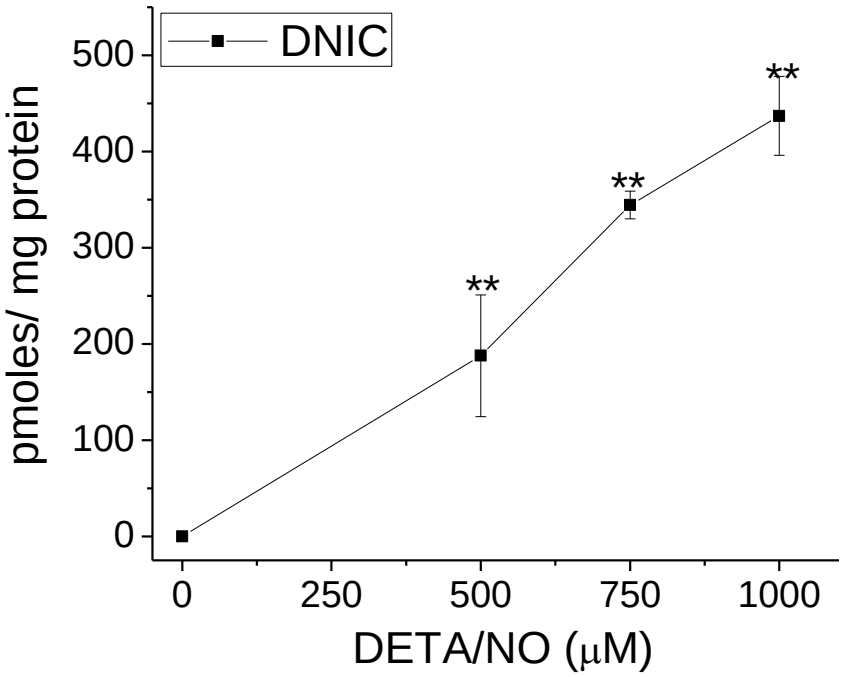


Figure 15 Dose Response formation of DNIC. HCC 1806 cells were treated with DETA/NO (0-1mM) for 4 hours. The cells were harvested and DNIC were measured using EPR analysis. The amount of DNIC is normalized to total amount of protein in the sample. **,$p<0.01$ with respect to untreated control. 100pmoles/mg protein ~ 5-10μM DNIC samples.

Stability of intracellular DNIC

We wanted to test the stability of DNIC after their formation. We treated different cancer cell lines (HCC 1806, MDA-MB-231, HT-29) with Sper/NO (1mM) for 1 hour followed by removal of •NO source by washing and supplementation with fresh growth media. Samples were harvested for DNIC analysis at various time points after •NO removal.

We observed that DNIC were stable for at least 2 hours after removal of •NO source in all cell lines. The half-life of DNIC formed was around 1 hour for all cell lines examined. (**Figure 16**)

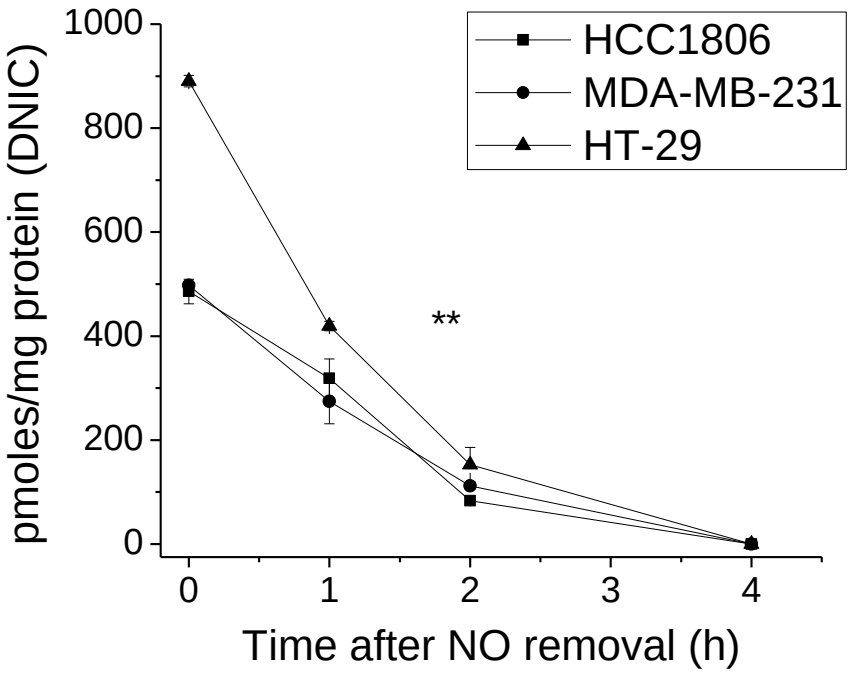


Figure 16 Stability of DNIC. EPR analysis of HCC1806, MDA-MB-231, HT-29 cells treated with •NO-donor Sper/NO (1mM) for 1 hour**.** Cell were washed and supplemented with fresh media. The time points indicate time after removal of •NO source. **,$p<0.01$ with respect to DNIC levels at 0 hour time point for respective cell lines. 100pmoles/mg protein ~ 5-10µM DNIC samples.

Endogenous DNIC formation in macrophages

Next we studied the formation of DNIC on exposure to endogenous •NO. The RAW 264.7 macrophage cells were infected with lipopolysaccharide (LPS, 1μg/ml) in order to induce inducible-nitric oxide synthase (iNOS) expression.

As oxygen is substrate for iNOS, we also varied oxygen levels in order to observe the effect of it on iNOS induction and overall •NO production. We observed time dependent increase in iNOS induction on treatment with LPS. Also, we observed higher level of iNOS induction at lower oxygen concentrations. (**Figure 17**)

In spite of higher iNOS levels at lower oxygen concentration we observed much less •NO production under these conditions. Nitrite is a stable end product of •NO metabolism and can be used as an indirect measure of total •NO production. The nitrite (proportional to total •NO) production increases with an increase in oxygen levels till 1-8%, after which it almost plateaus. (**Figure 17**)

We also measured DNIC in activated RAW 264.7 cells. In contrast to total •NO production, we observed much higher levels of DNIC at lower oxygen concentrations. The levels of DNIC observed were 2.5 fold higher in case of 1% oxygen compared to 21%. (**Figure 17**)

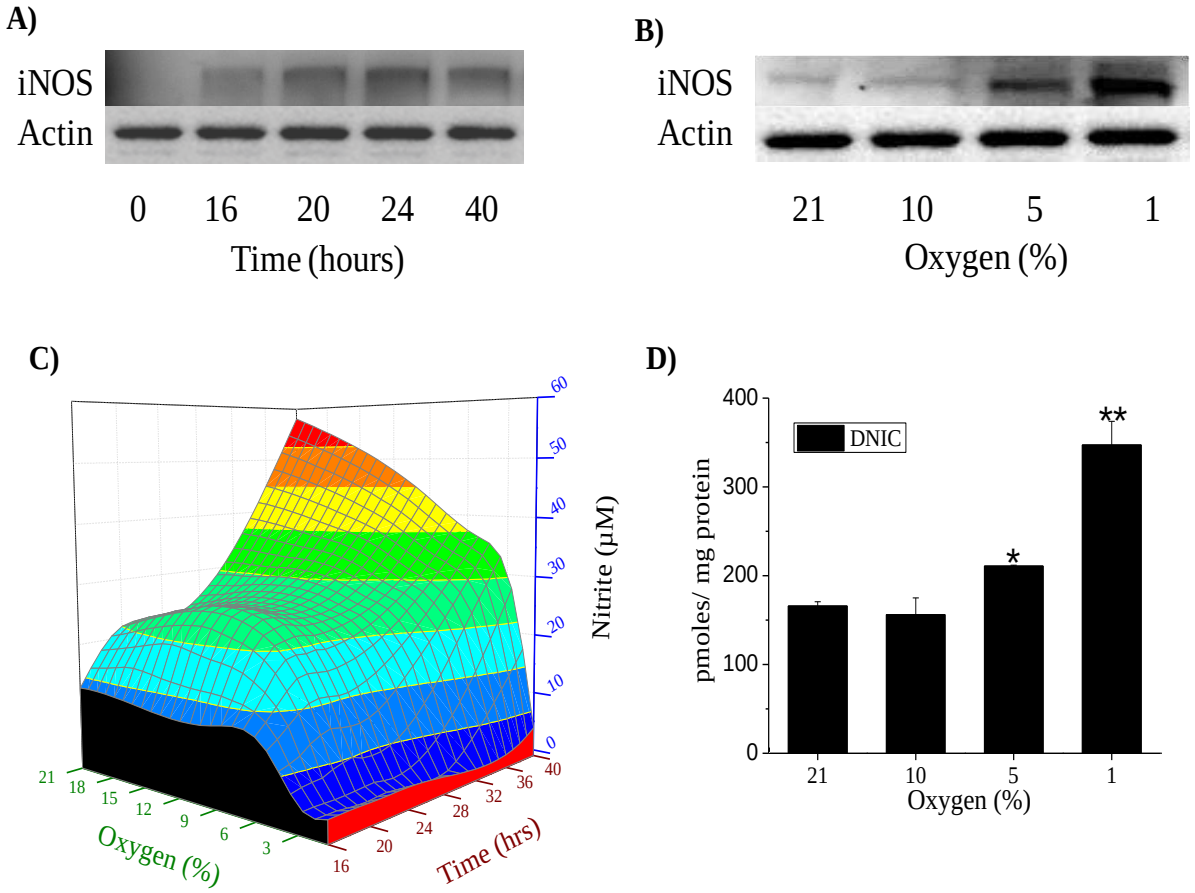


Figure 17 Endogenous •NO production and DNIC measurement: RAW 264.7 murine macrophage cells were treated with LPS (1μg/ml) for indicated time points. **A)** Western blot of iNOS at indicated time points after LPS treatment under normoxia (21% oxygen). **B)** Western blot of iNOS at different oxygen concentration after LPS treatment for 24 hours. **C)** Total amount of •NO produced (proportional of nitrite) at different time points and oxygen concentrations after LPS treatment. **D)** Measurement of DNIC levels at different oxygen concentrations after 24 hours of LPS treatment. *,$p<0.05$; **,$p<0.01$ with respect to 21% oxygen sample.

Next, we checked the effect of changing oxygen concentration on production of nitric oxide and DNIC formation. RAW 264.7 cells were treated with LPS at 1% and 21% oxygen concentration for 16 hours and cells were then switched from 1% to 21% and 21% to 1% for next 8 hours. The samples were collected for nitrite and DNIC analysis.

We observed higher levels of DNIC in LPS treated cells which were initially kept at 1% compared to 21%, although levels of nitrate/nitrite were lower under these conditions. (**Figure 18**)

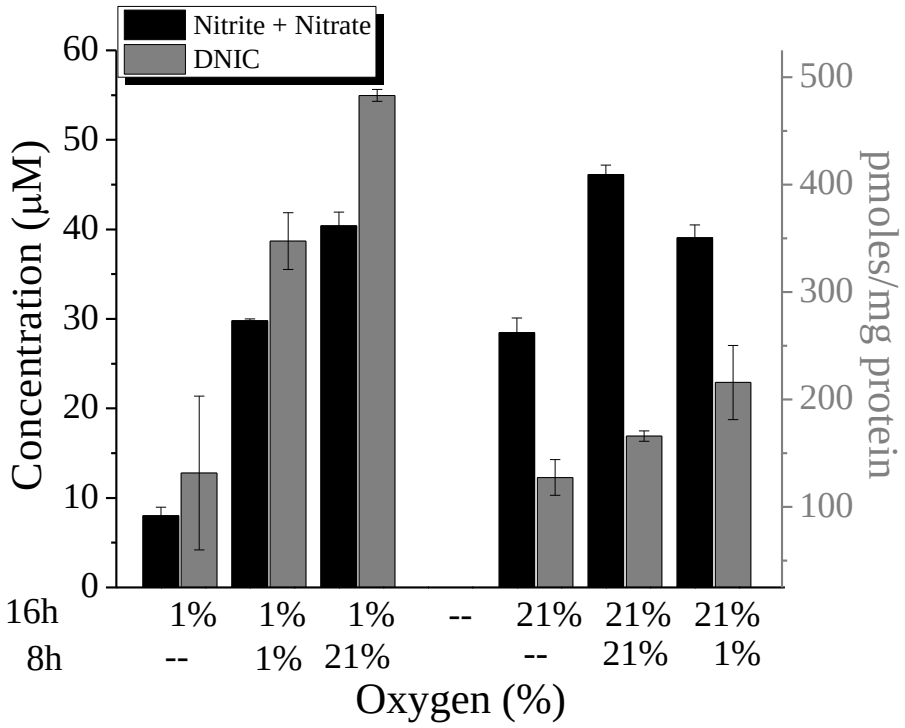


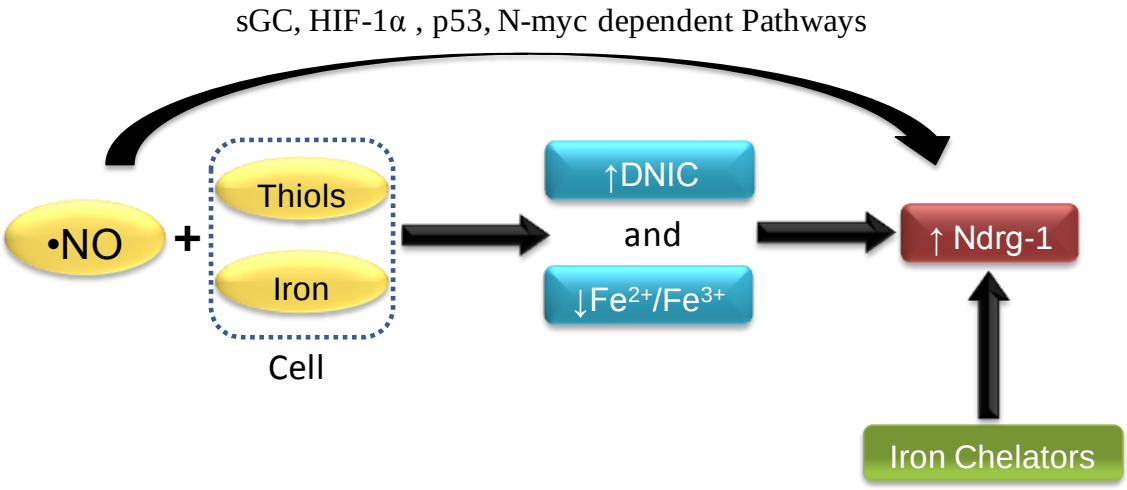
Figure 18 Effect of changing oxygen concentration on: Nitrate/Nitrite concentration (left panel) in media and DNIC production inside cells (right panel). RAW 264.7 cells were infected with LPS and incubated for at different oxygen concentrations (primary incubation of 16h followed by secondary incubation of 8h). Media was collected for measurement of Nitrate/Nitrite and cells were collected for DNIC measurement by EPR. Data represent mean ± S.E.M (n=3)

CHARTER 4

NITRIC OXIDE UP-REGULATES METASTATIC SUPPRESSOR GENE NDRG1

RATIONALE & HYPOTHESIS:

Inside the cell, one of primary interaction of nitric oxide occurs with iron and thiols leading to the formation of dinitrosyl iron complexes (DNIC)[33]. Formation of these complexes renders iron inactive towards various physiological functions it performs. We observed formation of these paramagnetic complexes on exposure of nitric oxide to cancer cells. Thus, •NO, due to its ability to form DNIC, behaves as an intracellular iron sequestering agent. Recently N-myc downstream regulated gene 1 (NDRG1), a well known metastasis suppressor gene, has shown to be up-regulated by iron chelation and HIF-1α stabilization[31,74]. We also observed HIF-1α stabilization in different cancer cells (HCC 1806, MDA-MB-231 cell lines) on exposure to •NO. Given the similarities between NDRG1 regulation and actions of •NO in cancer cells, we hypothesize that **exposure of nitric oxide to cells results in NDRG1 up-regulation, in an iron dependent manner**.



Scheme 2: Schematic representation of hypothesis for Chapter 4

RESULTS:

Time Course study of NDRG1 up-regulation

NDRG1 up-regulation was tested in HCC 1806 and MDA-MB-231 breast cancer cells. We treated the cells with a high concentration (1mM) of •NO-donor DETA/NO and harvested the cells at various time points for protein and mRNA analysis.

NDRG1 mRNA levels were measured using qRT-PCR analysis. We observed that mRNA levels start to increase by 4-6 hours in both HCC 1806 and MDA-MB-231 cell lines. Significant up-regulation was observed after 8 hours of treatment, with maximal effect at 24hours. Moreover, deactivated DETA/NO control (DC) did not show any considerable increase in mRNA levels in 24hours. (**Figure 19**)

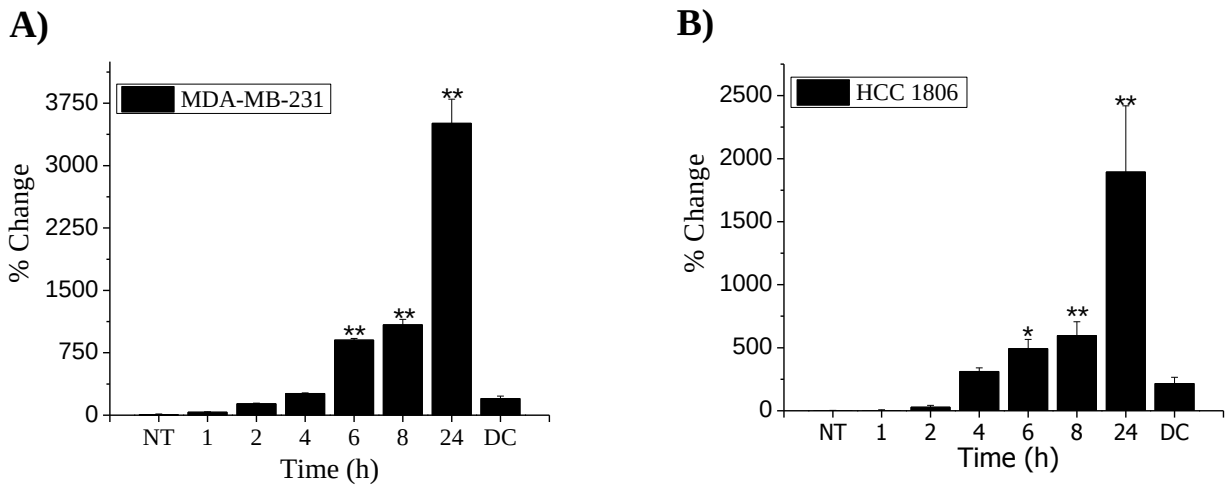


Figure 19: Time Course study of NDRG1 mRNA. qRT-PCR analysis of NDRG1 mRNA in MDA-MB-231(**A**) or HCC1806 (**B**) cells treated with •NO (1mM DETA/NO) for different time points (0-24 hours). NT= no treatment, DC=deactivated control (1mM decomposed DETA/NO for 24 hours)**.** *,$p<0.05$;**,$p<0.01$ with respect to untreated controls which are set arbitrarily to 0% change. (n=12)

NDRG1 protein levels were measured by western blot analysis. Increase in protein level followed similar trends as mRNA. Up-regulation was observed around 6-8 hours after initial •NO exposure and it becomes maximal at 24hours. Deactivated DETA/NO control (DC) did not show any considerable increase in protein levels in 24hours. (**Figure 20**)

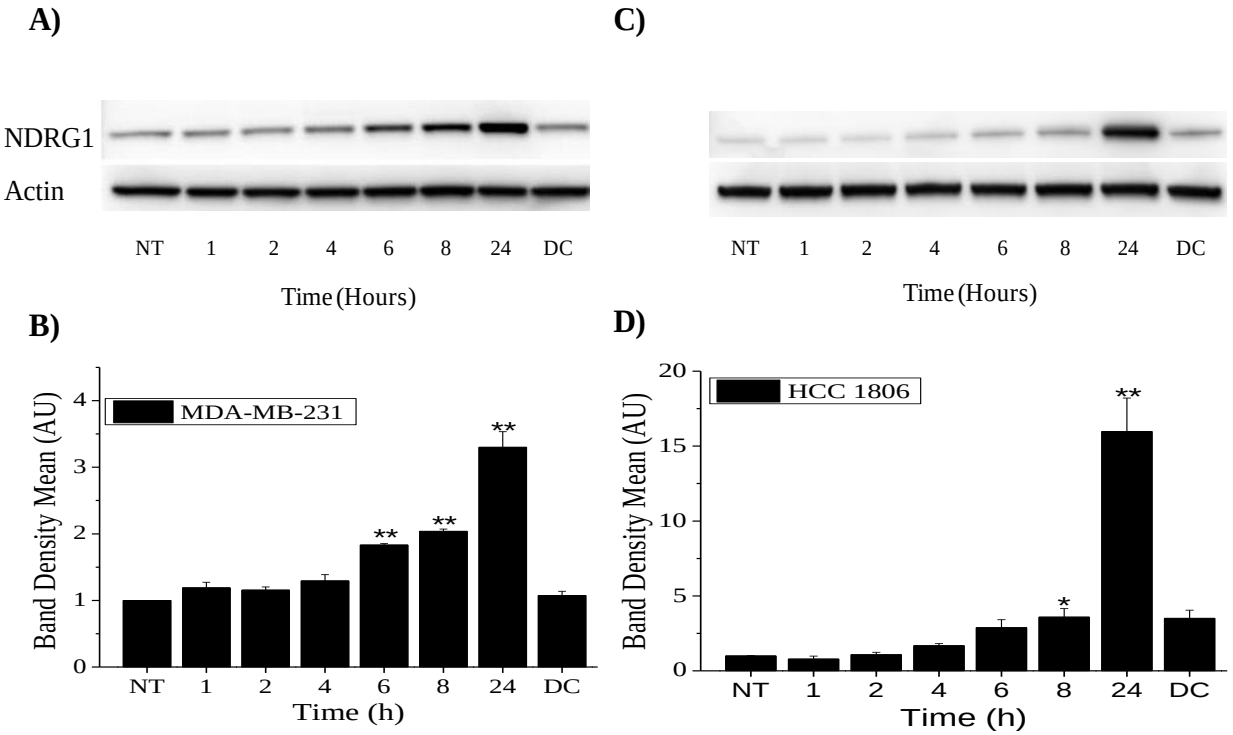


Figure 20 Time Course study of NDRG1 protein. Western blot and densiometric quantification for total NDRG1 protein in whole cell extract after time course (0-24h) of •NO treatment (1mM DETA/NO) in either MDA-MB-231(**A&B**) or HCC1806 (**C&D**) cells. NT= no treatment, DC=deactivated control (1mM decomposed DETA/NO for 24 hours)**.** *,$p<0.05$;**,$p<0.01$ with respect to untreated controls which are set arbitrarily to 1.0 . (n=3)

Top lane: NDRG1 protein, *bottom lane*: Actin (loading control).

Dose Response study of NDRG1 up-regulation

After establishing the time course for NDRG1 expression, we wanted to determine the dose of nitric oxide required to elicit the response. We treated HCC 1806 and MDA-MB-231cells with increasing concentration of DETA/NO (0-1mM) and harvested the cells for protein and mRNA analysis after 8hours.

NDRG1 mRNA levels start to increase with DETA/NO concentrations as low as 62.5 µM in MDA-MB-231 and 125 µM in HCC 1806 cells. Maximal effect was observed at around 500µM-1mM DETA/NO in both cell lines. (**Figure 21**)

Iron chelators are previously shown to up-regulate NDRG1. We also treated HCC 1806 cells with iron chelator HBED (200µM) in order to measure its effect in our cell lines. We observed similar up-regulation as obtained with maximal dose of DETA/NO. (**Figure 21**)

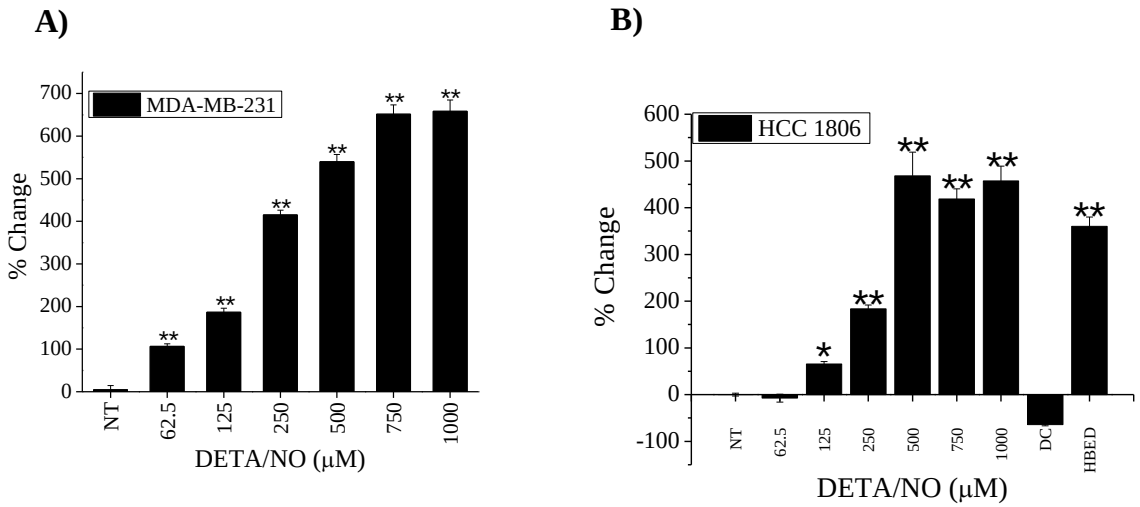


Figure 21 Dose Response study of NDRG1 mRNA. qRT-PCR analysis of NDRG1 mRNA in MDA-MB-231(**A**) or HCC1806 (**B**) cells treated with •NO donor DETA/NO (62.5-1000 µM) for 8 hours. NT= no treatment, DC= deactivated control (1mM decomposed DETA/NO for 8 hours), HBED= positive control (iron chelator HBED at 200µM for 8 hours)**.** *,$p<0.05$;**,$p<0.01$ with respect to untreated controls which are set arbitrarily to 0% change. (n=12)

We observed an increase in NDRG1 protein levels in a concentration dependent manner, with treatment as low as 125μM DETA/NO for both HCC 1806 and MDA-MB-231 cell lines. Maximum up-regulation occurred around 500-1000μM DETA/NO. Iron chelator HBED, which is used as a positive control, showed similar up-regulation for NDRG1 protein as maximal dose of DETA/NO. (**Figure 22**)

Deactivated DETA/NO (1mM decomposed DETA/NO for 8 hours) did not show any up-regulation in either NDRG1 or HIF-1α protein levels. (**Figure 22**)

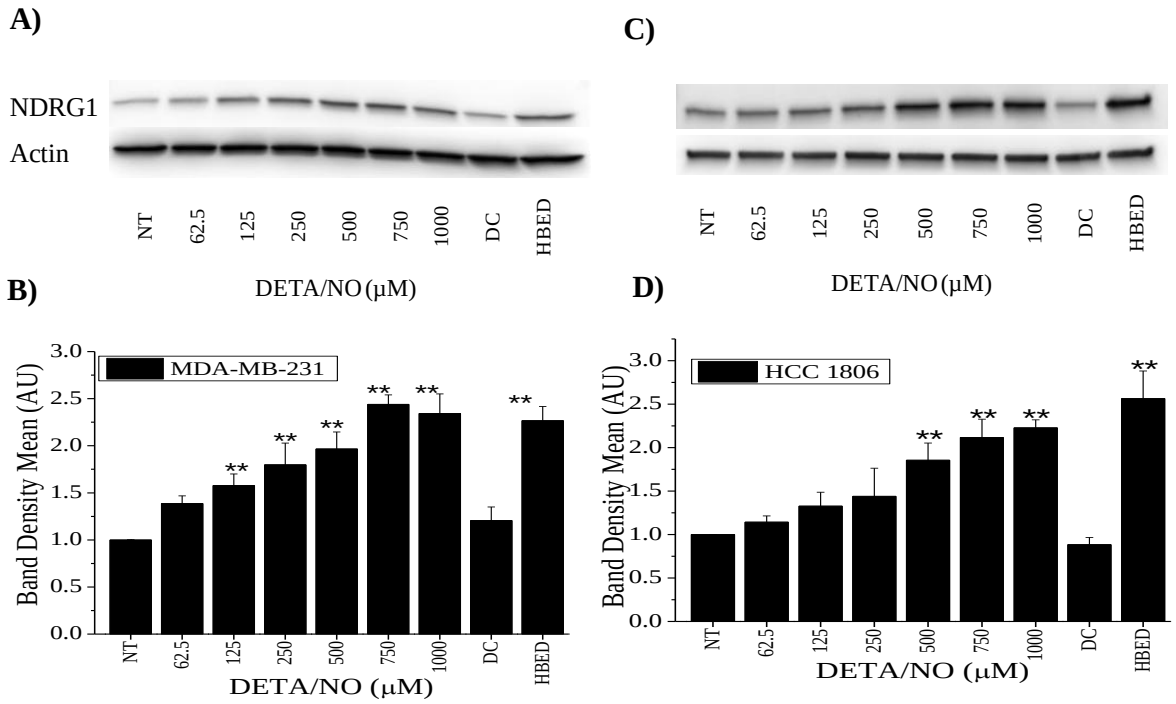
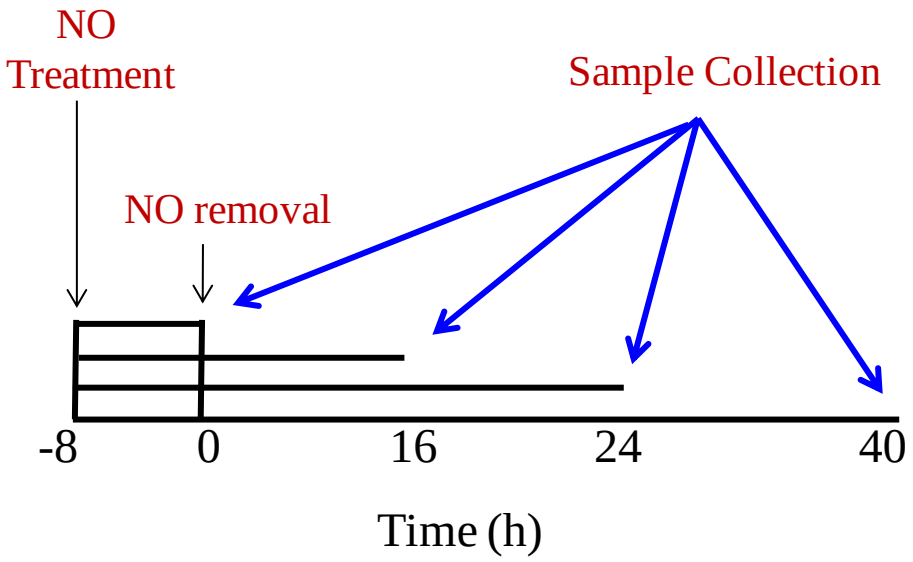


Figure 22 Dose Response study of NDRG1 protein. Western blot and densiometric quantification of total NDRG1 protein in whole cell extract after 8 hours of •NO treatment (62.5-1000 μM DETA/NO) in either MDA-MB-231(**A&B**) or HCC1806 (**C&D**) cells. NT= no treatment, DC= deactivated control (1mM decomposed DETA/NO for 8 hours), HBED= positive control (iron chelator HBED at 200μM for 8 hours)**.** *,$p<0.05$;**,$p<0.01$ with respect to untreated controls which are set arbitrarily to 1.0 . (n=3)

Top lane: NDRG1 protein, *bottom lane*: Actin (loading control).

Stability of NDRG1 gene products

We wanted to test the stability of NDRG1 gene products after •NO-mediated up-regulation. We treated cells with DETA/NO (500µM) for 8hours followed by removal of •NO source by washing and supplementation with fresh growth media. Samples were harvested for protein and mRNA analysis at various time points after treatment.



Scheme 3: Schematic representation of treatment and collection design of NDRG1 stability experiment.

NDRG1 mRNA showed no stability on removal of •NO source in both cell lines tested. We observed a significant decrease in up-regulation of NDRG1 mRNA levels after removal of •NO source for 16 hours, and it was back to endogenous levels by 40 hours. (**Figure 23**)

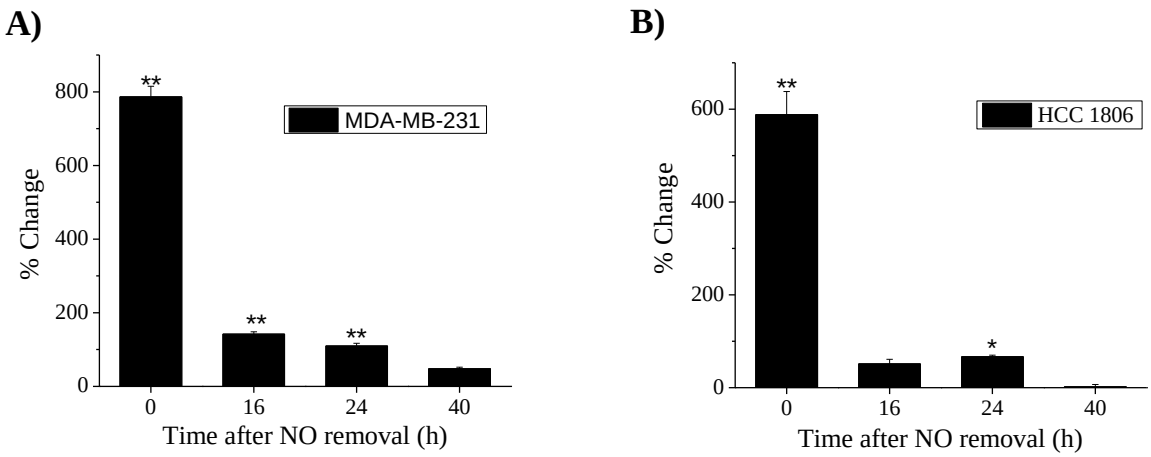


Figure 23 Stability of NDRG1 mRNA. qRT-PCR analysis of NDRG1 mRNA in either MDA-MB-231(A) or HCC1806 (B) cells. Cells were treated with •NO (500 μM DETA/NO for 8 hours). Cells were washed and supplemented with fresh media. The time points indicate time after removal of •NO source. Samples were collected 0-40 hours after removal of •NO source. Changes in mRNA are compared to no treatment control for each time point. *,$p<0.05$;**,$p<0.01$ with respect to untreated controls which are set arbitrarily to 0% change. (n=12)

On the other hand, we observed that, once expressed, NDRG1 proteins remains elevated for at least 40hours in both HCC 1806 and MDA-MB-231 cell lines. There was an increase in NDRG1 levels in no-treatment parallel control by 40 hours, possibly because of stress experienced by cells due to lack of growth factors and over confluence. Still, treated samples showed higher levels of NDRG1 protein compared to their respective no-treatment control. (**Figure 24**)

These results indicate that downstream phenotypic effects of NDRG1, such as inhibition of cell migration, may persist for days after treatment making it a therapeutically relevant target.

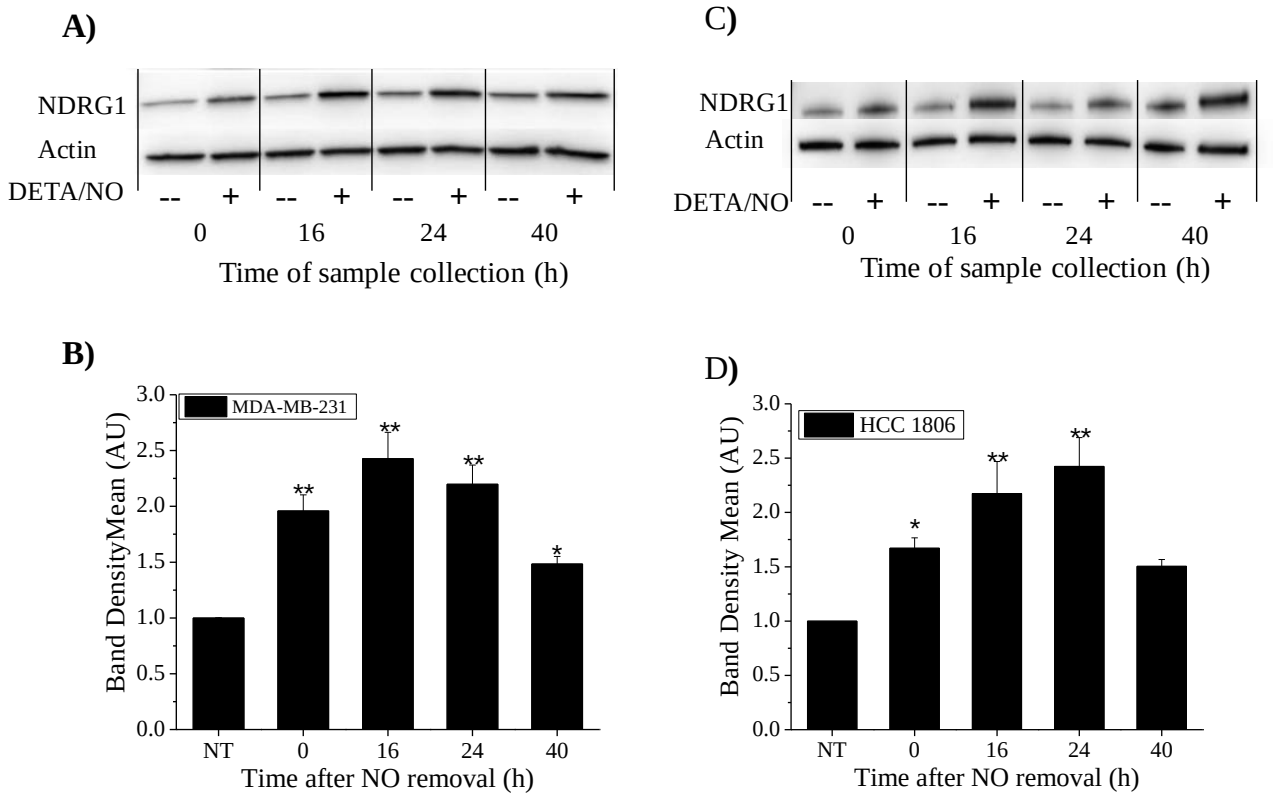
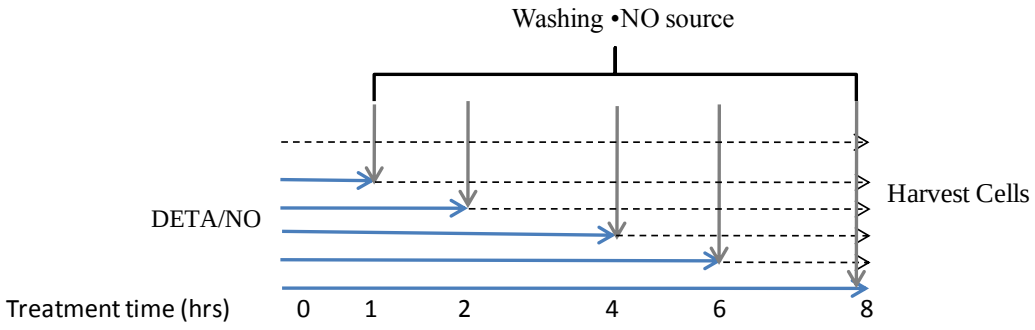


Figure 24 Stability of NDRG1 protein. Western blot and densiometric quantification of total NDRG1 protein in whole cell extracts from either MDA-MB-231(**A&B**) or HCC1806 (**C&D**) cells. Cells were treated with •NO (500 μM DETA/NO for 8 hours)**.** Cell were washed and supplemented with fresh media. The time points indicate time after removal of •NO source. Samples were collected 0-40 hours after removal of •NO source. Changes in protein are compared to no treatment control for each time point. *,$p<0.05$;**,$p<0.01$ with respect to untreated controls which are set arbitrarily to 1.0 . (n=3)

Top lane: NDRG1 protein, *bottom lane*: Actin (loading control).

Time dependent induction of NDRG1 protein

In Figure 20, we observed time dependent induction of NDRG1 protein, with a minimum of 6 hours of exposure to DETA/NO. We wanted to test whether this time is required for up-regulation of transcription/translation machinery after initial •NO exposure or a continuous treatment with •NO is required for induction of NDRG1. We compared the actual time after •NO exposure required for activation NDRG1 protein. The cells were treated with DETA/NO for the indicated time (1-8 hours) followed by removal of •NO source. Cells were supplemented with fresh serum free media till total of 8hours of treatment. Cells were then harvested for western blot analysis.



Scheme 4: Schematic representation of treatment and collection design for experiment to study time dependence in upregulation of NDRG1 by •NO.

NDRG1 protein was induced after 4 hours of exposure with DETA/NO in both HCC 1806 and MDA-MB-231 cell lines. There was time dependent increase from 4-8 hours with maximal effect at 8hr time point. (**Figure 25**)

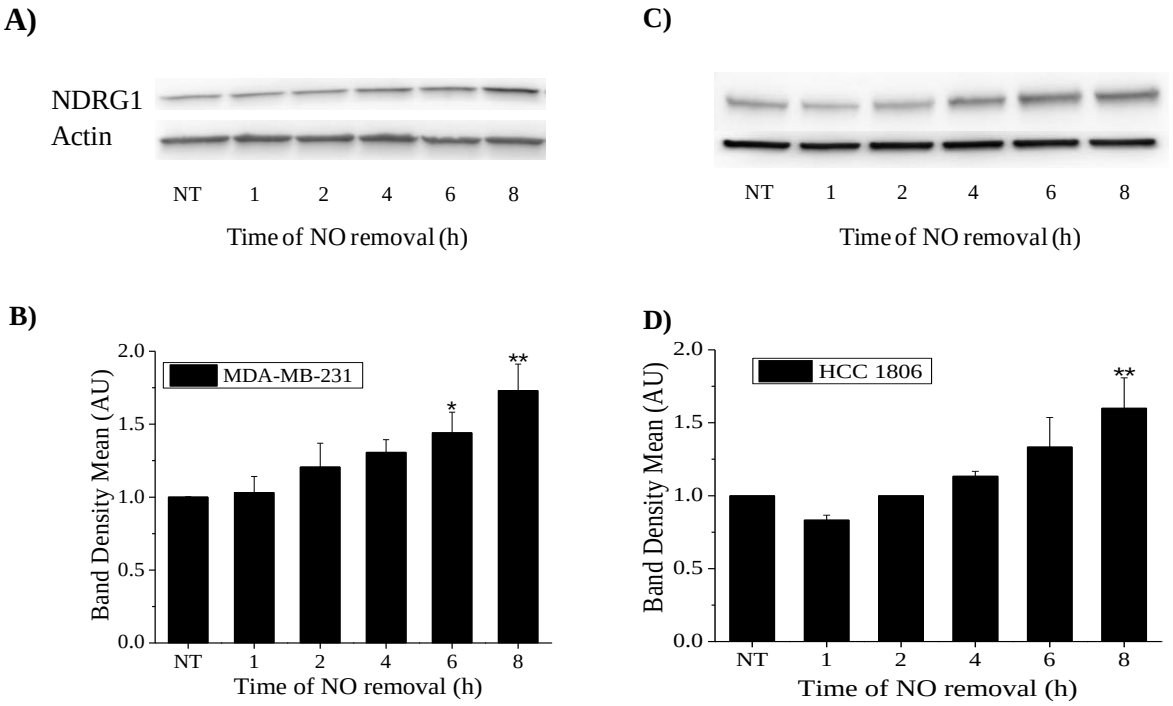


Figure 25 Time required for induction of NDRG1 protein after initial •NO exposure. MDA-MB-231(**A&B**) or HCC 1806 (**C&D**) cells were treated with •NO (500 µM DETA/NO) for indicated time points, followed by removal of •NO source and addition of serum free media. All samples were harvested for western blot analysis 8 hours after initial treatment. Western blot and densiometric quantification of total NDRG1 protein in whole cell extract.
*,$p<0.05$;**,$p<0.01$ with respect to untreated controls which are set arbitrarily to 1.0 . (n=3)

Potential Pathways responsible for •NO mediated NDRG1 up-regulation

Soluble guanylyl cyclase (sGC) pathway:

Many effects of •NO are mediated by its ability of activate soluble guanylyl cyclase (sGC) [161]. We wanted to determine the role of this protein in •NO-mediated NDRG1 up-regulation. We pretreated MDA-MB-231 and HCC 1806 cells with 1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ, 20µM; a chemical inhibitor of sGC[162]) for 30min followed by DETA/NO (500µM) for 8hours. No significant decrease in expression was observed on treatment with the inhibitor.

These results suggest that •NO mediated NDRG1 regulation is **independent of sGC-cGMP pathway**. (**Figure 26**)

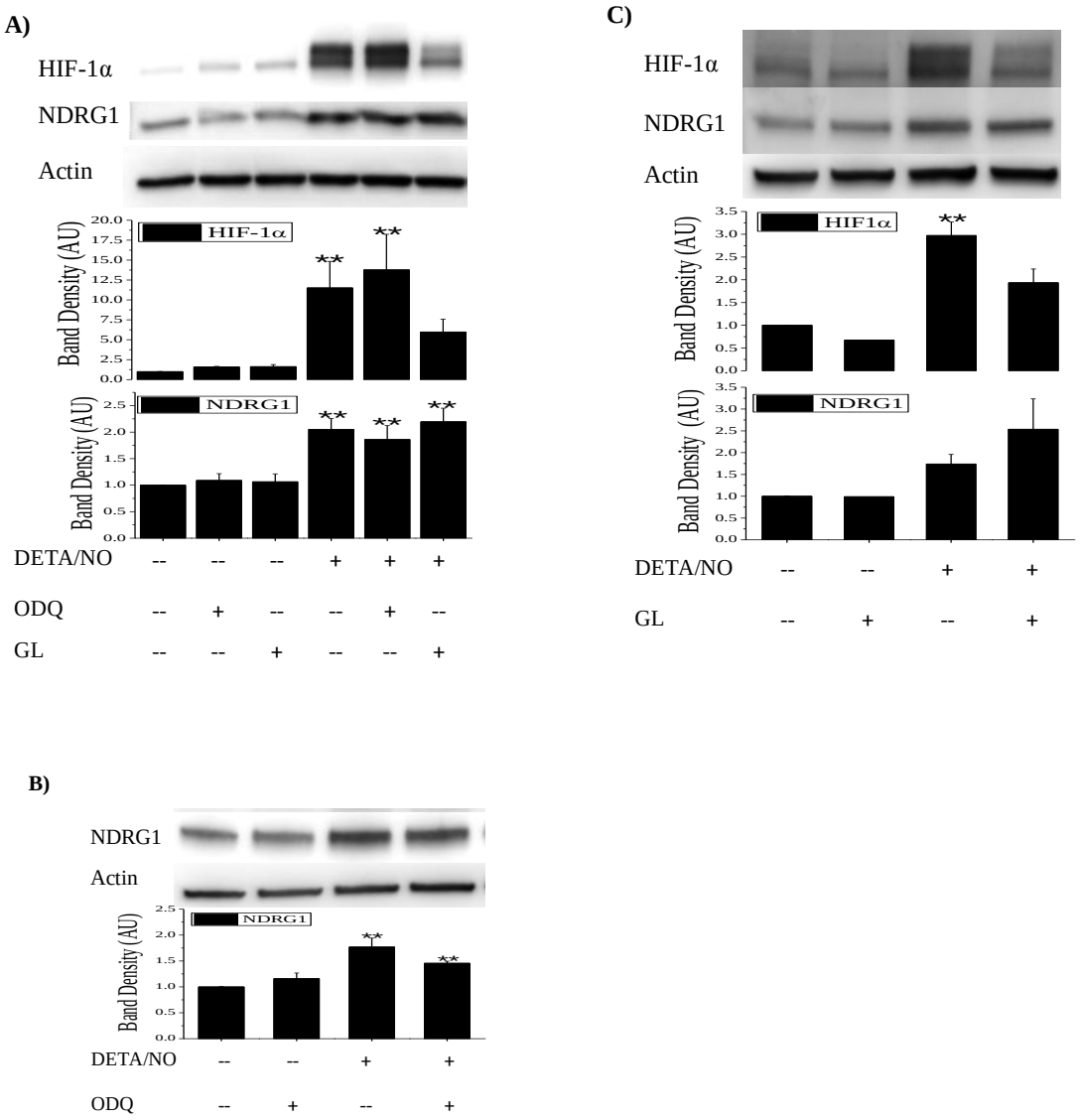


Figure 26: Western blot analysis demonstrating •NO up-regulates NDRG1 by a mechanism independent of sGC and HIF-1α A) MDA-MB-231 were pre-treated with either 1H-[1,2,4]Oxadiazole[4,3-a]quinoxalin-1-one (ODQ;20µM) or geldanamycin (GL;10µM) followed by •NO (500µM DETA/NO for 8 h). **B)** HCC 1806 cells were pre-treated with ODQ (20µM) followed by •NO (500µM DETA/NO for 8 h). **C)** HCC 1806 cells were pre-treated with GL (10µM) followed by •NO (500µM DETA/NO for 8 h). Western blot and densiometric quantification of total NDRG1and HIF-1α protein in whole cell extract. *,$p<0.05$;**,$p<0.01$ with respect to untreated controls which are set arbitrarily to 1.0 . (n=3)

In A) & C) *Top lane*: HIF-1α protein, *middle lane*: NDRG1 protein, *bottom lane*: Actin (loading control). In B) *Top lane*: NDRG1 protein, *bottom lane*: Actin (loading control)

HIF-1α dependent pathway:

There are reports that NDRG1 up-regulation by iron chelators is mediated by HIF-1α accumulation. •NO is also known to stabilize HIF-1α, so it might be possible that NDRG1 up-regulation is mediated through HIF-1α pathway. In order to determine the role of HIF-1α in •NO-mediated NDRG1 up-regulation, we pretreated both MDA-MB-231 and HCC 1806 cells with geldanamycin (GL, Hsp90 inhibitor, that has been known to induce rapid degradation of HIF-1α protein[163,164]) followed by DETA/NO (500μM). Although there was a significant decrease in HIF-1α expression in GL treated cells, no decrease in NDRG1 expression was observed. **(Figure 26)**

In order to ascertain the results obtained from inhibitor experiments we performed specific knock-down studies using either siRNA or shRNA against HIF-1α mRNA.

MDA-MB-231 cells were treated with either control or HIF-1α specific siRNA. We observed almost 80% reduction in HIF-1α stabilization on DETA/NO treatment. Further, after exposure to •NO there was no significant decrease in NDRG1 levels in cells treated with HIF-1α siRNA compared to no-treatment. Control siRNA, which is a scrambled sequence control, did not affect either HIF-1α or NDRG1 protein levels. (**Figure 27**)

HCC 1806 cells were transfected with stable shRNA against HIF-1α and NDRG1 in order to knock it down. We call this HIF-1α knockdown as HCC 1806/H2 and NDRG1 knockdown as HCC 1806/N1. Wild type, HCC 1806/N1 and HCC 1806/H2 cells were treated with •NO-donor DETA/NO. There was almost 80% reduction in HIF-1α stabilization in HCC 1806/H2 cells compared to wild type, on DETA/NO treatment. Moreover, there was no significant decrease in NDRG1 levels in HCC 1806/H2 compared to wild type cells, on DETA/NO treatment. In

NDRG1 knockdown cells, HIF-1α protein was still strongly up-regulated in response to •NO, whereas NDRG1 protein, as expected, was undetectable. (**Figure 27**)

All the results obtained from inhibitor as well as HIF-1α knock-down studies indicate towards HIF-1α independent mechanism for up-regulation of NDRG1 by nitric oxide.

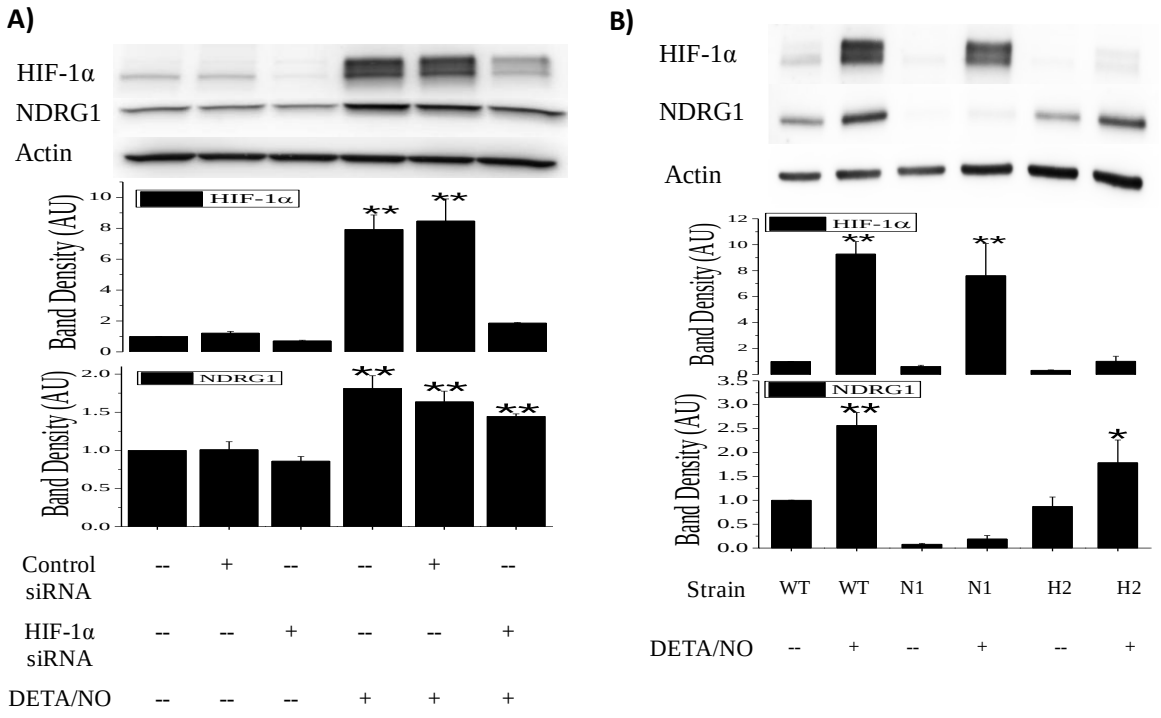


Figure 27 HIF-1α knock-down studies: A) MDA-MB-231 cells treated with either control or HIF-1α specific siRNA. After knockdown is achieved cells were treated with •NO (500μM DETA/NO for 8h). **B)** HCC 1806, HCC 1806/N1 and HCC 1806/H2 cells were treated with •NO (500μM DETA/NO for 8h). WT= HCC 1806, N1= HCC 1806/NDRG1 knockdown, H2= HCC 1806/HIF-1α knockdown cells. Western blot and densiometric quantification of total NDRG1and HIF-1α protein in whole cell extract. *,$p<0.05$;**,$p<0.01$ with respect to untreated controls which are set arbitrarily to 1.0 . (n=3)

Top lane: HIF-1α protein, *middle lane*: NDRG1 protein, *bottom lane*: Actin (loading control).

p53 dependent pathway:

p53 is a known tumor suppressor gene. Earlier reports have shown both p53 dependent as well as independent pathways in regulation of NDRG1. Nitric oxide is also known to activate p53 dependent pathways. So it was important to determine the role of p53 in the current study. HCC 1806 cell line is p53 null, thus the mechanism involving role of p53 in NDRG1 up-regulation was also ruled out[165]. Moreover, MDA-MB-231 cells are known to be p53 mutant and thus do not contribute via this mechanism[166].

MYC dependent pathway:

In deregulated conditions Myc proteins act as oncoproteins. Both, NDRG1 namesake N-myc (Neuroblastoma derived myelocytomatosis) as well as c-Myc is known to downregulate NDRG1 expression. Therefore, it was important to consider Myc dependent pathway in •NO mediated up-regulation of NDRG1.

Breast cancer cell line (BCCL) project done by Berkley laboratories showed that N-myc is absent in both HCC 1806 as well as MDA-MB-231 cells. We also confirmed the absence of N-myc protein as well as mRNA in HCC 1806 cell line. No detectable endogenous levels of N-myc protein were observed in western blot analysis. Moreover, DETA/NO treatments do not induce N-myc levels in HCC 1806 cells. (**Figure 28**)

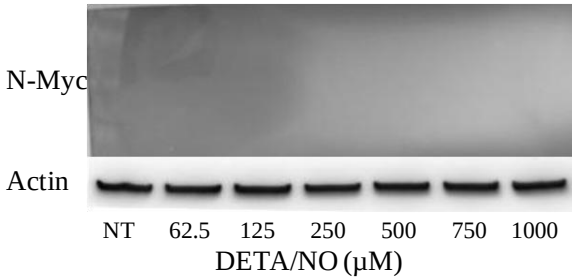


Figure 28 Role of N-myc in NDRG1 up-regulation. Western blot of HCC 1806 cells treated with increasing concentration of •NO (62.5-1000μM DETA/NO) for 8 hours.
Top lane: N-myc protein, *bottom lane*: Actin (loading control).

HCC 1806 cells treated with •NO-donor DETA/NO showed up-regulation of c-Myc at both protein as well as mRNA level. Since c-Myc is known to suppress NDRG1 levels, we concluded that •NO mediated up-regulation of NDRG1 occurs via some alternate mechanism. (**Figure 29**)

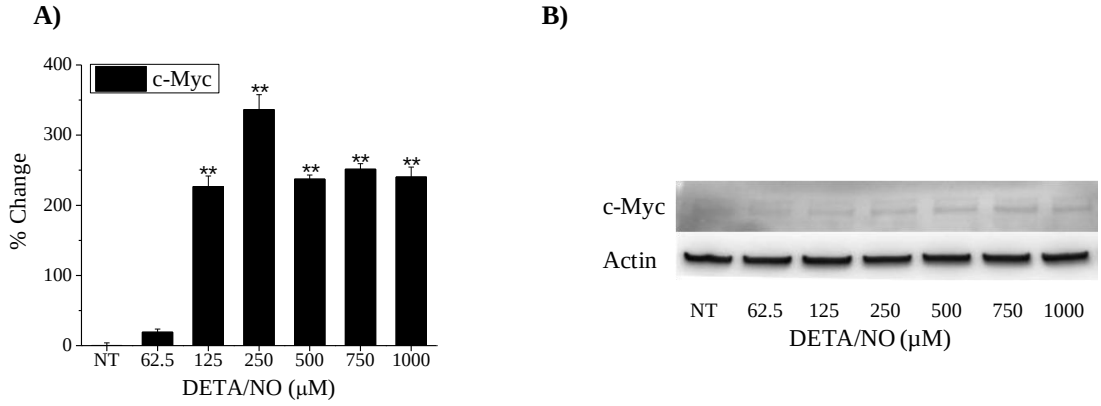


Figure 29 Role of c-Myc in NDRG1 up-regulation. A) qRT-PCR analysis of c-Myc mRNA in HCC 1806 cells treated with increasing concentration of •NO (62.5-1000μM DETA/NO) for 8 hours. **,$p<0.01$ with respect to untreated controls which are set arbitrarily to 0% change. (n=12) **B)** Western blot of c-Myc protein in HCC 1806 cells treated with increasing concentration of •NO (62.5-1000μM DETA/NO) for 8 hours.

Top lane: c-myc protein, *bottom lane*: Actin (loading control).

<u>Iron dependent pathway</u>:

The central theme of this proposal is that nitric oxide, by virtue of its property to form DNIC, acts similar to an iron chelator leading to NDRG1 up-regulation. We, therefore, wanted to see the role of iron in this up-regulation.

We measured the endogenous CIP levels in HCC 1806 cells. We also measured amount of DNIC formed after exposure to 500 μM DETA/NO for 8 hours. We observed DNIC levels to be similar to the CIP levels in HCC 1806 cells. These results indicate CIP to be major source of DNIC formation, under these cellular conditions. (**Figure 30**)

In order to determine the role of iron in •NO mediated up-regulation of NDRG1 we did iron supplementation studies. Ferric ammonium citrate (FAC, 150µg/ml) was used as an exogenous iron source. We first determined the effect of exogenous FAC supplementation on level of the chelatable iron pool (CIP). The HCC 1806 cells were incubated with FAC supplemented growth media for varying lengths of time (0-16 hours). There was time dependent increase in levels of the CIP on FAC supplementation. **(Figure 30)**

Next, we treated FAC supplemented cells with a •NO-donor DETA/NO (500µM for 8 hours) in order to measure the effect of iron supplementation on DNIC formation. There was an increase in DNIC levels with a parallel increase in the CIP levels. There was a significant increase in DNIC levels on iron supplementation for 16 hours. In all FAC supplemented cells treated with 500 µM DETA/NO, DNIC levels were always less compared to the elevated CIP levels. Only when cells were treated with higher concentration of DETA/NO (1mM for 8 hours) after 16 hours of FAC supplementation, we observed DNIC levels approaching the CIP values. (**Figure 30**)

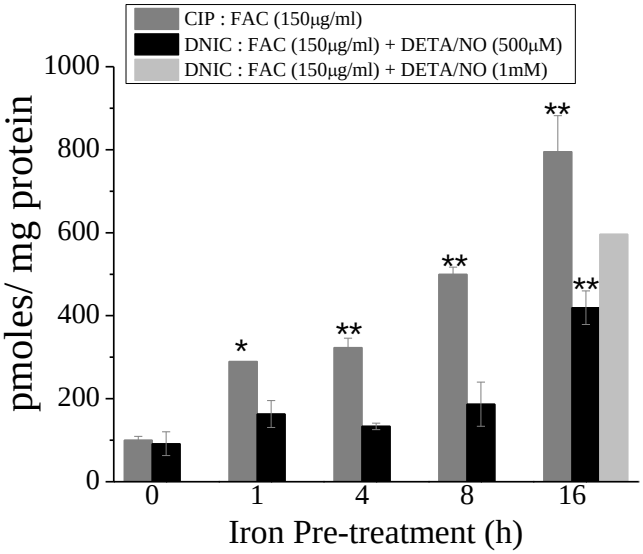


Figure 30: Iron supplementation. HCC 1806 cells were supplemented with ferric ammonium citrate (FAC, 150µg/ml) for indicated time points. CIP levels were determined 8 hours after FAC supplementation, on treatment with desferrioxamine (DFO, 1mM for 4 hours). DNIC levels were measured after treatment with DETA/NO for 8 hours. DNIC and CIP levels were measured using EPR spectroscopy. All values were normalized to amount of protein present in each sample. *,$p<0.05$;**,$p<0.01$ with respect to either CIP or DNIC untreated controls. ($n\geq3$)

Next, we determined the effect of this iron supplementation on nitric oxide mediated NDRG1 up-regulation. HCC 1806 cells were incubated with ferric ammonium citrate for the indicated time periods. The cells were then washed with DTPAC containing PBS to remove excess iron. The iron supplemented cells were then treated with •NO-donor DETA/NO (500 µM) for 8 hours. Cells were then harvested for protein and mRNA analysis.

There was time dependent decrease in NDRG1 mRNA levels. Decrease in NDRG1 mRNA was observed with as low as 1 hour of FAC supplementation, with maximal effect observed around 12-16 hours. Similarly, NDRG1 protein levels were also decreased in a time dependent manner on iron supplementation. These results show an inverse relationship between the CIP levels and NDRG1 levels in •NO treated HCC 1806 cells.

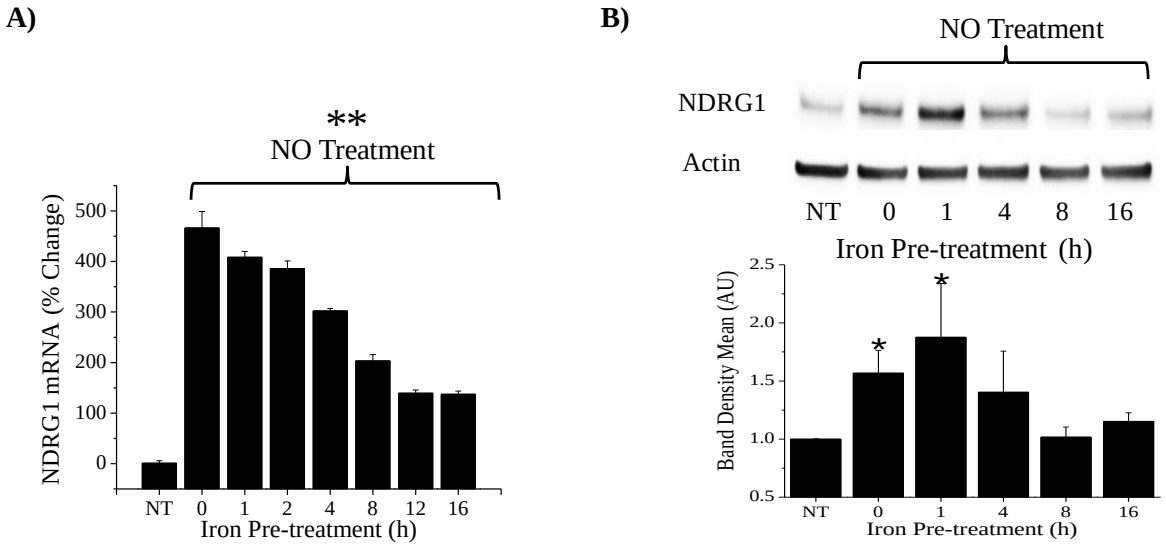


Figure 31 Effect of Iron Supplementation (Time Course) on NDRG1 mRNA and protein. HCC 1806 cells were incubated with FAC (150µg/ml) for indicated time points. The cells were then treated with •NO-donor DETA/NO (500 µM) for 8 hours. Samples were collected for mRNA and protein analysis. **A)** qRT-PCR analysis of NDRG1 mRNA. **B)** Western blot and densiometric quantification of total NDRG1 and HIF-1α proteins in whole cell extracts. NT= no treatment. *,$p<0.05$;**,$p<0.01$ with respect to untreated controls which are set arbitrarily to 0% change for mRNA and 1.0 for protein . (n=3)

*In **B)** Top lane*: NDRG1 protein, *bottom lane*: Actin (loading control).

We also measured the effect of iron supplementation on varying doses of DETA/NO. HCC 1806 cells were incubated +/- FAC for 16 hours, followed by treatment with increasing doses of DETA/NO for 8 hours. Samples were collected for mRNA analysis.

Iron supplementation of cells leads to decrease in endogenous levels of NDRG1. Moreover, on treatment with DETA/NO NDRG1 up-regulation is less in FAC supplemented cells compared to no supplementation at doses up to 750µM DETA/NO. Only at high dose of 1mM DETA/NO, at which we also observe DNIC levels approaching the CIP levels, we observed comparable up-regulation of NDRG1 mRNA. (**Figure 32**)

These results corroborate the hypothesis that nitric oxide mediated NDRG1 up-regulation at both mRNA and protein levels are dependent on the cellular CIP levels.

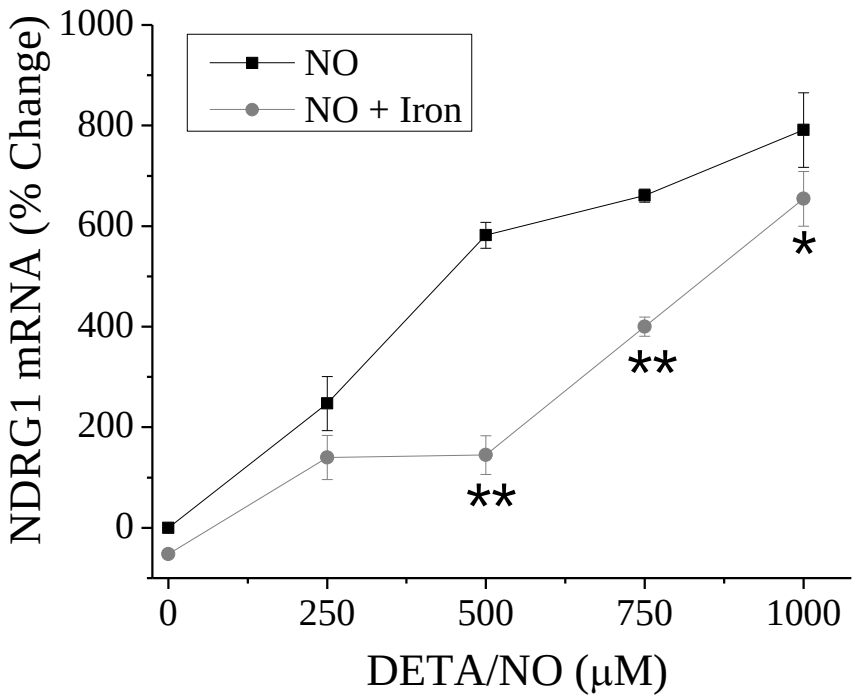


Figure 32 Effect of Iron Supplementation (DETA/NO Dose Response) on NDRG1 mRNA. HCC 1806 cells were incubated +/- FAC (150µg/ml) for 16 hours. The cells were then treated with increasing concentration of •NO-donor DETA/NO (0-1000 µM) for 8 hours. Samples were collected for qRT-PCR analysis of NDRG1 mRNA. *,$p<0.05$;**,$p<0.01$ indicate differences in iron supplemented samples with their dose matched controls. (n=12)

Other cell lines:

We also determined this •NO mediated NDRG1 up-regulation in a variety of other cancer cell lines. MCF-7 (non-metastatic breast cancer), HT-29 (Colon cancer), SKOV-3 (ovarian cancer) and SHSY5Y (neuroblastoma) cells were treated with •NO-donor DETA/NO (500µM) for 8 hours. Samples were collected either for protein or mRNA analysis.

All cell lines except SHSY5Y showed up-regulation of NDRG1 mRNA on treatment with DETA/NO. Interestingly, neuroblastoma cells have much higher expression of N-myc gene compared to other cell lines[167]. N-myc oncogene is known to be a suppressor of NDRG1. Therefore, N-myc might be responsible for suppressed NDRG1 levels in SHSY5Y cells. (**Figure 33**)

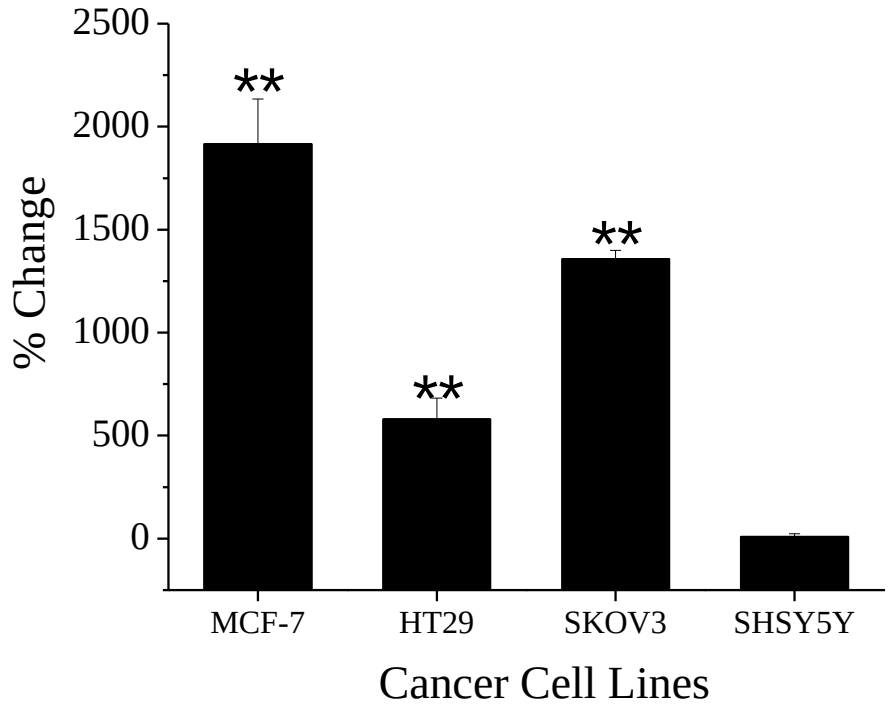


Figure 33 Up-regulation of NDRG1 mRNA in different cancer cell lines. MCF-7 (breast), HT-29 (Colon), SKOV-3 (ovarian) and SHSY5Y (brain) cancer cell lines were treated with DETA/NO (500 µM) for 8hours. qRT-PCR analysis of NDRG1 mRNA. *,$p<0.05$;**,$p<0.01$ with respect to individual untreated control. (n=9)

We observed similar results, as NDRG1 mRNA, for NDRG1 protein. There was up-regulation of NDRG1 protein in MCF-7, SKOV-3 and HT-29 cell lines, but not in SH-SY5Y cells. (**Figure 34**)

Together, these results show ubiquitous up-regulation of NDRG1 by nitric oxide in different cancer cell lines.

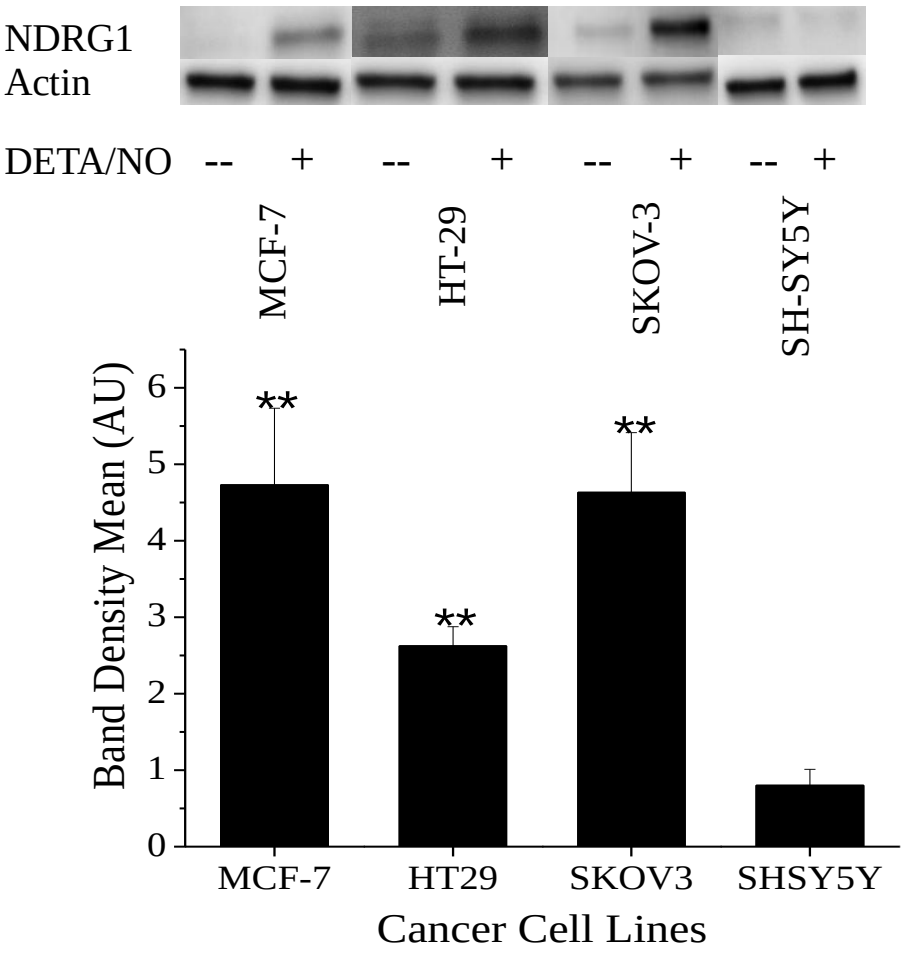


Figure 34 Up-regulation of NDRG1 protein in different cancer cell lines. MCF-7 (breast), HT-29 (Colon), SKOV-3 (ovarian) and SHSY5Y (brain) cancer cell lines were treated with DETA/NO (500 μM) for 8hours.Western blot and densiometric quantification of total NDRG1 protein. *,$p<0.05$;**,$p<0.01$ with respect to individual untreated control. (n=3)

Top lane: NDRG1 protein, *bottom lane*: Actin (loading control).

E-cadherin: known downstream target of NDRG1

Earlier reports have shown that E-cadherin is known to be up-regulated downstream of NDRG1. We wanted to ascertain the magnitude of E-cadherin up-regulation in our conditions. HCC 1806 cells were treated with increasing concentrations of DETA/NO (0-1000μM) for 8 hours. Cells were harvested for mRNA analysis.

The cells treated with DETA/NO do not show significant dose dependent induction of E-cadherin mRNA on •NO treatment. Significant increase in E-cadherin level was only observed at very high •NO concentration (1mM DETA/NO). (**Figure 35**)

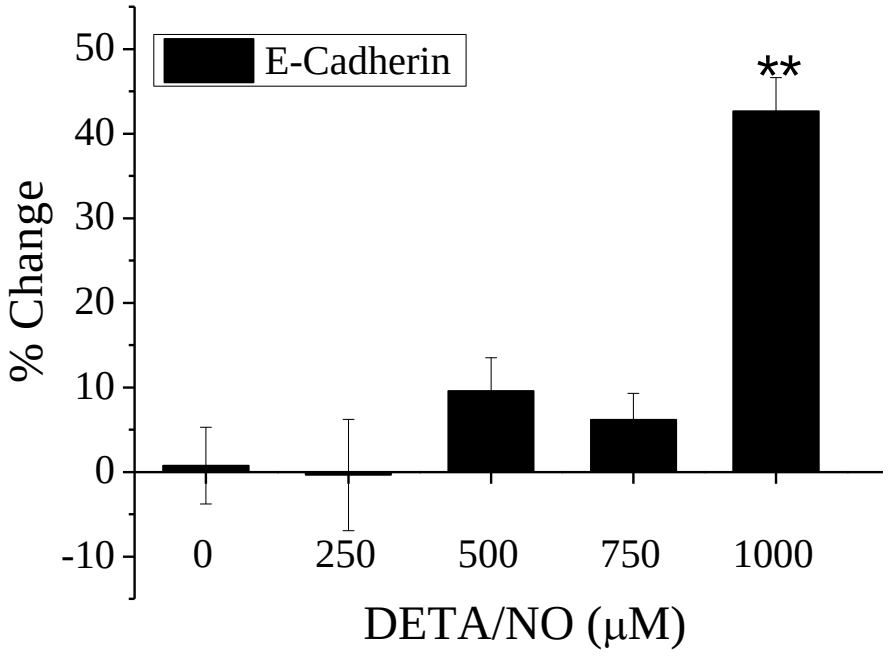


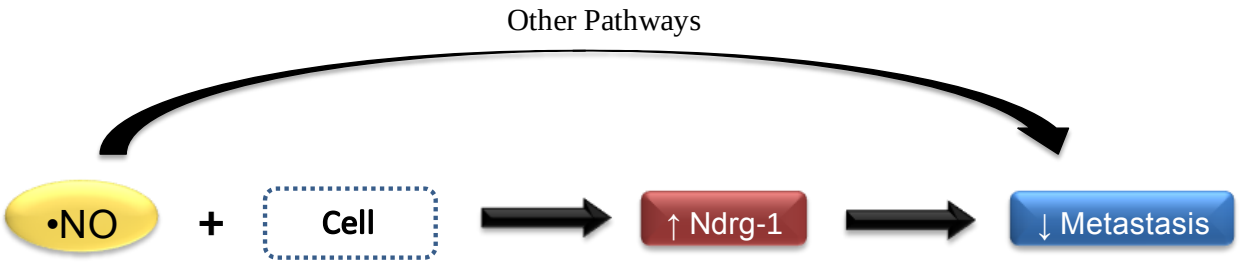
Figure 35 Dose Response Up-regulation of E-Cadherin mRNA. HCC 1806 cells were treated with increasing concentration of •NO-donor DETA/NO (0-1000 μM) for 8 hours. Samples were analysed by qRT-PCR for E-cadherin mRNA. *,p<.05;**,p<.01 with respect to untreated control. (n=9)

CHAPTER 5

EFFECT OF NITRIC OXIDE (•NO) ON TUMOR CELL MIGRATION

RATIONALE & HYPOTHESIS:

As discussed in the last chapter, •NO up-regulates NDRG1 gene in cancer cells by complexation of intracellular chelatable iron pool. As NDRG1 is a well known metastatic suppressor gene, we tested the ability of •NO to suppress tumor cell migration/invasion which is a critical element in metastasis process. It is also important to study the role of NDRG1 and HIF-1α in •NO mediated effects on cell migration. Moreover, effect of different •NO donors on tumor cell migration is also studied.



Scheme 5: Schematic representation of hypothesis for Chapter 5

RESULTS:

xCELLigence® DP system

To measure cancer cell migration/invasion and cytotoxicity we used an innovative new technology, the xCELLigence® system RTCA DP (Roche Diagnostics), which measures impedance-based signals to monitor real-time cell viability and cell migration/invasion. The system utilizes specially designed plate for cell viability (E-plate) and invasion/migration (CIM-plate). **Figure 36** describes the principle of impedance based measurement of cell growth/migration[168,169].

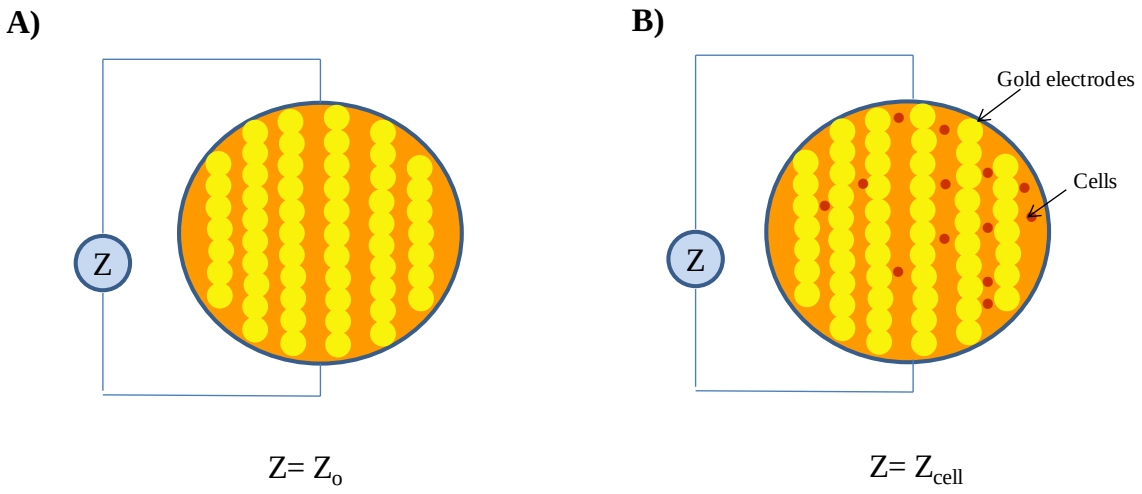


Figure 36 Principle of Impedance based xCELLigence RTCA DP system[168,169]. A) Background impedance (Z_o) of the media is measured before addition of cells. **B)** Impedance (Z_{cell}) is measured after addition of cells. Impedance increases with increase in number of cells. Change in impedance (Z_{cell} - Z_o) is converted into a function known as Cell Index. Gold Electrode arrays are placed either on bottom of plate (E-plate) or underside of membrane (CIM-plate).

E-plates are similar to regular 96-well tissue culture plate, but instead have 16 wells and gold electrode arrays at the bottom of each well for measuring impedance across plated cells. Change in impedance is converted to a function of cell growth/accumulation known as **CELL INDEX**[168].

We tested the ability of this technology to monitor cell growth. We cultured HCC 1806 cells at different densities. We observed that rate of cell growth/accumulation (cell index) is proportional to the starting number of plated cells (**Figure 37**). Wells plated with 1 x 10^4 cells showed most linear growth curve. Thus, we selected this density for future assays.

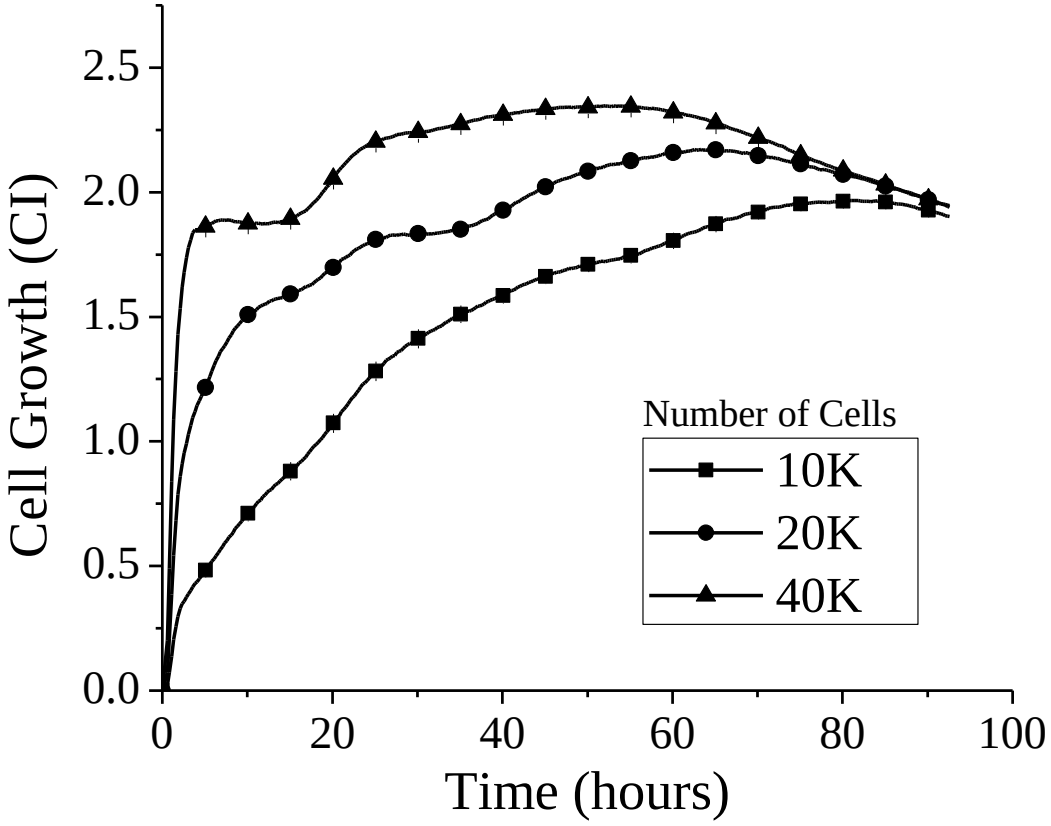


Figure 37 Validation of E-plate. HCC 1806 were plated in E-plate 16 at different starting cell densities (1-4 x 10^4 cells per well). Cell index (CI) was recorded for entire length of experiment (every 20 minutes) after initial background read.

CIM-plates are similar to transwell-chambers and measure movement of cells from an upper chamber to a lower chamber across the microporous polyethylene terephthalate (PET) membrane (pore size 8µm). Microfabricated gold electrode arrays are placed on the underside of the membrane to measure number of cells crossing the membrane[170]. Lower chamber usually contains a migration stimulant (10% FBS in our case).

We measured the ability of the system to differentiate migratory ability of HCC 1806 cells seeded with different number of cells (1-4 x 10^4 cells per well). We observed differential migratory ability based on seeding densities of cells. Wells plated with more number of cells showed higher migration (**Figure 38**). We selected cell density of 2 x 10^4 cells per cell for future assays. Moreover, difference between serum stimulated and non-stimulated cells was profoundly observed.

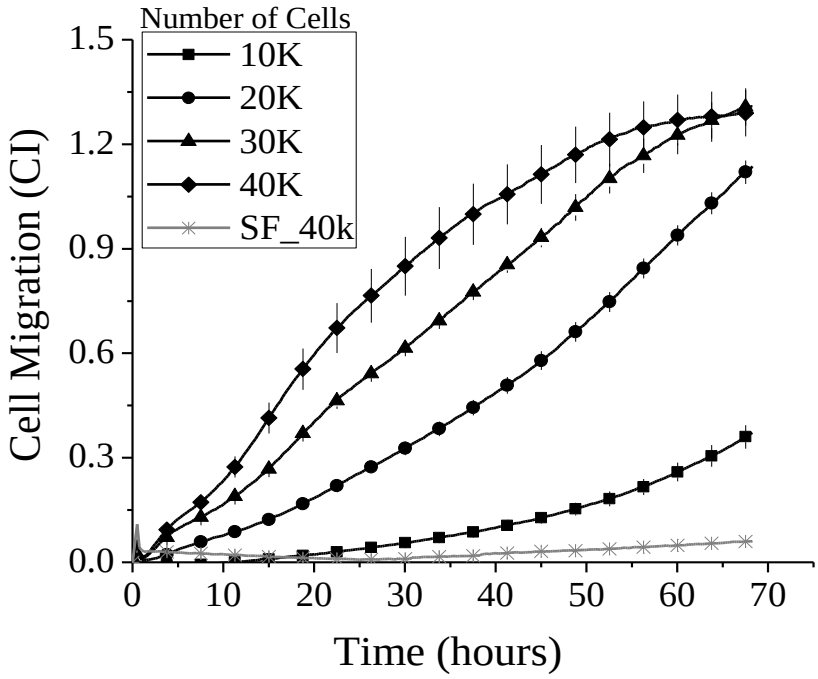


Figure 38 Validation of CIM-plate. HCC 1806 were plated in CIM-plate at different starting cell densities (1-4 x 10^4 cells per well) +/- 10% FBS in bottom well. Cell index (CI) was recorded for entire length of the experiment (every 20 minutes) after initial background read.

Effect of •NO on cellular migration

We tested the ability of •NO-donor DETA/NO on HCC 1806 cell migration. Migratory stimulant (10% FBS) was added to the bottom chamber followed by the addition of cells in serum free media in the top chamber. Six hours after initial seeding of cells in top chamber they were treated with increasing concentrations of DETA/NO. Migration of cells was measured across microporous membrane on DP system for 80 hours.

We observed 60-80% inhibition in migration in cells treated with 125-250 µM DETA/NO (**Figure 39**). This correlates well with NDRG1 protein up-regulation, which also occurs at about the same concentration. There was no observed suppression effect at low dose of DETA/NO (62.5 µM). Deactivated DETA/NO control (DC) does not show considerable inhibition in migration (data not shown).

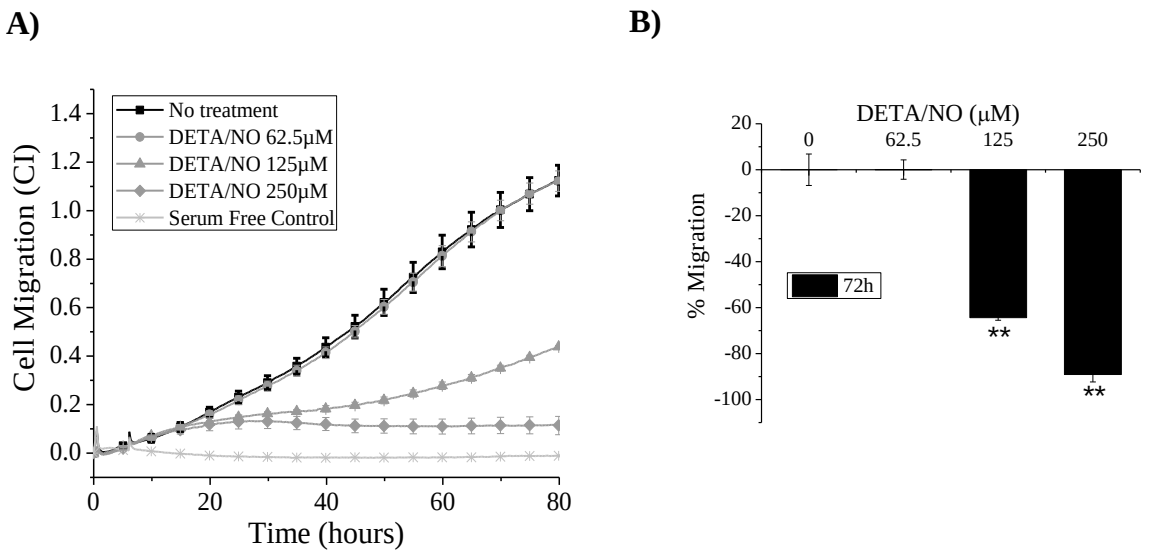


Figure 39 Effect of •NO on cellular migration. A) HCC 1806 cells (2 x 10^4 cells/well) were seeded in top chamber of CIM-plate. Measurement of migration (CI) on treatment with increasing concentration of •NO-donor DETA/NO (62.5-250µM) for entire length of experiment (every 20 minutes) .**B)** Percentage inhibition of migration on DETA/NO treatment compared to non treated cells after 72hours.**,$p<0.01$ with respect to untreated control. ($n\geq3$)

Role of NDRG1 and HIF-1α in •NO mediated migration suppression

In Chapter 4, we have shown nitric oxide mediated up-regulation of NDRG1 in HCC 1806 cells. NDRG1 is a known metastatic suppressor gene. We hypothesized that up-regulation of NDRG1 might be responsible for •NO mediated suppression of migration. Moreover, •NO also leads to stabilization of HIF-1α and thus activating HIF-1 responsive genes. HIF-1 is known to play an important role in tumor metastasis. Therefore, we also studied the role of HIF-1 in •NO mediated migration suppression.

In order to determine the role of NDRG1 and HIF-1α, we used HCC 1806 cell lines stably transfected with shRNA specific for either NDRG1 or HIF-1α. We named these cell lines as HCC1806/N1 and HCC1806/H2 for NDRG1 and HIF-1α knockdown respectively. First, we checked the efficiency of knockdown achieved by western blot analysis. There was no NDRG1 level detected +/- DETA/NO in HCC1806/N1 cells, but HIF-1α stabilization was observed on •NO treatment (**Figure 27**). Similarly, HCC1806/H2 cells showed no HIF-1α stabilization on •NO treatment, but NDRG1 up-regulation was observed (**Figure 27**).

We observed a significant increase in migratory ability of N1 cells compared to wild type HCC 1806 cells (**Figure 40**). This indicates importance of endogenous NDRG1 levels as a metastatic suppressor. We do not observe any significant change in migratory ability of H2 cells compared to wild type HCC 1806 cells (**Figure 40**). It was expected as HIF-1α is not stable under normoxic conditions and there is no HIF-1α protein present in normoxia. Therefore, knocking endogenous levels of HIF-1α at the mRNA level should not change phenotypic outcome (cell migration).

On treatment with •NO-donor DETA/NO (250 μM) we observed 25% decrease in migratory ability of N1 cells, 72 hours after the treatment. At the same time point, wild type HCC 1806 cells showed approximately 65% decrease in migration. Thus, it can be concluded that NDRG1 accounts for approximately 60% of •NO-mediated migration suppression. (**Figure 40**)

We do not observe any significant change in migration ability of H2 cells on •NO treatment (**Figure 40**). This was counter-intuitive as NDRG1 levels are up-regulated in H2 cells on •NO treatment. It also shows the importance of HIF-1α in •NO-mediated suppression of migration. We concluded that, HIF-1α levels are necessary for suppression of migration, but NDRG1 significantly potentiates this suppressive effect.

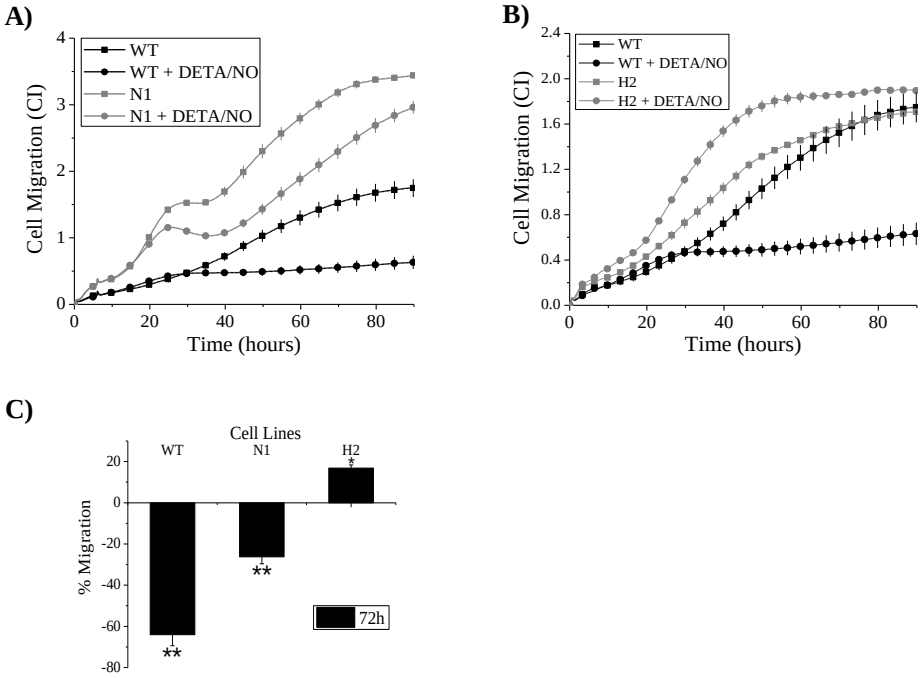


Figure 40: Role of NDRG1 and HIF-1. A) Measurement of migration (CI) on treatment of HCC1806 or HCC 1806/N1 cells with DETA/NO (250μM) for the entire length of experiment (every 20 minutes). **B) M**easurement of migration (CI) on treatment of HCC1806 or HCC1806/ H2 cells with DETA/NO (250μM) for the entire length of experiment (every 20 minutes). **C)** Percentage inhibition of migration on DETA/NO treatment compared to respective non treated cells in different cell lines after 72hours. *,$p<0.05$;**,$p<0.01$ with respect to individual untreated control. ($n\geq3$). WT = HCC 1806, N1= HCC 1806/NDRG1 knockdown, H2= HCC 1806/HIF-1α knockdown cells.

It was important to study the effect of nitric oxide on cell viability at concentrations at which we observe suppression in migration. If DETA/NO was cytotoxic at concentrations used, it will obviously decrease migration independent of anti-metastatic signaling. We treated all cell lines with •NO-donor DETA/NO under similar conditions as migration experiment.

We observed slight cytotoxic/cytostatic effects in HCC 1806 wild type cell lines, but not as significant as inhibition of migration. There was no decrease in cell growth kinetics in both N1 and H2 cell lines on treatment with DETA/NO. **(Figure 41)**

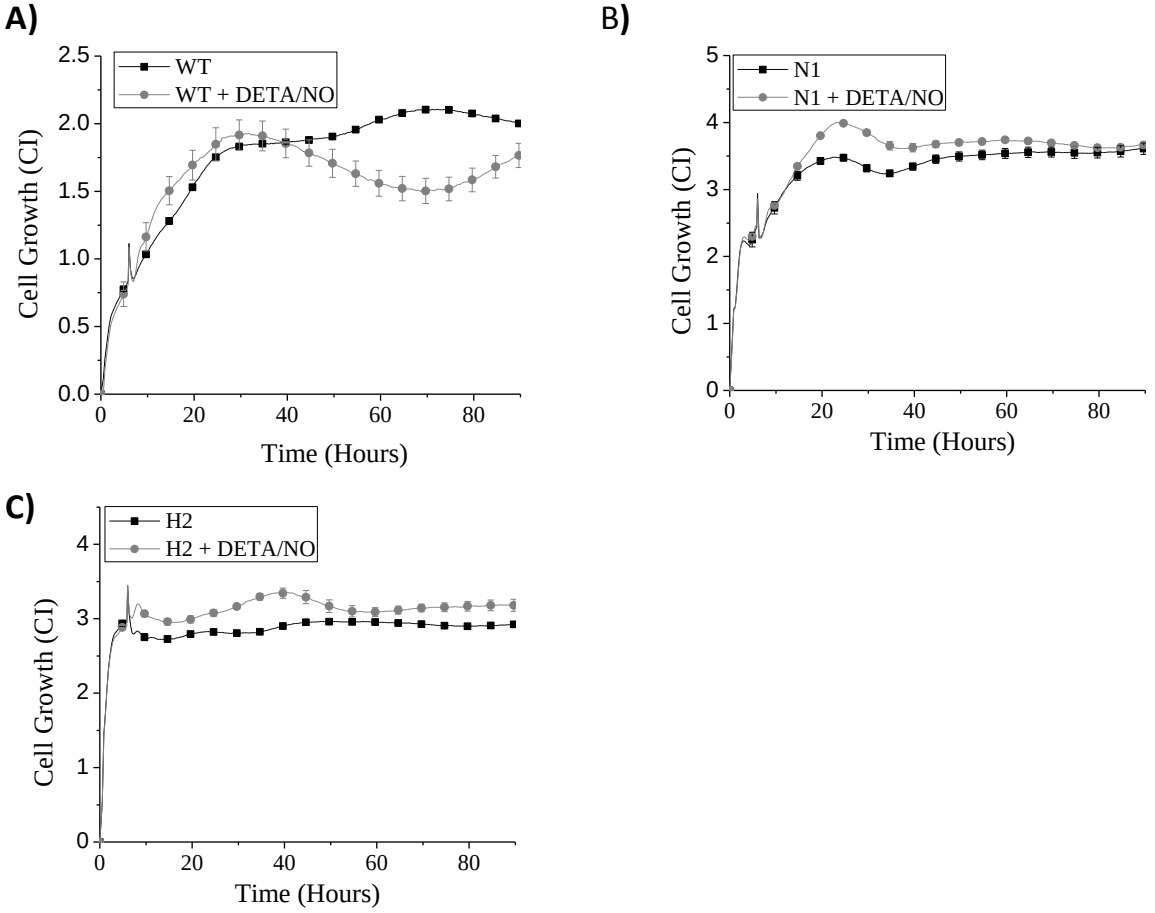


Figure 41: Cell Viability. Cells were grown in growth medium containing 5% FBS +/- DETA/NO (250µM) in E-plate 16. Continuous growth is monitored after every 20 minutes. **A)** HCC 1806 cells. **B)** N1 cells. **C)** H2 cells.

Role of Iron in •NO mediated migration suppression

In order to study the effect of iron on this •NO-mediated migration suppression, we artificially increased the cellular CIP levels by supplementation with FAC for 16 hours. The cells with or without iron supplementation were then treated with •NO-donor DETA/NO (500μM) for 8 hours in tissue culture dish. We observed significantly reduced up-regulation of NDRG1 in iron supplemented cells (**Figure 31**). These cells were then harvested and seeded into CIM plates for measurement of migration ability.

We observed decreased migration ability of non-iron supplemented cells treated with DETA/NO. There was no effect of DETA/NO on cell migration in cells supplemented with FAC. (**Figure 42**)

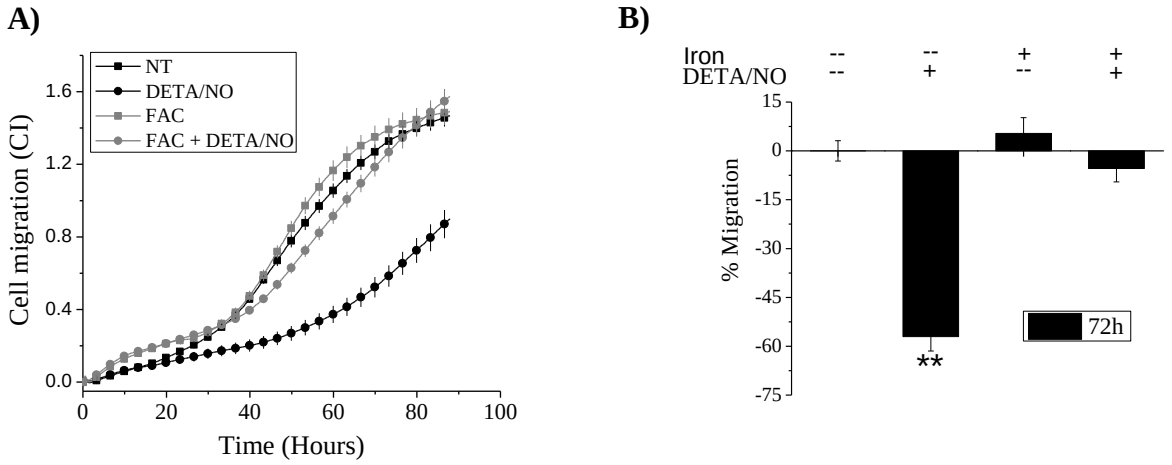


Figure 42: Iron supplementation. A) HCC 1806 cells +/- FAC (150μg/ml) treated with DETA/NO for 8 hours. Cells were then seeded in CIM-plates for measurement of migration (CI) for the entire length of experiment (every 20 minutes). **B)** Percentage migration of cells in **A).** **,$p<0.01$ with respect to untreated control. ($n\geq3$)

Effect of •NO on cell invasion

We performed cell invasion assay in HCC 1806 cells. The CIM-plates were coated with BD Matrigel™ Basement Membrane matrix from EHS mouse tumor[171,172]. Its major component is laminin, collagen IV, heparan sulfate proteoglycans and entactin/nidogen which block passage of normal cells and macromolecules, but can be invaded by invasive neoplasms[173,174].

Membrane in the top chamber of CIM plate was coated with 30µL of 1:30 dilution of Matrigel™ in serum free RPMI media. We observed about 40% inhibition in invasion ability of cells treated with DETA/NO compared to no treatment (**Figure 43**). Deactivated DETA/NO control does not show any inhibition in invasion (data not shown).

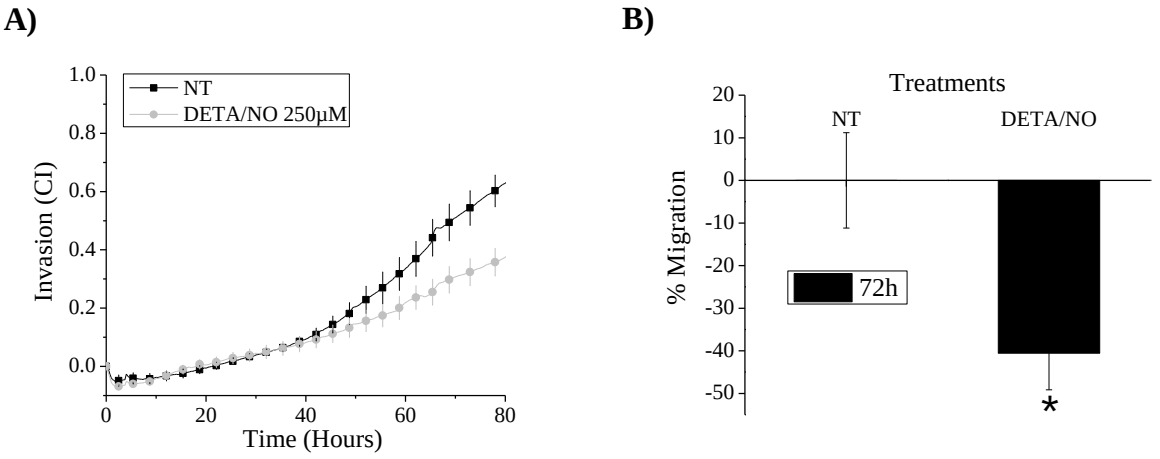


Figure 43: Cell Invasion. A) HCC 1806 cells seeded into Matrigel™ coated CIM-plate followed by treatment with •NO-donor DETA/NO (250µM). Measurement of cell invasion (CI) for the entire length of experiment (every 20 minutes) .**B)** Percentage invasion of HCC 1806 +/- DETA/NO (250µM). *,$p<0.05$ with respect to untreated control. ($n\geq3$)

Other •NO-donors

We also studied the effect of other •NO-donors on HCC 1806 cell migration. We used •NO-donor Sper/NO which has a shorter half life (~40 minutes) compared to DETA/NO (~20 hours)[175].

HCC 1806 cells were treated with increasing concentrations of Sper/NO (12.5-50μM) in CIM-plates. We observed suppression of migration even with low dose of 12.5μM Sper/NO. Maximal suppression was observed at 50μM Sper/NO concentration. There was no significant suppression with deactivated Sper/NO control (data not shown). No apparent cytotoxic effects were observed at these Sper/NO concentrations. (**Figure 44**)

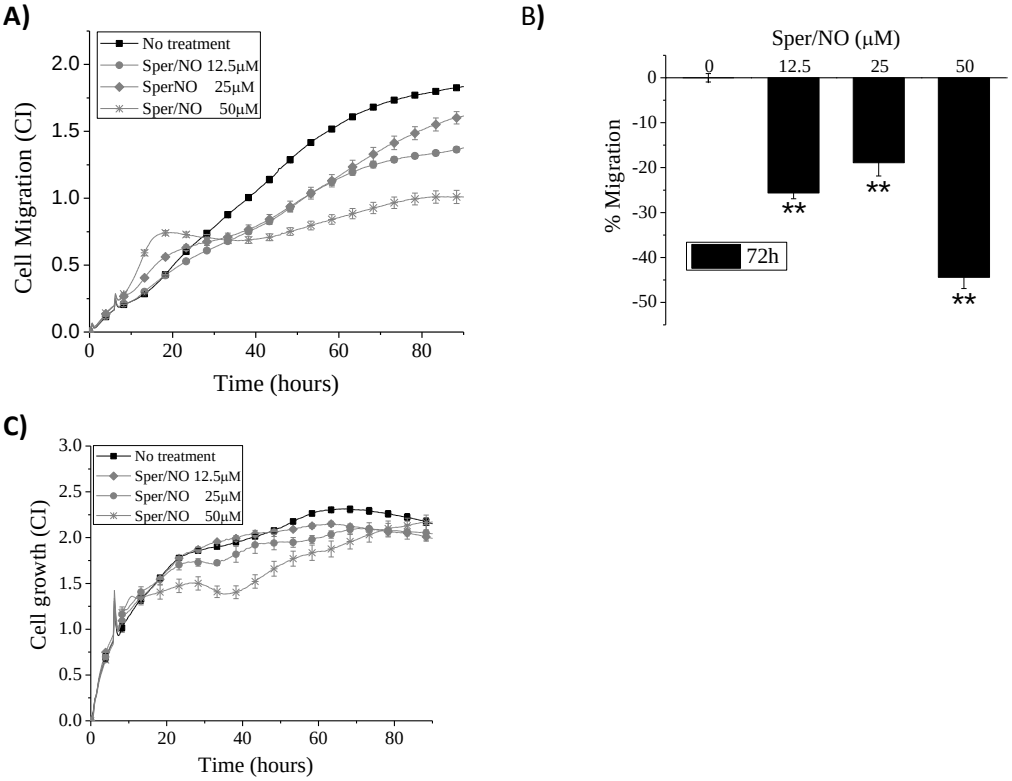


Figure 44: Effect of Sper/NO on HCC 1806 migration/viability. A) HCC 1806 cells treated with increasing concentrations of Sper/NO (12.5-50μM). Measurement of cell migration (CI) for the entire length of experiment (every 20 minutes) .**B)** Percentage inhibition in migration on Sper/NO treatment compared to non-treated cells. *,$p<0.05$;**,$p<0.01$ with respect to untreated control. ($n\geq3$) **C) M**easurement of cell growth (CI) of HCC 1806 +/- Sper/NO (12.5-50μM) for entire length of experiment (every 20 minutes).

Next, we used •NO-related novel compound, Blue acetate (BA). HCC 1806 cells were treated with increasing concentrations of BA (50-200μM) in CIM-plates. We observed 25% suppression of migration with 200μM BA. Vehicle (DMSO) control also showed significantly similar inhibition compared to 200μM BA. Thus, it was difficult to attribute this migration suppressive effect to •NO-donor. No apparent cytotoxic effects were observed at these BA concentrations. (**Figure 45**)

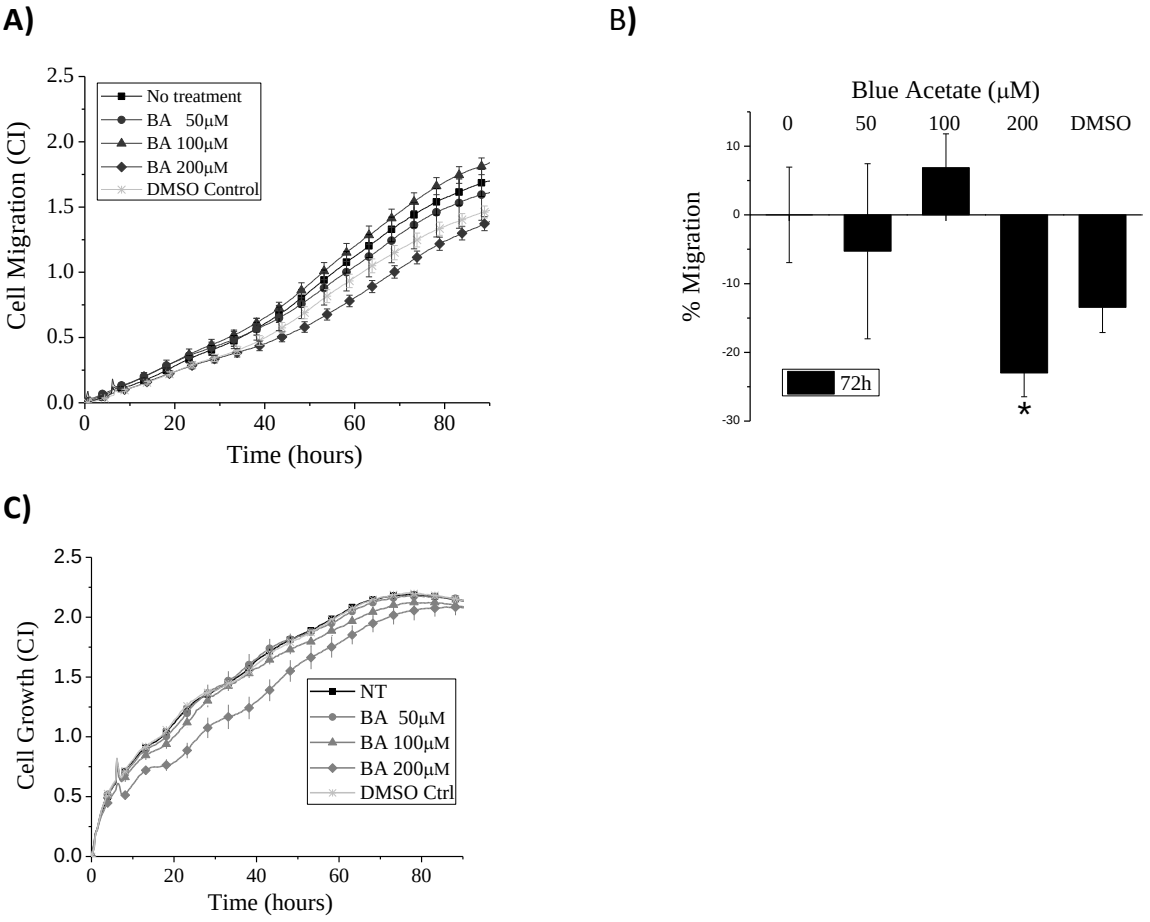


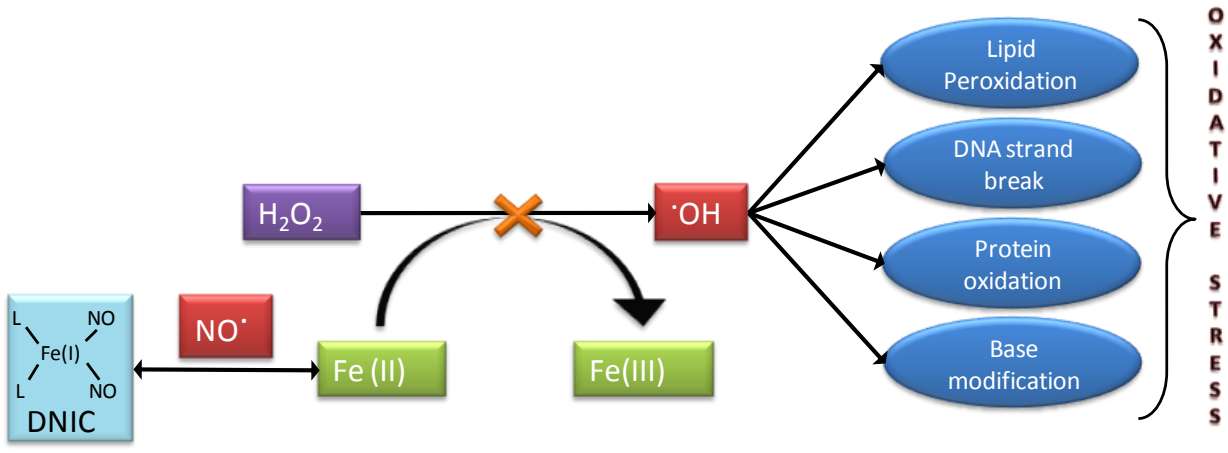
Figure 45: Effect of Blue acetate on HCC 1806 migration/viability. A) HCC 1806 cells treated with increasing concentrations of Blue acetate (50-200μM). Measurement of cell migration (CI) for the entire length of experiment (every 20 minutes) .**B)** Percentage inhibition in migration on Blue acetate treatment compared to non-treated cells. *,$p<0.05$with respect to untreated control. (n=3) **C)** Measurement of cell growth (CI) of HCC 1806 +/- Blue acetate (50-200μM) for entire length of experiment (every 20 minutes).

CHAPTER 6

ANTIOXIDANT EFFECTS OF NITRIC OXIDE

RATIONALE & HYPOTHESIS:

The chelatable iron pool (CIP) is a small but chemically significant fraction of total cellular iron. Due to its redox properties, the CIP is capable of generating highly oxidizing intermediates through its interaction with reactive oxygen species (ROS). These can lead to oxidative damage via DNA strand breaks, base modifications, protein oxidations, and lipid peroxidations. In chapter 3, we have shown involvement of CIP in formation dinitrosyl iron complexes (DNIC) in cancer cells when exposed to nitric oxide (•NO). Therefore, **we hypothesize that sequestration of cellular iron via DNIC formation may serve an antioxidant function by preventing deleterious redox reactions.**



Scheme 6: Schematic representation of hypothesis for Chapter 6

RESULTS:

Cytoprotective effects of •NO

We wanted to test the ability of nitric oxide to decrease/prevent the peroxide induced cytotoxicity. The HCC 1806 cells were pre-incubated with increasing concentrations of •NO-donor Sper/NO (10-100 μM) for 1 hour followed by incubation with hydrogen peroxide (200 μM) for 2 hours. Cells were washed and supplemented with fresh media. After 48 hours, cells were treated with 10% alamarBlue™ dye for 2 hours. Plates were read for absorbance at 570nm using 600nm as reference wavelength.

We observed dose dependent cytoprotective effect on pre-treatment with Sper/NO. At least 25μM Sper/NO was required to observe •NO mediated cytoprotective effect. (**Figure 46**)

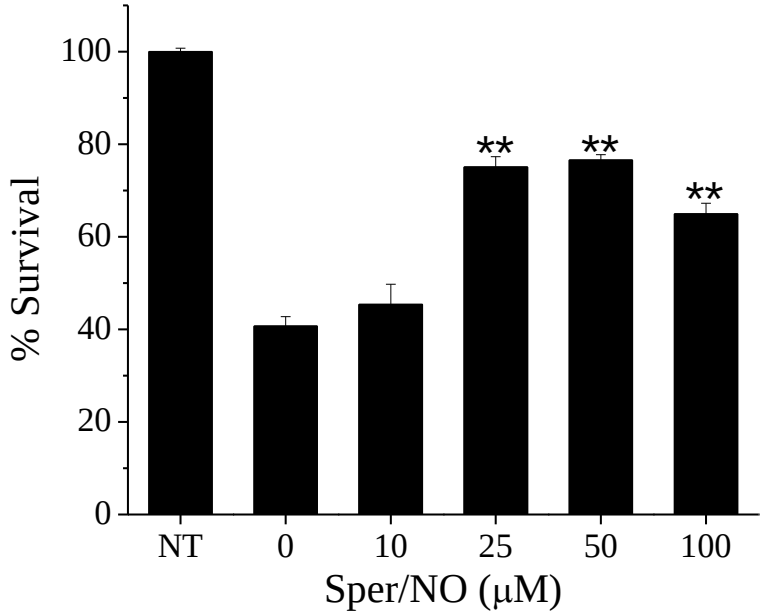


Figure 46 Cytoprotective effect of •NO. Percentage survival of HCC 1806 cells treated with increasing concentration of Sper/NO (0-100μM) for 1 hour followed by hydrogen peroxide (200μM) for 2 hours. Survival was measured 48 hours after removal of hydrogen peroxide. **,$p<0.01$ with respect to Sper/NO-untreated control.

Cytoprotective effects of Iron chelator

The central hypothesis of the proposal is that nitric oxide behaves similar to chemical iron chelators, by virtue of its property to form Dinitrosyl iron complexes. Therefore, we also tested the ability of chemical iron chelator to prevent peroxide mediated cytotoxic effects.

We treated HCC 1806 cells with increasing concentration of iron chelator Dipyridal (0-200 μM) for 1 hour, followed by treatment with hydrogen peroxide (200 μM) for 2 hours. The cells were washed and supplemented with fresh media. After 48 hours, we measured cell viability using alamarBlue™ dye.

We observed similar cytoprotective effects as Sper/NO on treatment with iron chelators. Thus, nitric oxide and iron chelators may be targeting same iron pool. (**Figure 47**)

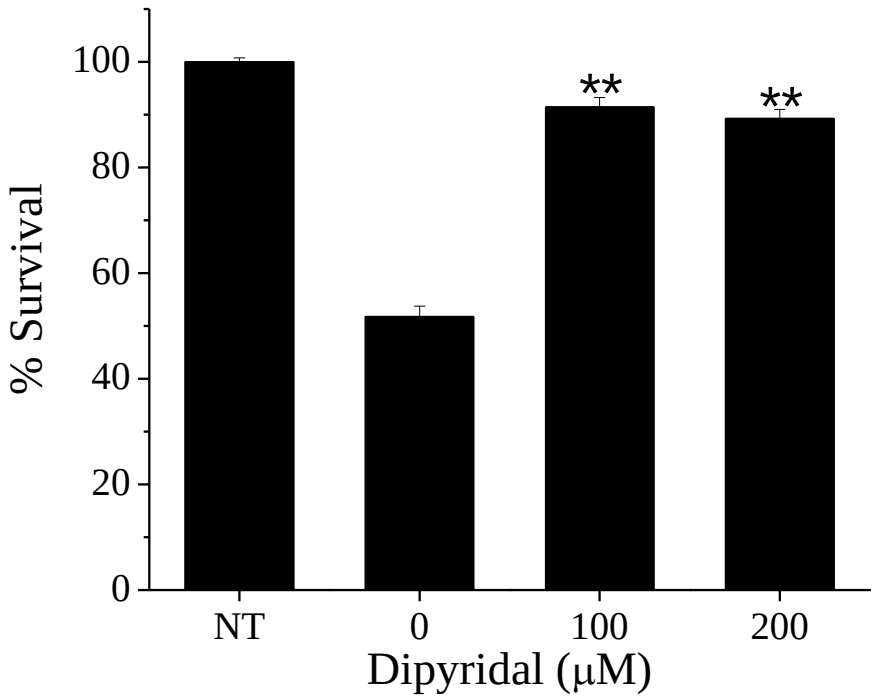


Figure 47 Cytoprotective effect of Iron chelator. Percentage survival of HCC 1806 cells treated with increasing concentration of Dipyridal (0-100μM) for 1 hour followed by hydrogen peroxide (200μM) for 2 hours. Survival was measured 48 hours after removal of hydrogen peroxide. **,$p<0.01$ with respect to Dypridal-untreated control.

Effect of exogenous •NO on oxidants (e.g., Hydroxyl radical) production

Hydroxyl radical (•OH) production is the main cause of cytotoxicity on treatment with hydrogen peroxide. Hydrogen peroxide itself is not that reactive with biomolecules, but •OH produced after Fenton type reaction reacts in almost diffusion controlled manner. These interactions are known to be main causes of peroxide mediated cell death.

We tested the ability of •NO to reduce the production of oxidants (e.g., •OH) on peroxide treatment. HCC 1806 cells were loaded with Dihyrorhodamine 123(DHR123) dye, which is an intracellular indicator of oxidants production (•OH). Cells were then treated with increasing concentrations of Sper/NO (0-1000μM) followed by treatment with hydrogen peroxide (200μM) for 2 hours.

We observed dose dependent decrease in oxidants (•OH) production on pretreatment with Sper/NO. There was no false positive fluorescence read from only Sper/NO treated cells. (**Figure 48**)

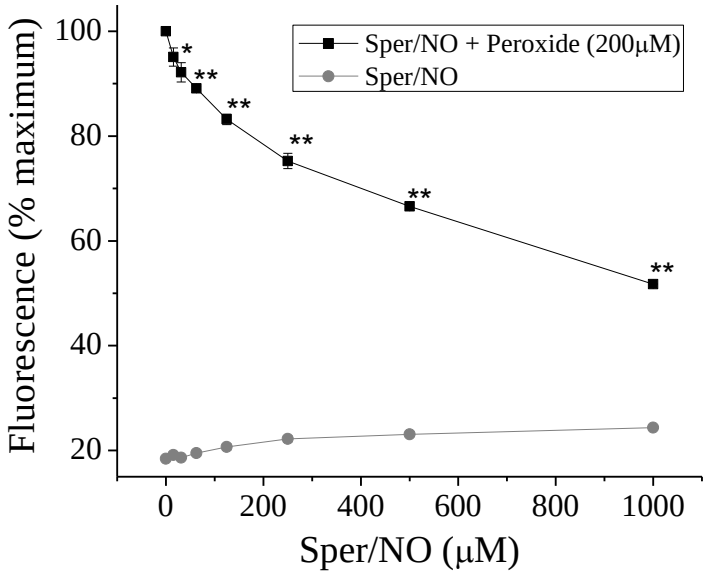


Figure 48 **Effect of exogenous •NO on oxidants production**. HCC 1806 cells were loaded with fluorescent dye, DHR 123 (100 μM). The cells were then treated with •NO-donor Sper/NO for 1 hour followed by hydrogen peroxide (200 μM) for 2 hours. Plates were read for fluorescence measurement. *,$p<0.05$;**,$p<0.01$ with respect to Sper/NO-untreated control.

Effect of endogenous •NO on oxidants (e.g.,•OH) production

We also studied the effect of endogenous •NO on oxidants (e.g., •OH) production. The RAW 264.7 macrophage cells were infected with lipopolysaccharide (LPS) in order to induce iNOS expression. Cells were then loaded with DHR 123 dye followed by treatment with hydrogen peroxide. After incubation for about 2 hours, fluorescent read was measured.

We observed significantly less production of oxidants (•OH) in LPS activated cells compared to non-activated cells. (**Figure 49**)

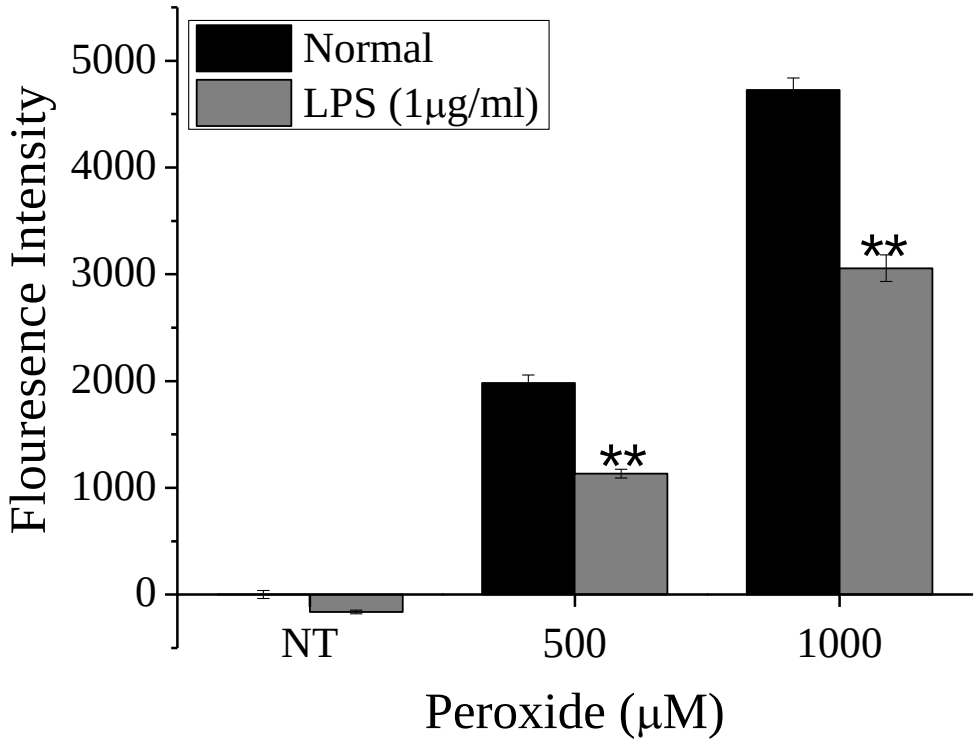


Figure 49 Effect of endogenous •NO on oxidants production. RAW 264.7 cells were activated with LPS (1μg/ml) for 16 hours. The cells were then loaded with fluorescent dye, DHR 123 (100 μM). The cells were then treated with hydrogen peroxide (.5-1mM). Plates were read for fluorescence.**,$p<0.01$ with respect to individual non-LPS treated control. (n=3)

Effect of Iron supplementation

We did iron supplementation studies in order to determine the role of the CIP in this •NO mediated cytoprotective effects. As shown in figure 30, supplementation of cells with ferric ammonium citrate leads to increase in the intracellular CIP levels. We treated HCC 1806 cells with FAC (150µg/ml) for 4 hours in order to increase iron concentration of the CIP. Cells were then treated with Sper/NO (0-50µM) for 1 hour followed by the addition of hydrogen peroxide (200µM) for 2 hours. Cells were washed and supplemented with fresh media for 48 hours. Cell viability was measured using alamarBlue™ dye.

We observed increased cell death in FAC supplemented cells compared to normal HCC 1806 cells. Moreover, higher dose of Sper/NO was required to achieve cytoprotective effect in case of iron supplementation. (**Figure 50**)

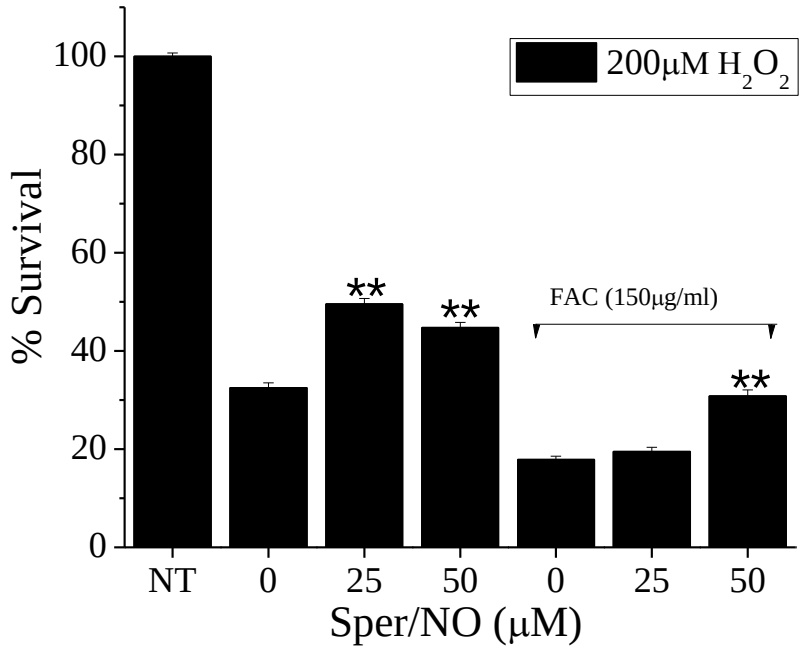


Figure 50 Role of Iron in •NO-mediated Cytoprotection: Percentage survival of HCC 1806 cells (+/- FAC) 48 hours after treatment with hydrogen peroxide +/- Sper/NO (0-50 µM). **,$p<0.01$ with respect to individual Sper/NO-untreated control.

We also measured the effect of iron supplementation of production of oxidants (e.g., hydroxyl radical). DHR 123 fluorescence was employed as an indirect measure of •OH levels. HCC 1806 cells were incubated with increasing concentration of FAC (0-125µg/ml) in order to elevate CIP to different levels. Cells were then loaded with DHR 123 dye. These dye loaded cells were then treated with Sper/NO for 1 hour followed by peroxide for 2 hours. The fluorescence was measured.

In HCC 1806 with no •NO pre-treatment there was a dose dependent increase in oxidants (e.g., •OH) production on iron supplementation. The fluorescence intensity of cells pre-treated with Sper/NO was considerably less as compared to non-treated cells. Further, there was no increase in oxidants (•OH) production on increasing the CIP levels by FAC supplementation in Sper/NO pre-treated samples. (**Figure 51**)

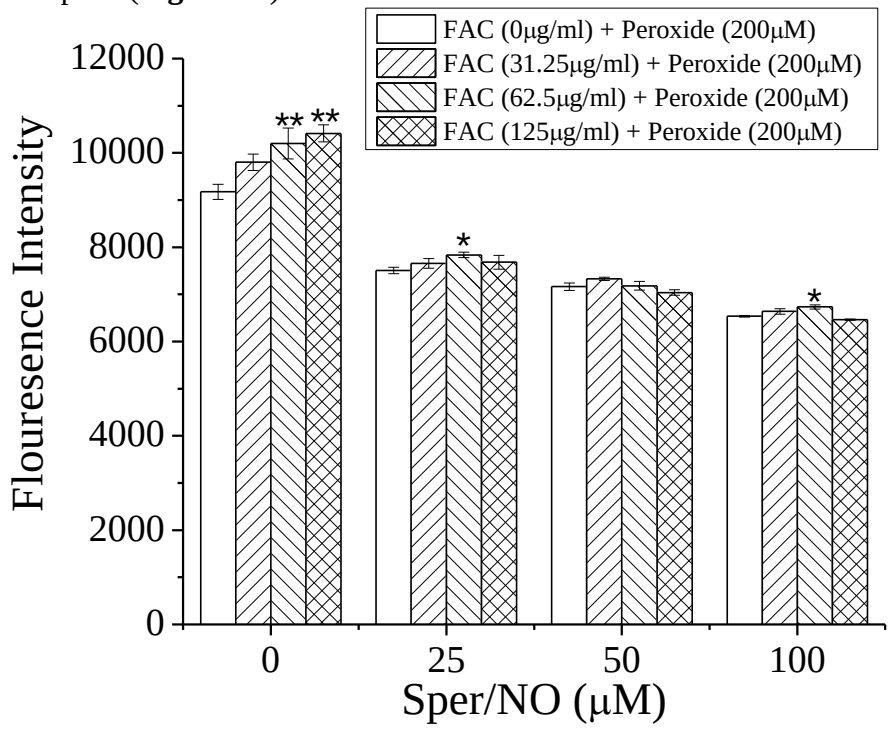


Figure 51 Role of iron in oxidants production: Oxidant (Fluorescence intensity) production of HCC 1806 cells (+/- FAC) 48 hours after treatment with hydrogen peroxide (200µM) +/- Sper/NO (0-50 µM). *,$p<0.05$;**,$p<0.01$ with respect to individual non-iron supplemented control.

Formation of DNIC

We also studied the formation of Dinitrosyl iron complexes under similar conditions at which we observe cytoprotective effects of •NO-donor Sper/NO. HCC 1806 cells were treated with increasing concentrations of Sper/NO (0-50 µM) for 3 hours. Cells were then harvested for determination of DNIC by EPR spectroscopy.

We observed dose dependent increase in level of DNIC on treatment with Sper/NO. At low dose of Sper/NO (10µM), at which we do not observe any cytoprotection, we do not observe detectable levels of DNIC. Concentrations at which we observed •NO-mediated cytoprotective effect against peroxide toxicity, there was formation of DNIC at levels similar to the CIP. (**Figure 52**)

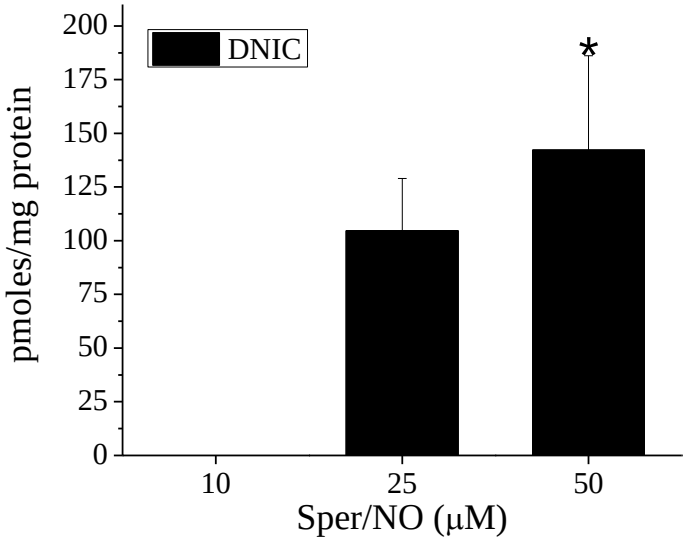


Figure 52 DNIC Formation: DNIC levels measured after treating HCC 1806 cells with •NO-donor Sper/NO (10-50 µM) for 3 hours. *,$p<0.05$ with respect to untreated control.

CHAPTER 7

DISCUSSION & CONCLUSION

DISCUSSION

Dinitrosyl Iron Complexes

DNIC due to their paramagnetic nature gives a characteristic EPR signal (g=2.03), which led to their identification in the biological system in late 1960s[176]. Later, these signals were observed in cancer cells, but lack of evidence for production of •NO in biological system halted further study of these complexes. In the 1980s there was a revival in interest towards these complexes following the discovery of endogenous •NO production[177] and concurrent detection of EPR signal corresponding to DNIC[178]. At present, detection of these signals by EPR is considered to be associated with production of •NO under different biological settings.

Major •NO adduct: In this study, we looked into physio-chemical characteristics of formation and decay of these complexes in different cancer cells. We observed that DNIC forms one of the most abundant •NO-derived adduct intracellularly, compared to other previously known species (**TABLE VI**) [37,179,180].

Intracellular compound	Concentration
DNIC	300-500 pmoles/mg protein
RSNO[37]	1-100 pmoles/mg protein
Total heme[180]	15-25 pmoles/mg protein
LNO_2[179]	249 ± 104 nM
3-NT[37]	Not detectable (< 1.5pmoles/mg protein)

TABLE VI: INTRACELLULAR CONCENTRATIONS OF MAJOR •NO ADDUCTS

Iron source: Previous studies have shown that the CIP is a major source of iron required for the formation of these complexes[5]. We have previously shown that the CIP is required for initiation of DNIC formation, as prior chelation of CIP with DFO completely ablates the DNIC signal[37]. Moreover, on treatment with higher concentrations of •NO, DNIC levels were shown to exceed the CIP. We observed similar results in different cancer cell lines under study. Thus, although the CIP is the primary source of iron for the formation of DNIC, at higher •NO concentrations other iron sources are also involved. Other potential sources of iron, in addition to the CIP, are iron storage proteins, iron-sulfur clusters, heme, and non-heme iron proteins.

Anionic ligands: The CIP is known to be loosely associated with either low or high molecular weight ligands intracellularly[15]. Based on principles of coordination chemistry, it is possible that binding of •NO to the CIP increases the strength of the bond between iron and its ligands. These anionic ligands are considered to be bound to iron through oxygen or sulfur atoms due to their abundance in biomolecules.

Glutathione (GSH) is one of the most abundant thiols in the cell and it can be a ligand for DNIC. Toledo *et al.* have shown that millimolar concentrations of GSH were unable to break the Fe-calcein (calcein is a weak iron chelator) bond *in-vitro*, implying that GSH is not a ligand for DNIC formation[5]. On the other hand, Ricci and co-workers have shown that glutathione S-transferase (GST) forms DNIC by replacing one of the GSH from GSH-DNIC[156]. As our and a number of other previous studies demonstrated stable DNIC under intracellular conditions[37,38], we expect at least one macromolecular ligand to be involved in formation of these complexes.

Stability: The stability of these complexes, like most •NO adducts, should be important in order to have biological significance. We observed that once the •NO source was removed, the DNIC

signal disappeared with a half-life of approximately 60min. We favor the term disappear over decay because at this stage of our investigation we cannot make inference on the mechanism of disappearance of DNIC signal. The loss in DNIC signal can be due to multitude of reasons: non-paramagnetic •NO-iron complexes, dimeric-DNIC, decay of DNIC and cellular export of DNIC.

Turella *et al* showed that GST extended the half life of DNIC in cell lysate from 2.8min to 4.5-8 hours[38]. Another study demonstrated that Cys-DNIC was less stable under ambient air, but persists for hours under deoxygenated conditions[33]. Watts *et al* suggested that there is an equilibrium between low and high molecular weight DNIC and the latter are exported out of the cell resulting in loss of the EPR signal[181]. These results show the complexity of the system and suggests towards cell type specific protein composition and/or clearance of DNIC, leading to different phenotypic consequences.

Endogenous DNIC: Apart from cancer cells, we also studied the formation of DNIC by endogenous •NO produced from macrophages. Tumor associated macrophages are known to produce •NO *in-vivo* and influence cancer phenotype and thus it was important to study the formation of DNIC under these conditions[182]. We observed formation of DNIC on production of •NO in LPS stimulated macrophages. As the tumor microenvironment is mostly hypoxic in nature and oxygen is a substrate for production of •NO by iNOS, we also studied the role of varying oxygen concentrations on total •NO production and DNIC formation[110,183]. We observed there was much higher production of total •NO under normoxic condition compared to hypoxia. In contrast, DNIC levels were higher in hypoxia compared to normoxia. This can be due to a higher rate of •NO metabolism, as it is also an oxygen dependent process[6]. Thus, although much higher amount of •NO was formed under normoxia, steady state •NO concentrations were lower compared to hypoxia. Moreover, varying oxygen concentrations during LPS activation, leads to

drastically differential production of •NO and DNIC. These results lead to the conclusion that tumor associated macrophages produce •NO in an oxygen dependent manner and DNIC formation can play an important role in their •NO mediated activity.

Biological significance: Formation of these complexes can have significant biological consequences in situations where iron has an important role. One of the important reactions where the CIP is involved, is Fenton Chemistry which leads to the formation of reactive oxygen species (hydroxyl radical)[19]. DNIC formation can bind up the chelatable iron pool and thus prevent its participation in reduction of hydrogen peroxide. This can be a part of the mechanism, by which •NO is known to exert its antioxidant effects.

Moreover, DNIC formation leads to an iron starved state similar to treatment with chemical iron chelators. However, in contrast to iron chelators, nitric oxide can also target other iron pools at higher concentrations. This iron starved condition created on exposure to •NO can trigger iron homeostasis mechanism involving IRP-IRE binding. A variety of genes contains these IRE sequences in their untranslated regions and can either trigger or repress translation depending on their position.

Nitric oxide may also sequester iron from the active site of various non-heme iron containing proteins (e.g., Iron and α-ketoglutarate dependent dioxygenase). This interaction can make iron unavailable for its catalytic function and makes the enzyme inactive towards its biological activity.

Finally, we can say that formation of Dinitrosyl iron complexes in the cells on exposure to nitric oxide is an important phenomenon and can lead to significant downstream effects, essentially by creating an iron starvation state.

Up-regulation of NDRG1 on exposure to •NO

NDRG1 is a well known metastasis suppressor gene. Previous studies have shown up-regulation of NDRG1 during hypoxia, treatment with hypoxia mimetics (Co^{2+} and Ni^{2+}) and metal chelators[31,59,74]. These results imply involvement of metal coordinated or oxygen binding protein. Chemical iron chelators primarily targets the chelatable iron pool (CIP) and render it unavailable for different biological processes. Divalent metal ions are also known to replace iron from the catalytic site of iron-requiring enzymes, and thus inhibiting their activity. Nitric oxide is a small free radical which reacts with only few intracellular targets. These targets are either other free radicals or transition metals. CIP is one of the important intracellular target of •NO. Nitric oxide interacts with the CIP and anionic ligands leading to the formation of DNIC. This interaction results in sequestration of the CIP, resulting in a cellular starvation state similar to chemical iron chelators. Further, it can lead to distinct downstream genotypic and phenotypic outcomes similar to chemical iron chelators, which are separate from previously well established •NO mediated pathways. For above stated reasons, we tested the ability of nitric oxide to up-regulate NDRG1 in a CIP dependent manner.

Nitric oxide is known to exert its effect intracellularly via a number of well known mechanisms. These include •NO-heme interactions, S-nitrosothiol formation and binding and destruction of Fe-S proteins[9,43,184]. It is also known to activate transcription factors e.g., p53 and HIF-1α[6]. The interaction of •NO with heme centers of proteins (e.g., soluble guanylyl cyclase) is one of the most important and intensively studied pathway by which •NO is known to exert majority of its effects. S-nitrosation of major protein residues can also be important, by having either direct or allosteric effects on protein function. Nitric oxide is also known to target Fe-S clusters of proteins (e.g., aconitase) at high concentrations. In Chapter 3, we observed that DNIC were one

of the most abundant intracellular •NO-adducts. Although, this interaction is studied in detail under synthetic laboratory conditions, there is little known about their role inside the cellular machinery. One of the possible implications of formation of these complexes is a creation of an iron starvation state, which can have significant downstream consequences.

Iron dependent pathway: On exposure of cells to •NO (500μM DETA/NO), DNIC were formed in an equivalent concentration as CIP. These results are consistent with previous studies, showing CIP as major iron source required for the formation of these complexes[5,37]. Iron chelators are known to up-regulate NDRG1[31]. We observed similar up-regulation of NDRG1 mRNA and protein on treatment with maximal dose of •NO.

In order to determine the role of DNIC formation in NDRG1 up-regulation, iron supplementation studies were performed. We were able to artificially manipulate the intracellular CIP levels on incubation with iron salt. There was an incremental increase in the CIP levels on increasing the incubation time with iron. We observed that elevation in the CIP levels led to decreased ability of •NO to up-regulate NDRG1 mRNA and protein. At the same time, the ability of DNIC to reach CIP levels was also diminished. Therefore, in iron supplemented cells, iron starvation state was not achieved on treatment with •NO. This leads to availability of free iron even after •NO-treatment, which leads to decrease in NDRG1 up-regulation.

We also observed decrease in endogenous levels of NDRG1 mRNA in cells incubated with iron. These results indicate a physiological upstream iron dependent modulation of NDRG1. In the same experiment, cells incubated with iron required higher amount of •NO in order to achieve similar NDRG1 up-regulation compared to cells with basal iron levels. Maximal NDRG1 up-regulation in iron supplemented cells was not achieved until the DNIC level approximates

elevated CIP levels. This again re-emphasizes the point that CIP/DNIC ratio is important determinant of •NO-mediated NDRG1 up-regulation and iron starvation state is required in order to achieve it.

Although, we showed that NDRG1 up-regulation by •NO is an iron dependent pathway, the exact mechanism is still not known. One of the important mechanisms by which the CIP controls translation of various proteins is through IRE-IRP pathway[21]. Saletta *et al* have shown the presence of stem loop structures in 3' untranslated region (UTR) of NDRG1 mRNA[185]. However, these stem loops were significantly different from previously characterized IREs and thus, authors concluded that they are not favorable for IRP binding[185].

In another study, Wong *et al* showed increased levels of H3K4 trimethylation in both the promoters and coding regions of NDRG1 on nickel treatment[186]. Nickel is a heavy metal known to replace iron from active sites of various iron dependent proteins and show effects similar to iron chelation. Nickel is also known to up-regulate NDRG1[59]. Histone methylation is known to be regulated by histone demethylases, which are iron and α-ketoglutarate dependent enzymes[187]. Therefore, it is possible that •NO-mediated NDRG1 up-regulation occurs in an iron dependent manner by increasing H3K4 tri-methylation via inhibition of histone demethylases. This hypothesis needs further investigation and can be a potential up-stream mechanism of CIP dependent regulation of NDRG1.

Other pathways: Both N-myc and c-Myc are known to down-regulate NDRG1[83]. Nitric oxide is also known to down-regulate N-myc and thus can be an important pathway for •NO mediated NDRG1 up-regulation[188]. Since our cell lines do not express N-myc, we ruled out this pathway. Also, we observed increased levels of c-Myc on •NO treatment. Although, elevated c-Myc levels

could be contributing to suppression of NDRG1, there are other compensatory pathways which are leading to overall NDRG1 up-regulation in our cell lines.

p53 and sGC are another important pathways through which nitric oxide is known to mediate its effects. We have shown that up-regulation of NDRG1 is independent of these pathways.

HIF-1 is a transcription factor which plays an important role in tumor metastasis[189]. Previously, NDRG1 has been known to be regulated by HIF-1α[74]. It is important to study role of this pathway, as •NO is also known to stabilize HIF-1α under normoxic conditions[6]. We observed HIF-1α independent up-regulation of NDRG1 in our cell lines.

Stability: It was important to assess the stability of these up-regulated gene products in order for this up-regulation to have any clinical significance. Although NDRG1 mRNA was not stable, protein showed remarkable stability once expressed (~ 40 hours). As protein is the functional product of the gene, it makes NDRG1 an important clinical target of interest. These results imply that short exposure to •NO can have long lasting phenotypic effects.

To summarize, these results show a novel pathway for •NO mediated up-regulation of metastatic suppressor gene NDRG1. Hence, NDRG1 can act as a potential therapeutic target for •NO related drugs. It is the first demonstration of biological effect of •NO-mediated chelatable iron dependent pathway via formation of DNIC.

Effect of •NO on Cancer Cell Migration/Invasion

Metastasis is an important clinical problem responsible for 90% of cancer related deaths[51]. Cancer cell migration and invasion are critical steps involved in metastasis-invasion cascade. We studied the effect of •NO on these phenotypic characteristics in highly aggressive breast cancer cells.

Many aggressive cancer cells are resistant to conventional chemotherapy[190]. Hence, development of new therapies against metastatic disease is required. In the current study we looked into regulation of metastatic suppressor gene NDRG1 by nitric oxide and its further effect on downstream phenotypic effects (migration/invasion).

Importance of NDRG1 as a metastatic suppressor: NDRG1 is known to be down-regulated in number of cancers (e.g., breast, prostrate, colon) and is defined as metastatic suppressor under these settings[64,76,77]. Thus, up-regulation of NDRG1 by a novel mechanism can be of considerable interest in these cancer types.

We observed a significant increase in metastatic potential of HCC 1806 breast cancer cells on knocking down NDRG1 mRNA. These results indicate the significance of basal NDRG1 levels in metastasis suppression. Moreover, exposure of cells to nitric oxide leads to up-regulation of NDRG1 as well as marked suppression of cell migration, indicating the role of NDRG1 in •NO-mediated metastasis suppression. Since various •NO-related drugs are already in clinic, these results may open a new area of therapeutic application in the field of tumor metastasis.

Role of NDRG1 and HIF in •NO mediated migration suppression: In NDRG1 knockdown cells, exposure to •NO results in much lower suppressive effect on cell migration compared to wild type cells. These results show major contribution of NDRG1 in •NO-mediated migration

suppression. However, these low suppressive effects are still considerable (~25%) and implies involvement of other genes apart from NDRG1 in this suppression. As metastasis is a complex process, with array of genes involved in its regulation, we concluded that NDRG1 forms a part of this system.

HIF-1α is also known to be involved in metastasis and stabilized by •NO under normoxic conditions. Therefore, we also studied its role in •NO mediated migration suppression. There was no migration suppression in HIF-1α knockdown cells, although, NDRG1 protein was up-regulated. These results indicated migration suppression in HIF-1α dependent manner.

Interestingly, in NDRG1 knockdown cells where HIF-1α was stabilized, there was a decrease in migration suppression by •NO. Thus, we concluded that the presence of both HIF-1α and NDRG1 protein was required for •NO-mediated migration suppression.

Cytotoxicity: It is also important to consider the cytotoxicity of nitric oxide to understand its true metastatic suppression effect as decrease in cellular migration can be due to increased cell death. In that situation, it is more of a standard cytotoxicity therapy, rather than suppression of metastasis. We observed no significant cytotoxic effects of •NO-donors at doses which showed suppression in metastasis. These results indicate selective inhibition of metastasis without effecting cellular viability. This is important, as metastasis is a true distinct phenotype of tumor cells compared to other normal cells in the body. Thus, it provides specificity to the drug against metastatic tumors.

Role of Iron: In the last section, we concluded that nitric oxide up-regulates NDRG1 via novel mechanism of sequestering chelatable iron pool. We also studied the role of chelatable iron pool in •NO-mediated migration suppression. We observed that artificial enrichment of the CIP

completely revert the migration suppression effect of •NO. This again strengthens the hypothesis that interaction of •NO with the CIP to form DNIC leads to the up-regulation of NDRG1 and suppression of migration. It also implies that multitude of other iron-independent, •NO-induced pathways are not involved in this migration suppression.

Nitric oxide vs Iron Chelators as anti-tumor agent: The basic hypothesis of this project is that •NO can act in a similar manner as chemical iron chelators, leading to an iron starvation state. Iron chelators are also known to have anti-tumor activity, however, because of their strong interaction with the CIP it poses significant biological problems. Iron is required for normal functioning of the cell cycle and its chelation for a prolonged period of time can result in deleterious non-specific effects in the growth of normal cells. Nitric oxide, on the other hand, sequesters iron for shorter lengths of time (~2-4 hours) via formation of the DNIC. As NDRG1 protein is stable after its up-regulation by •NO, it provides a lucrative therapy by transient sequestration of the CIP. Moreover, •NO is produced biologically via enzymatic synthesis suggesting that it may be better tolerated as a drug. Besides, number of •NO containing molecules are already in clinical use, increasing its chances as a potential drug.

The major problem with •NO is its plethora of other biological effects along with metastatic suppressor action. Being a small and highly diffusible molecule, it is difficult to selectively deliver it to the tumor. Hence, •NO based chemotherapy will pose its own undesirable effects and it is critical to analyze its beneficial vs. deleterious effects. Even then, this study sheds some light on metastatic suppressive effects of •NO under certain biological conditions.

Possibly failed mechanism: There are some cancer types, where increase in NDRG1 levels was observed in comparison to the normal tissues[79,99]. Up-regulation of NDRG1 in this situation may lead to failed mechanism of metastasis suppression.

Moreover, we have shown that the presence of both NDRG1 and HIF-1α is required for significant up-regulation of NDRG1 under normoxic conditions. Since, number of solid tumors are hypoxic in nature and •NO is known to destabilize hypoxia mediated HIF-1α stabilization[191], we expect to see no decrease in migration potential in these conditions.

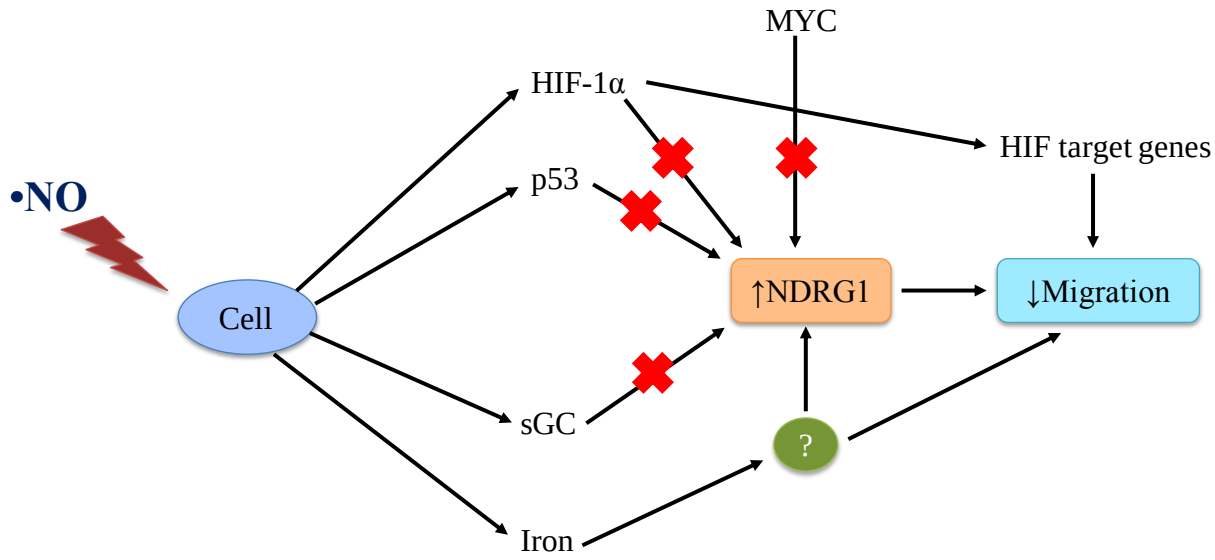
Other potential genes responsible for migration suppression: Although an important metastatic suppressor, it is highly unlikely that NDRG1 is the only gene responsible for migration suppression by •NO. NDRG1 knockdown cells still showed a substantial decrease in migration on •NO treatment. This NDRG1 independent migration suppression possibly results from a number of other potential genes modulated by nitric oxide.

Saletta *et al* observed 5 differentially regulated genes in cancer cells compared to normal cells, on treatment with chemical iron chelators[185]. These genes are APP, GDF15, CITED2, EGR1, and PNRC1[185]. It is possible that •NO also exerts part of its action through these genes by sequestration of the CIP.

HIF-1 is a transcription factor and stabilization of HIF-1α by •NO under normoxia can increase transcription activity of genes containing HIF response elements (HREs). This increase in transcriptional activity can be one of the possible reason for •NO mediated migration suppression. Moreover, HIF-1α itself is also regulated by iron dependent protein prolyl hydroxylases.

Endogenous •NO: Nitric oxide is known to be produced by tumors as well as surrounding stromal cells via iNOS activity[108]. Under these conditions of endogenous •NO production, both NDRG1 and HIF-1α can get up-regulated by •NO. Thus, in certain cancers and situations this can present a mechanism by which nitric oxide can endogenously modulate the tumor phenotype.

To summarize, these results provide a novel mechanism by which •NO can mediate its migration suppression effect. As migration is one of the initial and critical step in metastasis, we believe that •NO can be an effective metastatic suppressing agent.



Scheme 7: Schematic representation of pathways studied for NDRG1 upregulation and migration suppression by nitric oxide

Antioxidant effects of •NO

Hydrogen peroxide (H_2O_2) mediated oxidative stress is known to play an important role in cancer development[192]. Cancer cells are known to produce much higher level of H_2O_2 leading to generation of hydroxyl radical (•OH) via Fenton reaction, which can further cause number of deleterious effects e.g., DNA strand break[192]. These alterations in DNA and genetic instability caused by hydrogen peroxide mediated oxidative stress (via production of •OH) can be one of the potential mechanism by which hydrogen peroxide can initiate carcinogenesis.

Nitric oxide is shown to have oxidant as well as antioxidant properties, depending on the cellular conditions. There are reports demonstrating cytoprotective effects of nitric oxide against hydrogen peroxide mediated insult[143]. In this project, we studied one of the possible mechanisms for the observed cytoprotection.

DNIC are known to be formed when cells are exposed to nitric oxide. We and others have shown that the CIP as major iron source for the formation of these complexes. The CIP is a redox active pool of iron, which is known to play a catalytic role in Fenton type reactions. These Fenton type reactions are mainly responsible for hydrogen peroxide mediated damage, via generation of hydroxyl radical. We proposed, DNIC formation as a potential mechanism for •NO-mediated anti-oxidant effects against hydrogen peroxide.

HCC 1806 cells pretreated with •NO showed significantly less toxicity against hydrogen peroxide compared to non-treated cells. According to our hypothesis nitric oxide behaves similar to chemical iron chelators, as it targets same iron pool for the formation of DNIC. Thus, we hypothesized that •NO-donor and chemical iron chelators work in a similar manner. We observed similar cytoprotective effects with iron chelator dipyridal.

Hydroxyl radical production is an important indicator of H_2O_2 mediated oxidative stress. We observed decreased level of hydroxyl radical on pre-treatment with •NO-donor. Moreover, endogenously produced •NO from activated macrophages also showed a significant decrease in hydroxyl radical production on treatment with H_2O_2. As a large amount endogenous •NO production is known to occur via up-regulation of iNOS in various pathological conditions, it may play an important antioxidant in those situations.

Artificial increase in the CIP led to increased level of hydroxyl radical production and increased cell death. These results showed the importance of free chelatable iron in hydrogen peroxide mediated oxidative stress. In addition, •NO pre-treatment resulted in no further increase in hydroxyl radical production with increasing the CIP levels. These observations point towards a mechanism by which •NO mitigates the effect of the excess CIP, most probably by the formation of DNIC.

We also observed DNIC formation at •NO doses at which we observe cytoprotective effects. We observed a strong correlation between formation of DNIC and decrease in hydrogen peroxide mediated oxidative stress.

We concluded that DNIC formation is an important intracellular event, when cells are exposed to •NO. This can be, at least in part, a potential mechanism by which nitric oxide is shown to exert its anti-oxidant effects.

CONCLUSION

These results demonstrate that nitric oxide, via its interaction and sequestration of chelatable iron pool, results in number of biological important effects. We observed up-regulation of metastatic suppressor gene NDRG1, suppression of tumor cell migration and anti-oxidant effects against peroxide on exposure of cells to nitric oxide. These interactions represent a novel mechanism by which •NO can exert its action intracellularly by sequestration of the CIP, via formation of DNIC. This is a first demonstration of the functional role of DNIC formation in the cells. As nitric oxide is produced by a variety of tumor types and it is also generated by several classes of experimental chemotherapeutics, these results will help us better understand its role in different cancer settings.

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Hickok JR*, **Sahni S*** (*equal contribution), Mikhed Y, Bonini MG, Thomas DD.Nitric oxide suppresses tumor cell migration through N-Myc downstream regulated gene-1 (NDRG1) expression: the role of chelatable iron. J Biol Chem. 2011 Oct 5

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- **S Sahni**, J Hickok, S Hong and DD Thomas. DNIC: Potential mechanism for NO antioxidant properties. International Conference on Recent Advances in Free radical Research, Natural Products, Antioxidants and Radioprotectors in Health and 9th Annual Conference of Society for Free Radical Research-India (Jan 11-13, 2010)
- Hong Shen, Jason Hickok, **Sumit Sahni**, Douglas D Thomas. Dinitrosyliron Complex-dependant Protein Nitrosothiol Formation and Degradation in Macrophages. SFRBM's 17th Annual Meeting (Nov17-21,2010)
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