श्री चित्रा तिरुनाल आयुर्विज्ञान और प्रौद्योगिकी संस्थान, त्रिवेंद्रम, तिरुवनन्तपुरम - 695 011, केरल, भारत
SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM
THIRUVANANTHAPURAM - 695 011, KERALA, INDIA
(एक राष्ट्रीय महत्व का संस्थान, विज्ञान और प्रौद्योगिकी विभाग, भारत सरकार)
(An Institution of National Importance, Department of Science and Technology, Government of India)
टेलीफोन नं./Telephone No.: 0471-2443152 फैक्स/Fax: 0471-2446433, 2550728
ई-मेल/E-mail: sct@sctimst.ac.in वेबसाइट/Website: www.sctimst.ac.in

PROJECT COMPLETION REPORT

1. **Project number:** 5132

2. **Title of the project:** Studies on matrix metalloproteinsase (MMP) gene transcription by nitric oxide: mechanism of MMP gene induction in human colon cancer cells

3. **Funding Agency Name:** DBT

4. **Project Reference Number provided by the Funding Agency:** No.BT/PR4201/Med/14/513/2004

5. **Principal Investigator (Name & Address):** Dr. G. Srinivas, Scientist D, Department of Biochemistry, SCTIMST, Trivandrum, Kerala

6. **Co-Investigators (Name & Address):** Dr. Priya Srinivas, Scientist B, Cancer Biology, RGCB, Trivandrum 695014

7. **Implementing Institution:** SCTIMST

8. **Collaborating Institutions:** Nil

9. **Date of Commencement**: 17/11/2004

10. **Duration**: 3 YEARS

11. **Date of completion**: 16/11/2008

12. **Objectives approved:** Experiments on tumor cell proliferation and invasiveness by exogenous nitric oxide donor using cell viability assay, wound healing assay and transwell migration assay
    - Determination of different MMP expression by NO (by RT-PCR and in culture supernatants).
    - Transfection of MMPs promotor-luciferase fusion plasmids to study the MMPs promotor activity in presence of exogenous nitric oxide.
    - To study the signaling molecules involved in nitric oxide mediated MMPs upregulation using phospho MAPK/JNK western blot.

13. Deviation made from original objectives if any, while implementing the project and reasons thereof: Yes, Due to technical and scientific challenges, certain modifications were made in the study objectives; however, the overall theme remained unchanged. Initially it was thought to use iNOS overexpressing cells by transfection by iNOS plasmid. That however, led to extensive cell death; probably by the increased reactive nitrogen species and thus those cells could not be used for experiments with metastasis. It was initially suggested that MMP-1 promoter constructs will be used for the study. But our results suggested that MMP-2 and 9 and not MMP-1 was activated by SNAP. We have, by this study, shown that NO regulates metastasis by activating specific cellular signaling pathways leading to increased migration and invasion of tumor cells.

14. Field/Experimental work giving full details of summary of methods adopted, data collected supported by necessary tables, charts, diagrams and photographs:

Maintenance of cell lines

Human colonic adenocarcinoma cell line (WiDr) was grown at 37^0C in a humidified atmosphere containing 5% CO_2. Cells were cultured in DMEM, Dulbecco's Modified Eagle Medium (Cat. no: D5523, Sigma- Aldrich, St. Louis USA), which is supplemented with10% heat inactivated fetal bovine serum (Cat. no: 3302-P290310, PAN Biotech, Germany), 100 units/ml penicillin and 100 units/ml streptomycin. At first, healthy cells were seeded into T25 cm^2 tissue culture flask and allowed to become 80% confluent. When cells were grown to confluence, the medium was removed, and washed once with PBS (phosphate buffered saline), 0.25% Trypsin-EDTA solution was added, and incubated for 3-5 min at 37^0C. Fresh medium (with 10% serum) was added and cells were dispersed gently by a pipette. A known number of cells were dispensed into new flasks or microtitre plates for further experiments. All the cells were routinely maintained in complete medium, which contained DMEM, 10% FBS (Fetal Bovine Serum), with antibiotics.

Measurement of *in vitro* NO production

The NO production in three colon cancer cell lines (SW 480, SW620, WiDr) was detected by using DAF-2DA (Cat. no: D23844, Calbiochem, USA) a non-fluorescent cell permeable reagent that can measure free NO and NOS activity in living cells. Cells were incubated with 10 µM Diaminofluorescein–2 Diacetate (DAF-2DA). Once inside the cell the diacetate groups on the DAF-2DA reagent are hydrolyzed by cytosolic esterases,thus releasing DAF-2 and sequestering the reagent inside the cell. Production of NO converts the non-fluorescent dye, DAF-2, to its fluorescent triazole derivative, DAF-2T. Cells were seeded in 96 well plates at a seeding density 1 x 10^4 cells/well (10,000 cells/well). The cells were washed in PBS and then DAF 2 DA of a final concentration 10 µM was added to the cells and incubated for 30 min at 37ºC in CO_2 incubator. Finally thecells washed to remove the excess dye and visualized using fluorescence microscope having an excitation 480 nm and emission 515 nm.

Griess assay

Griess assay is one of the most popular and simplest methods used to detect the NO concentration. Nitrite accumulation, an indicator of nitric oxide production, was measured in cell culture supernatants using the Griess reagent. Samples of culture supernatants were mixed with an equal volume of Griess reagent (Cat.no:G4410, Sigma- Aidrich, USA) and incubated at room temperature for 10 min.The absorbance at 570 nm was measured in a spectrophotometer. The nitrite concentration was calculated from a $NaNO_2$ standard curve.

Cell viability assay

MTT assay is a colorimetric assay based on metabolic activity of viable cells. SNAP (s-nitroso-n-acetylpenicillamine), a nitrosothiol derivative, releases nitric oxide under physiological conditions thus making it a useful tool for studing physiological and pharmacological actions of NO. Cytotoxicity of SNAP (Cat. no: N3398, Sigma, USA) was determined by MTT assay. The amount of formazan crystal formed correlates with the amount of viable cells and can be used for the cell proliferation assay.

The WiDr cells were seeded into a 96-well microtitre plate at a density of 1 x 10^4 cells/well (10,000 cells/well) and incu incubated for 24 h. After 24 h, cells were incubated with varying concentration of SNAP (NO donor) (3.625 µM to1000 µM) in 2.5% DMEM medium for 4 h and were replaced with 10% DMEM without SNAP at 37˚C. Thereafter, the cell proliferation was assayed by adding 1mg/ml of MTT dissolved in DMEM to each well and incubated for 2.5 h in dark. The yellowish MTT is reduced to dark coloured formazan by viable cells only. The supernatant was aspirated and the MTT formazan crystals formed by metabolically viable cells were dissolved with 100 µL of acidified isopropanol (0.04M HCl). The color developed is measured with an ELISA plate reader

(ELX-800 Bio tek, USA) at measuring wavelength: 570 nm and reference wavelength: 630 nm. The cell proliferation was expressed as percentage over control.

Transwell migration inhibition assay

Migration of WiDr cells was determined with the modified Boyden's chamber method. Response of the cells were assayed using 24 well migration chamber with an upper well having a membrane of 8 µm pore size and 12 mm of diameter. WiDr cells were seeded into the upper well of each chamber (1 x 10^5 cells) with and without SNAP and ODQ (1H-[1, 2, 4]oxadiazolo-[4,3-a]quinoxalin-1-one) (Cat. no: O3636 Sigma- Aidrich, USA) and 500 µL of 10% medium were added to the lower chamber. Cell migration was assayedby counting the number of cells that had migrated from the upper side to the lower sideof the filter membrane after 48 h of incubation. Migrated cells were fixed with cold methanol and stained with 0.5% crystal violet solution. Cells on the upper side of the membrane were carefully removed by using a wet cotton swab. Migrated cells were documented using inverted light microscope.

Wound migration assay

Scratch wound- healing motlity assays are commonly used to assess the effect ofmolecule on the cellular proliferation and migration associated with wound closing. Whenwounded or scratched, cell monolayer responds to the disruption of cell-cell contact by healing the wound through a combination of proliferation and migration. Wound healingassay was performed by introducing a wound on monolayer culture of WiDr cells using pipette tip and the wound is allowed to heal for 24h with and without SNAP and ODQ. Representative pictures were taken using an inverted light microscope.

Cyclic GMP competitive ELISA kit

It is a competitive immunoassay for the quantitative determination of cyclic GMP in cell culture supernatants using cGMP competitive ELISA kit (Cat.no: EMSCGMPL, Thermo scientific, USA). Cells were treated with SNAP and co incubated with ODQ for 2 h and 4

h. After that, the cells were lysed and the supernatant were taken. To a microtitre plate (Anti-Rabbit IgG precoated), supernatant containing cGMP and alkaline phosphatase conjugated cGMP is added. The assay is based on the competition between cGMP inthe sample and cGMP-AP for a limited amount of cGMP monoclonal antibody bound to the microtitre plate. As the concentration of cGMP in the samples increases, the amount of cGMP-AP captured by the coating antibody decreases. Following incubation and washing steps, any cGMP-AP bound to the plate is detected with addition of a p-NPP substrate. The colour formed is inversely proportional to the amount of cGMP present in the sample, i.e., an inverse relationship between optical density and the amount ofcGMP in the sample. The colour developed is measured with an ELISA plate reader(ELX 800, Biotek, USA) at measuring wavelength: 405 nm and reference wavelength: 570 nm. A standard curve is prepared from cGMP standard and sample concentration is then determined.

RNA isolation and RT-PCR

The cells were grown in a 60mm diameter dish, and incubated with SNAP (15 µM) for different time points at 37˚C and 5% CO_2. After treatment, total RNA was isolated using Pure Link RNA Mini Kit (Cat. No: 12183-018A, Invitrogen, USA) following manufacture's protocol. 4 µg of total RNA was reverse transcribed in total volume of 25 µl, including200 units of moloney murine leukemia virus reverse transcriptase, 8 units of RNase inhibitor, 0.5 µg of random primer, 2.5 mM dNTP and 1X Moloney murine leukemia virus reverse transcriptase buffer (Promega, Madison, WI, USA). The reaction was carried out at 37°C for 90 min. The products were subsequently used for RT-PCR amplification. 1µl of RT products was brought to a volume of 20 µl containing 0.8 mM MgCl2, 2.5 mM dNTPs, 5 pM of both upstream and downstream PCR primers, 1X taq buffer and 1unitsof GoTaq DNA polymerase (Promega, Madison, WI). Amplification was carried out with I Cycler (Bio-Rad).The amplified PCR products were visualized by

electrophoresis on 2% agarose gel in 1X TBE buffer after staining with ethidium bromide. The UV illuminated gels were documented.

Gene	Primer sequence	Product size
MMP1	5’-GAGCAGATGTGGACCAT -3’ 5’-ACCGGACTTCATCTCTGTCG- 3’	645bp
MMP-2	5’- CAGGCTCTTCTCCTTTCACAAC-3’ 5’- AAGCCACGGCTTGGTTTTCCTC -3	400bp
MMP-9	5’- TGGGCTACGTGACCTATGACAT -3 5’- GCCCAGCCCACCTCCACTCCTC -3’	173bp
MMP 3	5’-CTCACAGACCTGACTCGGTT-3’ 5-’ CACGCCTGAAGGAAGAGATG-3’	294bp
MMP 7	5’- TACAGTGGGAACAGGCTCAGG-3’ 5’-GGCACTCCACATCTGGGCT-3’	181bp
MMP8	5’-TGGACCCAATGGAATCCTTGC-3’ 5-‘ATAGCCACTCAGAGCCCAGTA-3’	544bp
MMP10	5’CGACAGAAGAGGTTCGTGCT3’ 5’CTTGGATAACCTGCTTGTACCTCAT3’	209bp
MMP11	5’CGACAGAAGAGGTTCGTGCT3’ 5’CTTGGCTGCTGTTGTGTGTGCT3’	710bp
MMP 13	5’-AAGATGCATCCAGGGGTCCT-3’ 5’-GTCCAGGTTTCATCATCATCA-3’	626bp
MMP 15	5’-CCATATGTCCACCATGCGTT-3’ 5’-ATGATGGCATTGGGGTTGCT-3’	627bp
TIMP1	5’- GGGGACACCAGAAGTCAACCAGA-3’ 5’- CTTTTCAGAGCCTTGGAGGAG	400bp
TIMP2	5’- GTTTTGCAATGCAGATGTAG-3’ 5’-ATGTGGAGAAACTCCTGCTT-3’	540bp

Gelatin Zymography

Cells were seeded into the 60 mm plate and treated with 15 μM SNAP for (0h, 6h, 8h, 12h, 24h). Subsequently, the conditioned medium was collected and gelatin zymographywere performed to examine the activities of MMP-2, MMP-9. Samples were mixed with loading buffer and electrophoresed on 10% SDS-polyacrylamide gel containing 0.1% gelatin. Electrophoresis was performed at 100 V for 3 h. Gels were then washed twice in Zymography washing buffer (2.5% Triton X-100 in double-distilled H_2O (pH 7.5) at room

temperature to remove SDS, followed by incubation at 37ºC for 36 h in Zymography reaction buffer (40 mM Tris–HCl (pH 7.5), 10mM CaCl2, 0.02% NaN3), stained with Coomassie blue R-250 (0.125% Coomassie blue R-250, 50% methanol, and 10% acetic acid) for 1h and destained with destaining solution (20% methanol, 10% acetic acid, and 70% double-distilled H_2O). Non-staining bands representing the levels of the latent form of MMP-2 and MMP-9 were quantified by densitometer measurement.

Western blot

Cells were cultured to confluence in 100 mm plates and treated using the drug at the concentration and time course stated. At the end of treatment, cells were harvested by scraping in ice-cold PBS. Cells were washed twice with PBS and pelleted by centrifugation. The total cellular protein was extracted with Radio Immuno Precepitation Assay buffer (50 mM Tris HCl, pH 7.4, 150mM NaCl, 1% Triton X 100, 1% Sodium deoxycholate, 0.1% SDS, 1mm PMSF, 1mM sodium orthovanadate, 1mM EDTA, 5µg/ml aprotinin and leupeptin) and stored in –70C^0. Protein samples were subjected to electrophoresis in 10% SDS polyacrylamide gel, separated proteins were electro blotted to nitrocellulose membrane, and the blot was blocked for 1h at room temperature with blocking buffer (0.1% TBST with 5% fat free dried milk powder). The membrane was then incubated with primary antibody (1:1000 dilution), at 4ºC overnight. The membrane was washed with TBST, and incubated with horseradish peroxidase-conjugated secondary antibodies (Cat. No: 31430, Pierce, USA) (1:2000 dilution) for 2h. The membrane was washed again and then incubated with ECL western blotting detection reageont (Cat.no:32106, Pierce, USA). The antibodies used were Phospho ERK (Catno:4370 Cell Signaling Technology), Phospho JNK (Cat:no:4668, Cell SignalingTechnology) Phospho p38 (Cat no:4511, Cell Signaling Technology) β Actin (Cat.no:A 5316, Sigma, USA).

MMP-2 reporter assay

MMP-2 luciferase plasmid and deletion mutant was obtained as kind gift from C.D.Kim, School of Medicine, Korea. Luciferase reporter assays were performed by Dual Luciferase Reporter Assay System (Cat.no: E1910, Promega, Madison, WI) after 24 h post-transfection and 4h treatment following the manufacturer's instruction. Cells were washed with phosphatebuffered saline (PBS) and lysed in lysis buffer. Cell debris was removed by centrifugation at 10000 rpm for 1 min. Twenty microliters of the supernatant were mixed with 100 ml of luciferase substrate and measured by luminometer (Sirius, Berthold detection systems, Germany). Fire-fly luciferase activity was normalized for transfection efficiency using Renilla Luciferase activity (pRL-SV40).

15. Detailed analysis of Results

Exogenous nitric oxide production

Initially we screened SW 480, SW620, HCT 115, Colo 320, Colo 205 and WiDr cell lines for endogenous nitric oxide production. Out of which we selected SW480, SW 620, WiDr for the preliminary experiment. Among the three colon adenocarcinoma cell lines (SW480, SW620, and WiDr) WiDr showed low endogenous level of nitric oxide production (Figure 1). Hence we used WiDr cells for our all future experiments with nitric oxide donor.

Cytoprotective effect of SNAP on WiDr

Cell viability assay showed that SNAP at different concentrations for 4h exhibited a specific dose-dependent effect on colon cancer cell line WiDr. The results clearly indicate SNAP

at 15 μM for 4 h increased the proliferation by ~25% (Figure 2). MTT assay provided preliminary information about the effect of lower concentration of nitric oxide donor in the proliferation of WiDr cells. Above 62.5 □M of SNAP, the released NO exerted cytotoxic effects.

Estimation of nitric oxide production using modified Griess assay

Nitrite released by 15 μM of SNAP was analyzed by Griess method and found that it was

175 nM (Figure 3). This amount was equal to the physiological concentration of endogenous nitric oxide released from the tissues.

SNAP induced migration of WiDr cell was inhibited by ODQ

The migration inducing capacity of nitric oxide was proved by modified Boyden chamber migration assay. Treatment of SNAP for 4h induced migration in WiDr cells, but this SNAP induced migration was inhibited when the SNAP was co treated with ODQ, a known guanyl cyclase inhibitor (Figure 4). This result suggests that, NO induced cell migration through a cGMP dependent mechanism. This was confirmed further by using scratch wound healing motility assay. Treatment of SNAP for 4h effectively increased themigration of cells into the wounded area. Similarly treatment with ODQ inhibited SNAP induced migration (Figure 5). In order to prove the involvement of cGMP and proteinkinase G, we used a specific inhibitor (KT 5823) to inhibit the NO induced pathways. KT 5823, (proteinkinase G inhibitor) inhibited SNAP induced cell migration when cells were co treated with SNAP and KT 5823, indicating the involvement of proteinkinase G in SNAP induced WiDr cell migration. 8BrcGMP, a cGMP analogue induced cell migration comparable to the action of SNAP, specifies that the mode of action of SNAP is through cGMP pathway. This pathway was further confirmed by inhibition of 8BrcGMP induced migration by KT 5823. This correlate with the results obtained from the modified Boyden chamber migration assay.

SNAP induced overexpression of MMPs mRNA

MMPs are a family of key zinc-dependent enzymes involved in the degradation of extracellular matrix in cancer metastasis; we examined the expression of several important MMPs induced by 4h exposure of SNAP in WiDr cells. Semiquantitative RT- PCR revealed that the expression of MMP-2 and MMP-9 was increased in a timedependent manner in SNAP treated WiDr cells compared with control cells. SNAP induced a differential expression of MMPs in WiDr cells. The results point to the fact that not all MMPs are get activated in presence of SNAP in WiDr cells. Among the ten MMPs,MMP-2 and 9 showed maximum expression at the same time MMP-3 and MMP-10 also showed upregulation but it is lesser compared to MMP-2 and MMP-9. All other MMPs showed not much variation on SNAP treatment (Figure 6 A & B). The results suggest possible involvement of the above said MMPs in colon cancer migration. ODQ inhibited SNAP induced activation of MMP2/9mRNA which suggests the involvement of cGMP in SNAP induced activation of MMPs (Figure 7A & B).

Involvement of cGMP in SNAP induced migration

By cGMP competitive ELISA, it was found that the levels of cGMP were increased on treatment with SNAP (2h and 4h) and the levels considerably decreased once co incubated with ODQ. It can be understood that on SNAP treatment, the cGMP levels go high in the cells and upon incubation with ODQ, the levels are brought back to that of theuntreated control. From the standard curve (Figure 8), cGMP concentration obtained is given below:

	Parameters	Concentration cGMP (pmols)
1	Control	0.13 ±.014
2	SNAP (2h)	0.35±.014
3	SNAP (4h)	0.85±.035
4	SNAP + ODQ (4h)	0.15±.014

Thus this experiment gives indications that effector functions of NO may be through the modulation of cGMP mediated signaling event

TIMP1/2 m RNA levels down regulated by SNAP

Partial regulation of MMPs in the biological system is by tissue inhibitors of matrix metalloproteinases (TIMPs). We examined the mRNA levels of TIMP1/2 in SNAP treated cells and found that there is a time dependent down regulation of both TIMP1/2 (Figure 9). Thus SNAP upregulates MMP2/9 partially via by the down regulation of TIMP1/2. They act as key MMP2/9 inhibitors in the biological system via 1:2 stochiometric binding.

SNAP Induced regulation of MMP-2 via AP1

To further investigate the requirements of the transcription factors for SNAP induced MMP-2 expression, the cis-reporter plasmids for wild type MMP-2, AP-1 mutated MMP-2 and NF-κB mutated MMP-2 was transfected into WiDr and then the reporter activity was measured. As shown in figure 10 SNAP increased the transcription activity of WT MMP-2 in WiDr compared to the untreated cells. Moreover, the increased MMP-2 promoter activity by SNAP was abolished to a significant extent in mutated AP1 binding site in the MMP-2 promoter region, but not by mutation of the NFkB binding site demonstrating participation of AP1 in SNAP induced MMP-2 transcription.

SNAP induced gelatinase activity

Gelatin zymography assay revealed that there is a basal level of gelatinase activity in WiDr cells. On SNAP treatment a marked increase of MMP-2 and MMP9 activity in atime dependent manner in the conditioned medium of WiDr cells (Figure 11 A & B).

SNAP upregulates pERK and downregulates pP38 and pJNK signaling in WiDr cells

To determine whether ERK signaling is required for SNAP induced MMP-2/9 expression in WiDr cells the lysates from the same cells were immunoblotted for the activated forms of ERK1/2 with an antibody specific for phosphorylation on residues Thr202 and Tyr204. Levels of activated pERK were increased at the same time there is a decrease in pP38 and pJNK in SNAP-treated cells (Figure12 A & B), whereas levels of β actin protein were unchanged. These results demonstrate that signaling via MAPK is necessary for SNAP induced MMP-2/9 expression in WiDr cells. ODQ decreased SNAP induced activation of ERK1/2 (Figure13 A & B). Thus it confirms the involvement of cGMP in SNAP induced phosphorylation of ERK1/2 and further upregulation of MMPs.

Figure 1

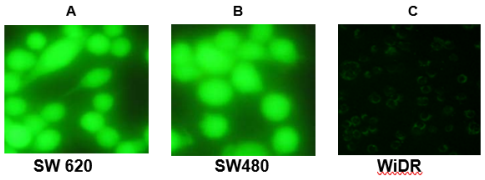


Figure 2

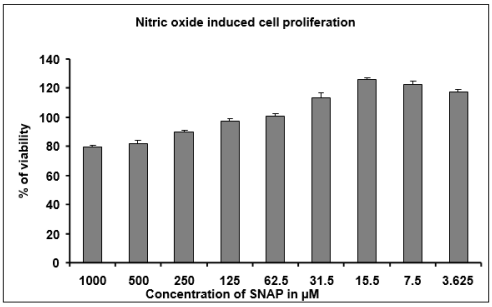


Figure 3

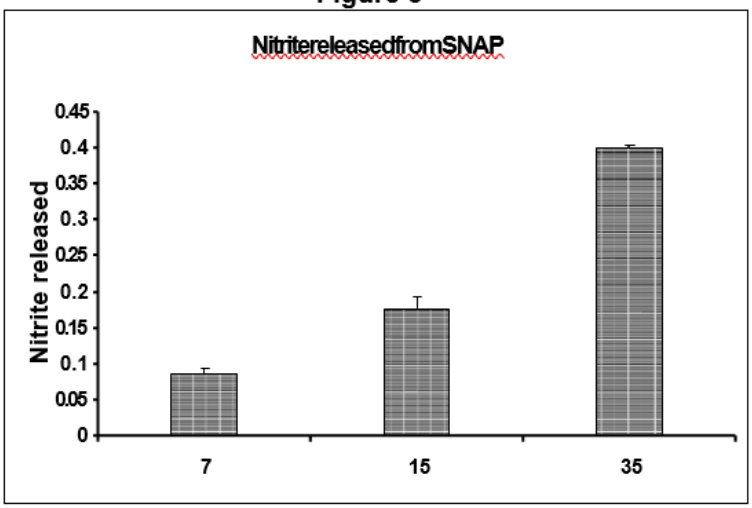


Fig.1

Endogenous nitric oxide formation

Representative photograph of DAF-2DA-preloaded in three colon adenocarcinoma cell lines incubated for 15min. A. SW 620 shows high level of endogenous NO production. B. SW 480 shows high level of endogenous NO production. C. WiDr cells show very low level of endogenousNO production. The photographs are representative of at least three separate experiments with similar outcomes.

Fig.2

SNAP increases the proliferation of WiDr cells

WiDr cells were cultured with various concentrations of SNAP (3.625 µM -1000µM). After 2 days, cell proliferation was measured by MTT assay. Results represent the mean ±SD of three experiments performed in triplicate. SNAP increases proliferation of WiDr cells in a concentration dependent manner.

Fig.3

SNAP induced transwell migration was inhibited by ODQ

A. Migration experiments were carried out in Transwell migration plates equipped with 6-mm inserts with 8-µm pore size. The same initial cell number (5×10^4) was used in all experiments. After 24h at 37°C, cells on the bottom membrane surface were stained with crystal violet (A) Control WiDr cells showed normal transwell migration. B. SNAP (15µM) treated cells showed increased migration and which is inhibited by ODQ. C. Cells in 5 contiguous microscope fields were counted at 20X magnification in Olympus inverted microscope.

Figure 4

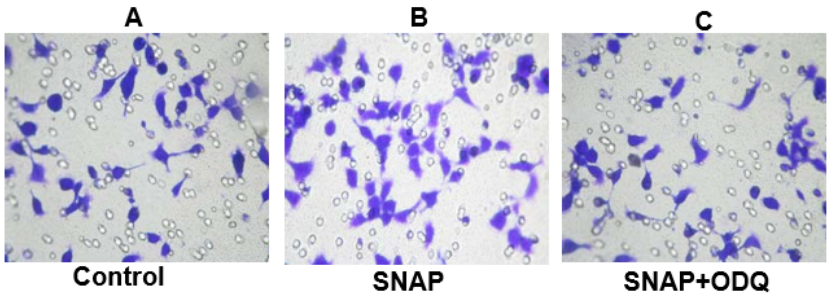


Figure 5

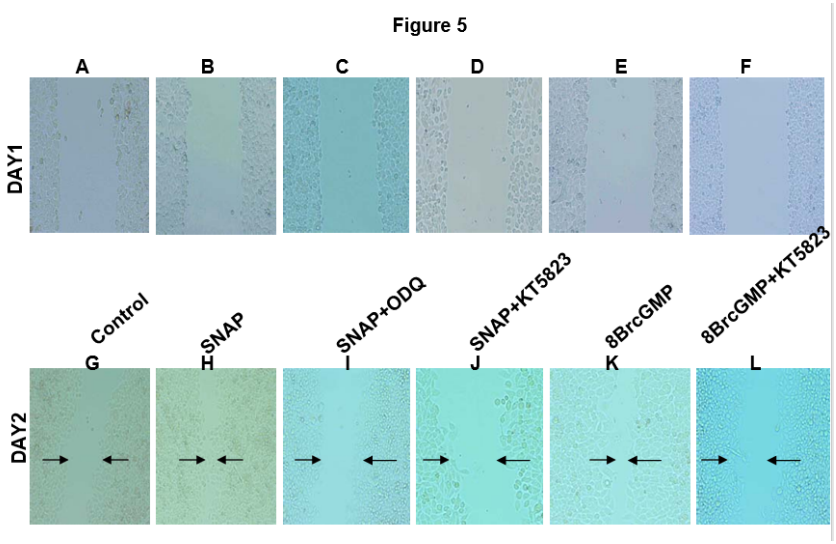
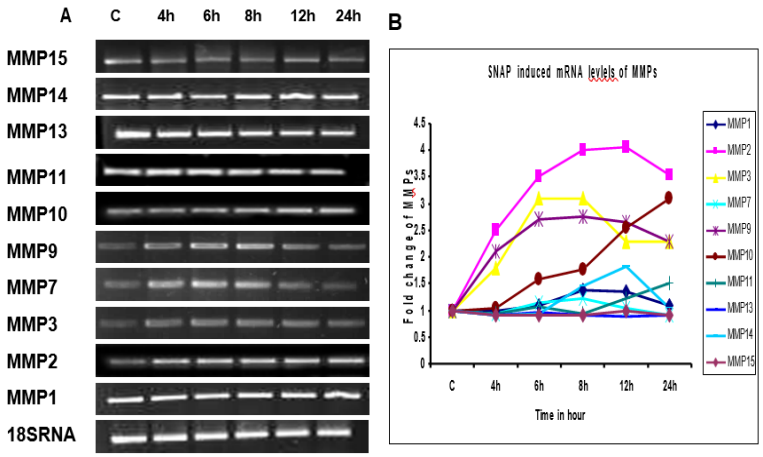


Figure 4 and 5

SNAP treated WiDr cells in scratch wound migration assays

A-F. Images of scratch in confluent monolayer cells in day 1(G)/ Control cells showing normal migration.H. SNAP treated cells showing increased migration. I. ODQ; Cgmp inhibitor decreases SNAP induced migration. J. KT-5823 (180nM), a PKG inhibitor decreases SNAP induced migration. K. 8 BrcGMP (1mM), cGMP analogue increases migration. L. KT-5823 decreased 8BrcGMP induced migration

Figure 6



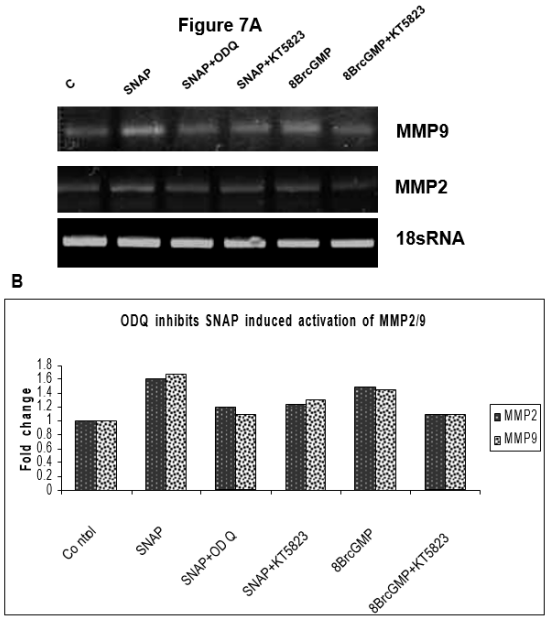


Fig.6 Levels of expression of MMPs in WiDr exposed to SNAP

A. WiDr cells exposed to SNAP at the concentrations indicated for different time points. The level of expression of the mRNA of MMP2 and 9 were increased in SNAP treated cells detected by RT-PCR in different time course. The mRNA levels of different MMPs was normalized with β actin mRNA. Each experiment has been carried out at least three times. B. Line graph representationof mRNA levels was measured by quantity one (Biorad) designed densitometry values from the above and two other representative RT-PCR from independent experiments.

Fig.7 SNAP increased MMP2/9 mRNA levels was inhibited by ODQ

ODQ inhibited SNAP induced activation of MMP2/9 mRNA levels. Further we confirmed the role of cGMP pathway in SNAP induced activation of MMP2/9mRNA levels using KT5823 which againinhibits SNAP induced activation. 8BrcGMP, a cGMP analogue up regulates MMP2/9 expression which is inhibited by KT5823. (7B) Histographic representation of mRNA levels was measured by quantity one (Biorad) designed densitometry values from the above and two other representative RT-PCR from independent experiments.

Figure 8

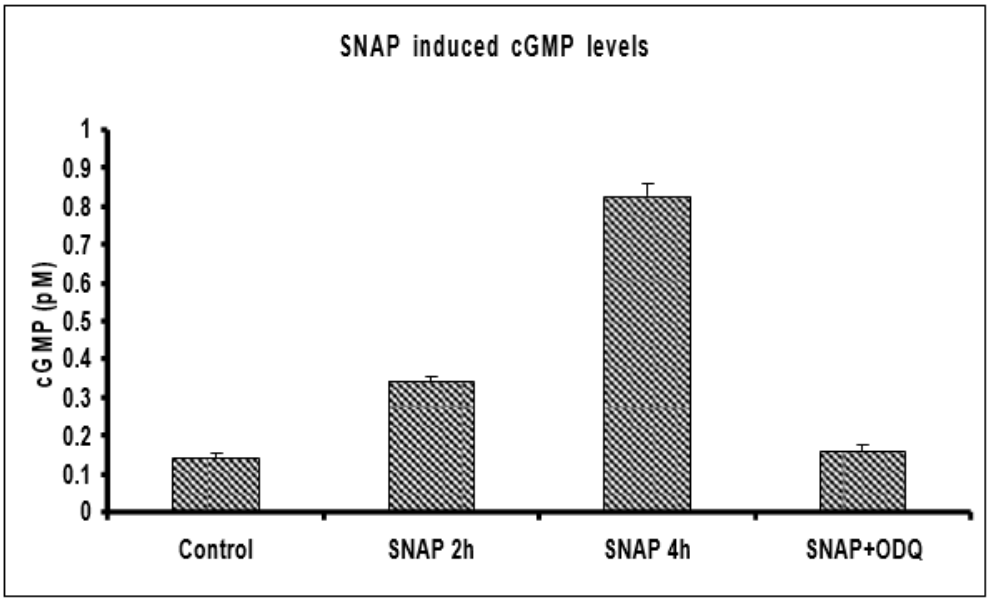
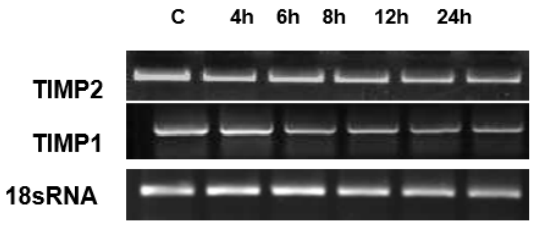


Figure 9 A



B

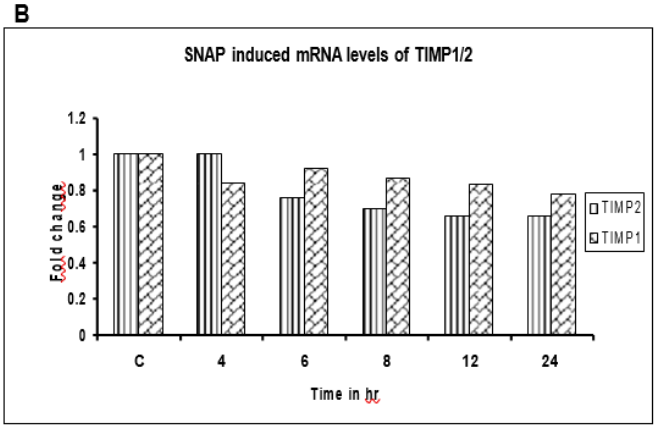


Fig.8 SNAP increased cGMP levels was inhibited by ODQ

SNAP (15µM) increased cGMP levels in a time dependent manner. There is a considerable increase in cGMP levels on SNAP treatment in 2h and 4h increased cGMP levels is partially reverted on ODQ treatment.

Fig.9 SNAP induced down regulation of TIMP1/2

A. WiDr cells exposed to SNAP (15µM) induced down regulation of mRNA levels of TIMP1/2 in atime dependent manner. B. Histographic representation of mRNA levels was measured by quantity one (Biorad) designed densitometry values from the above and two other representative RT-PCR from independent experiments.

Figure 10

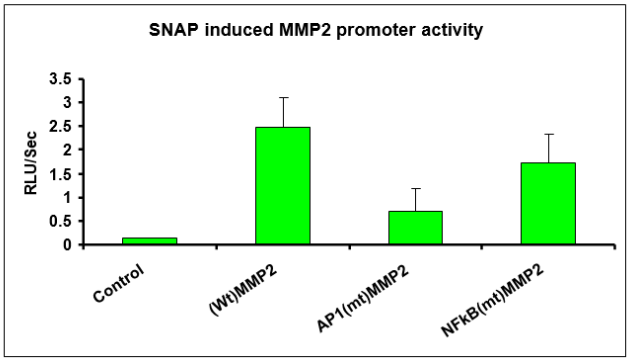
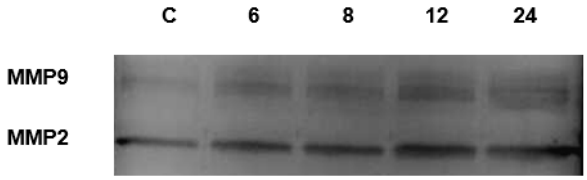


Figure11 A



B

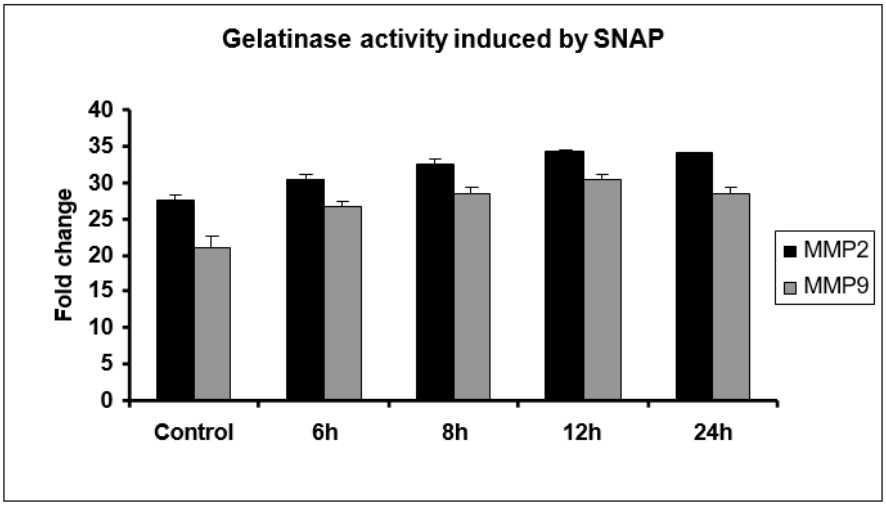


Fig.10 SNAP enhances transcriptional activity of MMP-2 in WiDR

WiDr cells were transfected with wild-type (WT), or mutant promoter for NF-κB, and AP-1 binding sites for 36h, and then MMP-2 promoter activity was measured in cells stimulated with SNAP (15μM) for 12h by fire fly luciferase. Promoter activity was assayed and normalized to the Renilla luciferase activity. Luciferase activity is represented as the mean ±SD from three independent experiments.

Fig.11 SNAP increases gelatinolytic activity of MMP2/9

A. Gelatin zymography was performed to detect MMP 2/9 activity in culture supernatant after SNAP (15μM) in a time dependent manner. Gelatinolytic activity increased in a time dependent manner compared to control. The figure is a representative of three independent experiments. B. Histographic representation of activity of MMP-9 and MMP-2 was measured by quantity one (Biorad) designed densitometry values from the above zymography and two other representative zymograms from independent experiments.

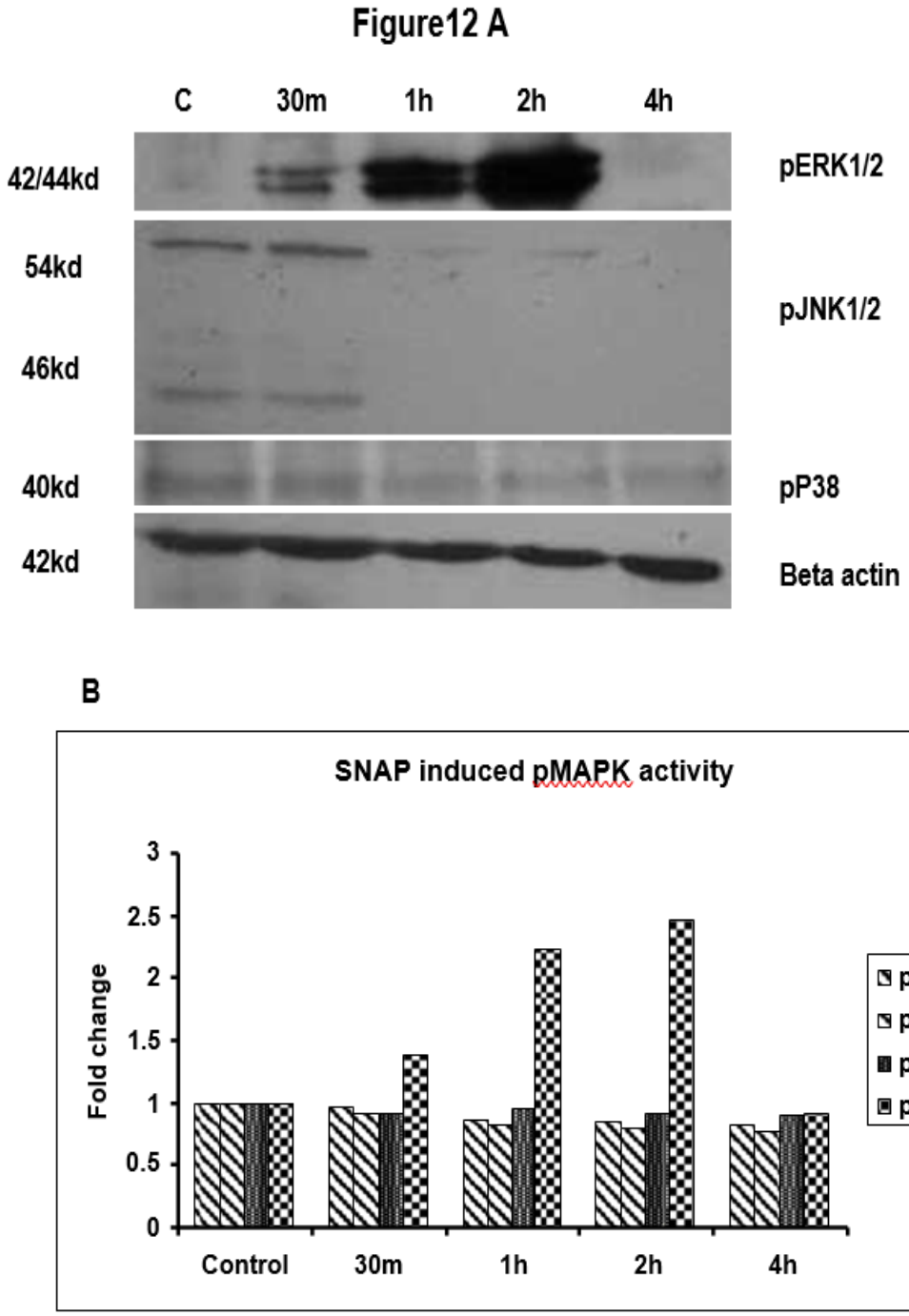
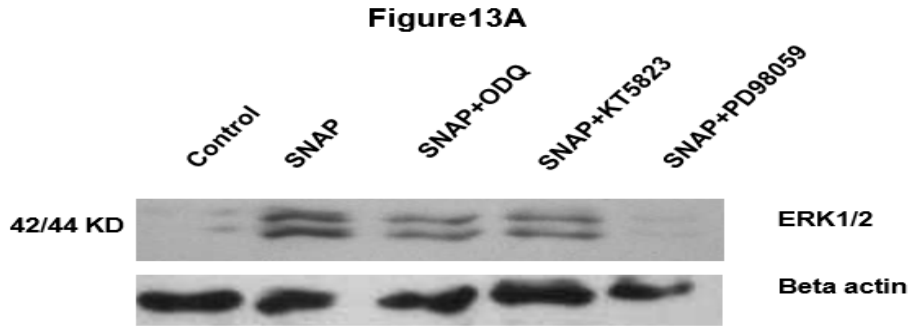


Fig.12 SNAP induced MAPK activity in WiDr cells

A. Representative Western blot analysis of phosphorylated-ERK1/2 (pERK1/2), phosphorylated JNK(pJNK) phosphorylated P38 (pP38) levels in control and SNAP treated WiDr cells in different time points .Protein lysates were then prepared at the indicated times and subsequently Western blotted for pERK, pJNK, pP38. Blots were stripped and re-probed for β actin. The accompanying graphs are densitometric representations of the western analysis showing relative pERK, pJNK, pP38: β actin levels at the indicated times. ODQ, a cGMP inhibitor inhibits partially the SNAP induced ERK phosphorylation. KT5823, a protein kinase G inhibitor inhibits SNAP induced ERK1/2 phosphorylation. B. Corresponding graph represents densitometry representations of thewestern blot analysis showing pMAPK: beta actin levels.



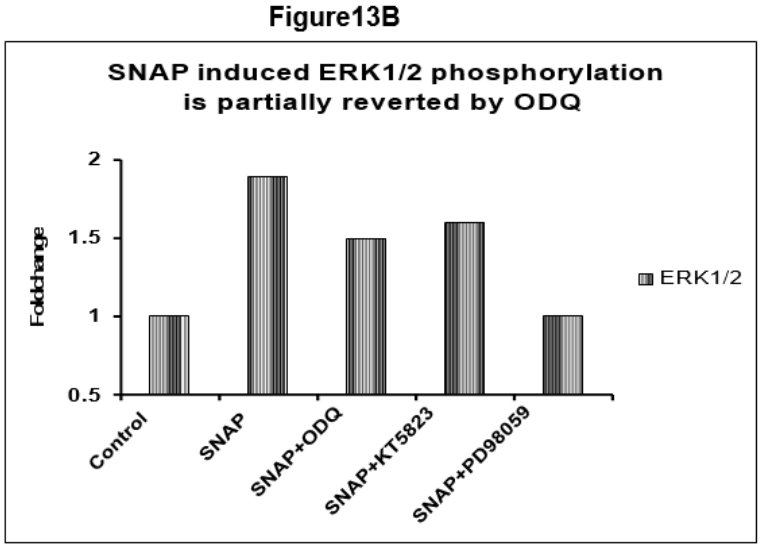


Fig.13 SNAP induced activation of ERK1/2 is via cGMP

A. ODQ inhibits partially the phosphorylation of ERK1/2 in WiDr cells. Involvement of cGMP pathyway is again confirmed using KT 5823, a protein kinase G inhibitor. Further the specificity ofERK1/2 activation was confirmed by PD98059 (10μM), an ERK1/2 inhibitor. B. Corresponding graph represents densitometry representations of the western blot analysis showing pERK1/2: beta actin levels.

15.Science and Technology benefits accrued:

a. List of Research publications with complete details: 2

1. Suboj Babykutty, Priya S, Priya Srinivas, Asha S Nair, K Chandramohan, Srinivas Gopala. Insidious role of nitric oxide in migration/invasion of colon cancer cells by upregulating MMP-2/9 via activation of cGMP-PKG-ERK signaling pathways. ***Clinical and Experimental Metastasis*** 29: 471-492, 2012.
2. Suboj Babykutty, Priya Prasanna Sathiadevan, Nandini RJ, Suresh Kumar MA, Mangalam S Nair, Priya Srinivas, **Srinivas Gopala**. Nimbolide retards tumor cell migration, invasion and angiogenesis by downregulating MMP-2/9 expression via inhibiting ERK1/2 and reducing DNA binding activity of NF-κB in colon cancer cells. ***Moleclar Carcinogenesis***, 51: 475-490, 2012.

b. Manpower trained on the project:

i. Research Scientists or Research Associates : nil

ii. No. of PhD's produced : 1

iii. Other Technical Personnel trained :

c. Patents taken, if any : Nil

d. Products developed, if any : Nil

16. Procurement of/Usage of Equipment:

a. Details of Equipment:

Sl.No.	Name of equipment	Make/Model	Cost (Rs.)	Date of Installation	Utilisation	Remarks regarding maintenance breakdown
1	Luminometer	Berthold Technologies	8850 Euro	12/12/2005	Using	working

b. Suggestions for disposal of equipment(S): equipment in working condition

(Name and Signature of PI)