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Emodin mediated down regulation of matrix metalloproteinases (MMPs) in SNP treated colon cancer cells

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ABSTRACT

The degree of over expression of MMP2 and MMP9 (gelatinases A and B) has been noted to correlate with the stages of disease or prognosis and demonstrated early in the transition from colon adenoma to adinocarcinoma. Elevated levels of distinct MMPs detected in tumor tissue or serum of patients with advanced cancer and they are the major prognostic indicators in cancer. Inhibition of MMPs has been explored as a therapeutic goal for almost two decades. Nitric oxide (NO), a free radical plays an important role in signaling pathways regulate MMP expression. MMPs are under the control of NO system. The present study demonstrated the role of exogenous NO levels in the regulation of MMP 2 and 9 in colon cancer cell line WiDr and its inhibition with emodin (a naturally occurring anthraquinone).

Keywords: Matrix metalloproteinase, colon cancer, emodin, sodium nitroprusside, nitric oxide.

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INTRODUCTION

Cancer cells from malignant tumors move and continue to duplicate forming new tumor, a process called metastasis. Tumor invasion is controlled by various Matrix metalloproteinases (MMPs) that degrade extra cellular matrix (ECM) components [4, 10]. These enzymes are manufactured by cells within the tumor associated stroma. Proteolytic enzymes involved in the degradation of ECM, play a relevant role in tumor progression by providing a permissive micro environment for tumor invasion and metastasis. Gelatinase A (MMP2) and gelatinase B (MMP9) play a key role in the proteolytic cascade leading ECM cleavage during metastasis in colon carcinoma. [1]. Colon cancer is the third most common form of cancer and the second leading cause of death among cancer patients. Nitric oxide (NO) is a free radical generated by a family of nitric oxide synthases (NOs). NO has important role in the regulation of tumor angiogenesis and metastasis. The presence of NO in the tumor microenvironment promotes metastasis [5]. NO activates MMPs and the subsequent loss of ECM integrity would promote angiogenesis. The ability of NO to inhibit apoptosis at low levels and promote the apoptosis cascade at high concentrations indicates dual role of this free radical in tumor biology. Emodin, a naturally occurring anthraquinone present in roots and barks of Chinese herbs such as *Polygonum cuspidatum* and *Rheum officinalae* having anti-inflammatory and antitumor activity [11]. Emodin can induce apoptosis in several cancer cells. Recently, several scientific studies of its biological activity have been performed regarding the anti proliferative effect of emodin on several human cancers such as multiple myeloma and lung squamous cell carcinoma [7]. The presence of hydroxyl groups in position 1, 3 and 8 of the aromatic ring system is essential for the purgative action of the compound [2]. Most of the pathological conditions involve hyper activity of MMPs and the protective effects could be related to the presence of emodin. In the present study we took emodin to evaluate its anti metastatic ability in colon cancer cells.

MATERIALS AND METHODS

Cell culture

Cells of human colonic adenocarcinoma cell line WiDr was grown at 37ºC in a humidified atmosphere containing 5% CO_2. Cells were cultured in DMEM (Dulbecco's modified eagle medium), which is supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin. When the cells were grown to confluency, the medium was removed and washed once with PBS (phosphate buffered saline), 0.25% Trypsin-EDTA solution was added and cells were dispersed gently by a pipette. A known number of cells were dispensed in to new microtitre plates for further experiments.

MTT-Cell proliferation assay

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To find out the non cytotoxic concentration of exogenous nitric oxide releaser - sodium nitroprusside (SNP) and emodin in colon cancer cell line. MTT assay [9] is based on the metabolic activities of the viable cells. In this assay a tetrazolium salt MTT (3-(4,5-Dimethyl-2-thiazolyl))-2,5-diphenyl-2H-tetrazolium bromide) was used. Briefly, WiDr cells were seeded in to a 96-well microtitre plate. Exogenous NO donor SNP and drug emodin were added in different concentrations in quadruplicate (62.5 to 500 µM for SNP and 6.25 to 50 µM for emodin). Six wells contained cells in drug free medium to determine the control cell survival and the percentage of live cells after culture. Stock concentrations of drug were prepared in dimethyl sulfoxide and diluted concentrations were made in the medium added. The cells were incubated for 48 hrs at 37ºC. A fresh medium was added along with 20 µl MTT to each well. The plates were kept for 2½ hrs incubation in dark. The formazan crystals formed were solubilized with acidified isopropanol. The plates were kept for 10 min to solubilize the crystals. The colour developed was quantitated with an ELIZA plate reader at 570 nm.

Wound healing assay

This experiment was performed to detect the directional cell migration *in vitro* under the influence of SNP and emodin [8]. The assay detects the directional cell migration *in vitro* under the influence of SNP and emodin. Cells were cultured in a 24 well plate. After 24 hrs a wound was created in the cell monolayer using a micropipette tip. Cells were treated with and without 300 µM SNP, 300 µM SNP and 6.25 µM emodin. Images were viewed at 0 hr and after 24 hrs using light microscope.

Gelatin Zymography

The protein expression of MMP2 and 9 was detected by gelatin zymography [6]. Since gelatinases are secreted proteases, the cell culture supernatant was taken for this assay. Cells were grown in 100 mm plates and treated with and without 300 µM SNP, 300 µM SNP and 6.25 µM emodin for 24 hrs. After incubation, cell supernatant was taken, filtered and centrifuged at 5000 rpm for 30 min at 15ºC. 7.5 µl of the concentrated sample was mixed with equal amount of 2x non reducing sample buffer and electrophoresed on 10% SDS-PAGE co-polymerized with gelatin (2 mg/ml). Following electrophoresis, gel was washed with rinse buffer to wash away SDS, thus allowing the gelatinase to renature. It was placed in reaction buffer for 24 hrs to facilitate gelatin degradation by gelatinase. Then the gel was incubated for 45 min in Coomassie brilliant blue stain and destained. The gelatinolytic activities were detected as white bands.

RESULTS

A. Cytotoxic profile of SNP on WiDr cell line.

The result was expressed as the mean percentage of control ± SD of quadruplicate determinations from the independent experiments. The cytotoxic profile showed that SNP at a concentration below 300 µM showed a little effect on cell viability, hence we used 300 µM for

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further experiments to evaluate the role of NO in modulating the expression of MMP2 and MMP9 in the above cell line.

Table 1: Cytotoxicity of SNP on WiDr

SNP (µm)	OD values		Average	SD
62.5	86	88	87	1.414
125	80	84	82	2.828
250	75	77	76	1.414
500	64	60	62	2.828

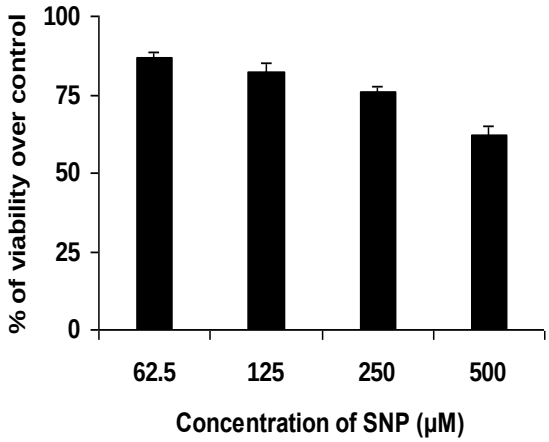

Fig-1. MTT assay of SNP on WiDr

B. Cytotoxic profile of Emodin on WiDr cell line

Emodin at a concentration below 6.25 µM showed a little effect on cell viability and a concentration above this showed a considerable cytotoxic effect on WiDr cell line. Therefore, a concentration of 6.25 µM is used for further experiments.

Table 2: Cytotoxicity of emodin on WiDr

Emodin (µm)	OD values		Average	SD
6.25	79	66	72.5	9.192
12.5	67	54	60.5	9.192
25	52	48	50	2.828
50	35	33	34	1.412

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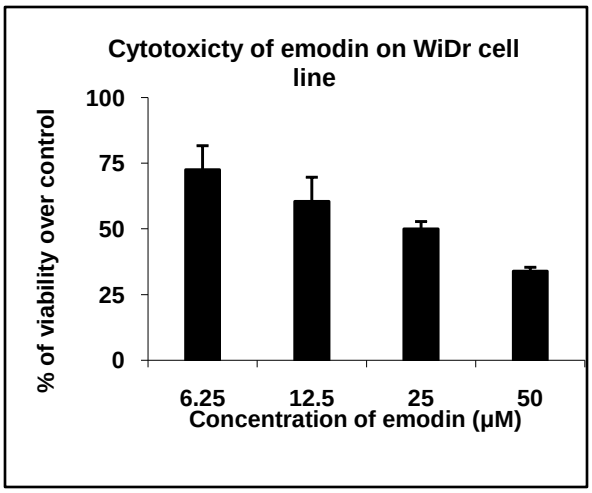

Fig-2. MTT assay of emodin on WiDr

C. Inhibition of SNP induced migration by emodin

Ability of WiDr cells to migrate under the influence of SNP and emodin was visualized using wound healing assay. Migrations of SNP treated cells (300 µM) showed a considerable increase in migration compared to control. Emodin at a concentration 6.25 µM exhibited a significant inhibition of SNP induced migration.

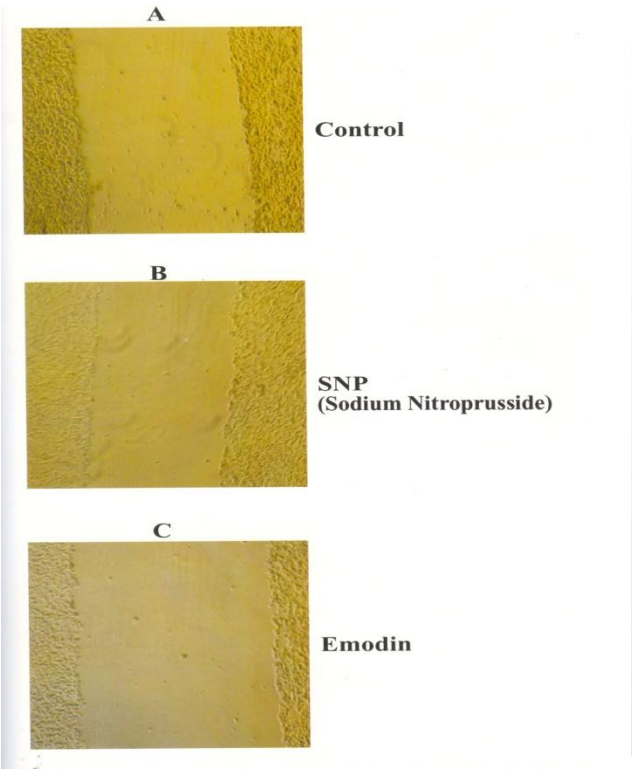

Fig- 3. Wound healing assay of SNP induced migration of WiDr cells by Emodin at 0 hr.

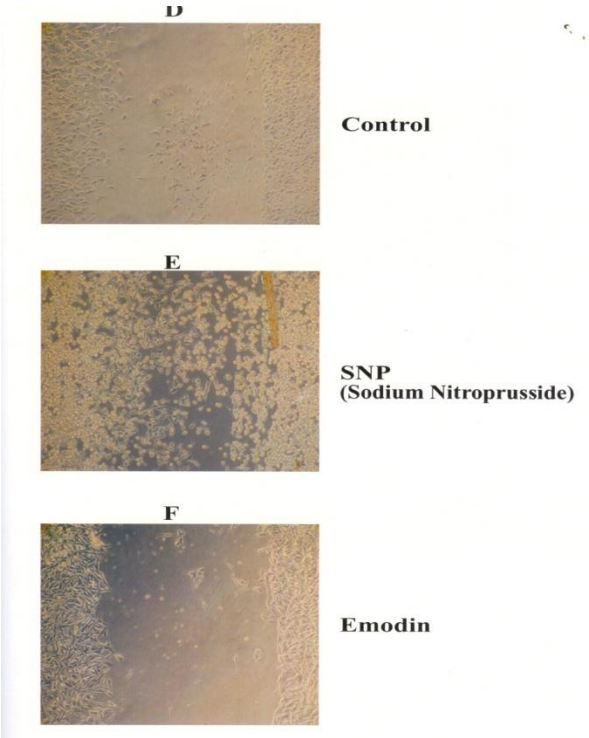

Fig- 4. Wound healing assay of SNP induced migration of WiDr cells by Emodin after 24 hrs.

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D. Effect of emodin on MMP2 and MMP9 protein expression

The gelatinolytic bands present in SNP treated lane showed much clear bands in a concentration dependent manner compared to the control. This indicates that SNP (300 µM) treatment for a period of 24 hrs increased MMP2 and 9 expression in WiDr cell line compared to that in the untreated cell. Treatment of emodin at a concentration of 6.25 µM decreased SNP induced protein level of MMP 2 and 9.

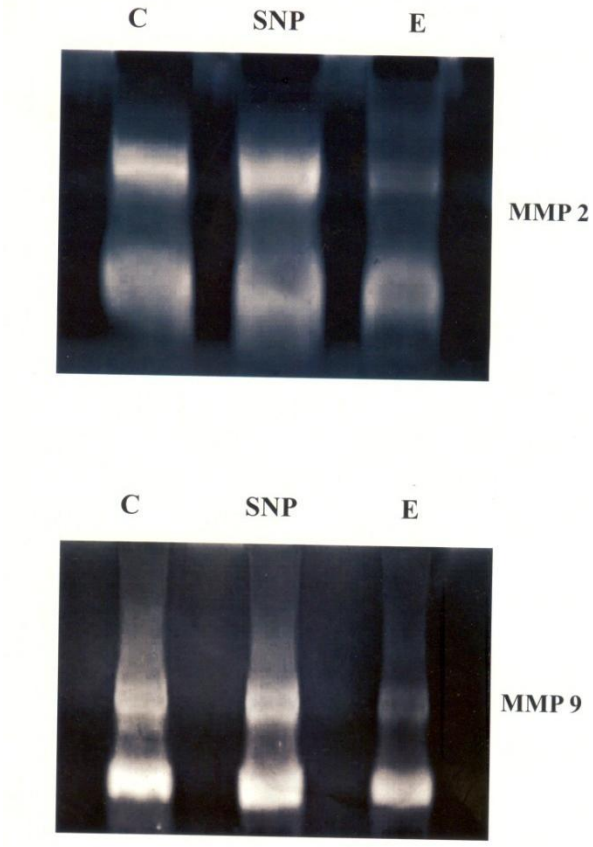

Figure 5. Zymographic analysis for detecting the effect of emodin on MMP2 and MMP9 expression in WiDr cell line

C-Control, SNP-Sodium nitroprusside, E-Emodin

DISCUSSION

Formation and expression of MMPs in tumor cells play an important role in tumor progression [12]. Initially we studied the least cytotoxic concentration of SNP and emodin in WiDr cells using cell viability assay. NO released from SNP at higher concentration can kill the cells [3]. So we used the derived concentrations for further experiments. Our preliminary observation was wound healing assay in order to detect the directional cell migration *in vitro* under the influence of SNP and emodin. The assay gave an indication of increased cell migration

in SNP (300 µM) treated cells and its subsequent inhibition in emodin (6.25 µM) treated cells. Further we confirmed our results at the protein level using gelatin zymography in three different conditions such as untreated, SNP treated and SNP treated+emodin. Increased gelatinolytic activity of MMP2 and MMP9 in SNP treated culture supernatant shows the role of NO in increasing prometastatic events leading to the digestion of extracellular matrix components. Decreased gelatinolytic activity in emodin treated cells shows the involvement in the down regulation of MMP2 and MMP9, and thereby inhibiting cell migration. Therefore NO, MMP2, MMP9 and emodin axis could be a potent therapeutic target in colon cells. Further characterization of emodin mediated down regulation of MMP2 and MMP9 in colon cancer was important subject for future research.

CONCLUSION

MMP 2 and 9 (gelatinase A and B) are very important in colon cancer progression. Inhibition of these MMPs by synthetic MMP inhibitors (MMPIs) has been considered to be an effective approach to block colon cancer. NO, plays a lead role in signaling pathways have an important role in the regulation of colon cancer. Tumor cell derived NO promotes the expression of angiogenic factors. Emodin, suppresses the NO mediated up regulation of MMP2 and 9. Emodin can be targeted as an effective anti metastatic agent in NO induced tumor progression. Therefore elucidation of critical pathways in metastasis, where emodin could exert its inhibitory effects should make it a perfect fit for anti tumor therapy.

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