RESEARCH ARTICLE

Induction of Apoptosis by Eugenol and Capsaicin in Human Gastric Cancer AGS Cells - Elucidating the Role of p53

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Abstract

   <u>Background</u>: Loss of function of the p53 gene is implicated in defective apoptotic responses of tumors to chemotherapy. Although the pro-apoptotic roles of eugenol and capsaicin have been amply reported, their dependence on p53 for apoptosis induction in gastric cancer cells is not well elucidated. The aim of the study was to elucidate the role of p53 in the induction of apoptosis by eugenol and capsaicin in a human gastric cancer cell line, AGS. <u>Materials and Methods</u>: AGS cells were incubated with or without various concentrations of capsaicin and eugenol for 12 hrs, in the presence and absence of p53 siRNA. Cell cycling, annexin V and expression of apoptosis related proteins Bax, Bcl-2 ratio, p21, cyt c-caspase-9 association, caspase-3 and caspase-8 were studied. <u>Results</u>: In the presence of p53, capsaicin was a more potent pro-apoptotic agent than eugenol. However, silencing of p53 significantly abrogated apoptosis induced by capsaicin but not that by eugenol. Western blot analysis of pro-apoptotic markers revealed that as opposed to capsaicin, eugenol could induce caspase-8 and caspase-3 even in the absence of p53. <u>Conclusions</u>: Unlike capsaicin, eugenol could induce apoptosis both in presence and absence of functional p53. Agents which can induce apoptosis irrespective of the cellular p53 status have immense scope for development as potential anticancer agents.

Keywords: Apoptosis - p53 - eugenol - capsaicin - AGS cell line

Introduction

   Evasion of cell death is one of the important hallmarks of malignant transformation and hence provides an attractive opportunity for intervention. To this day, induction of apoptosis is considered to be a key strategy in the treatment of cancer. A host of literature suggests that the success of many anticancer therapies lies in their ability to generate a potent pro-apoptotic stimulus. However, due to the severe side-effects of conventional chemotherapies, phytochemicals from dietary sources are receiving increasing prominence as potential sources of more compatible anticancer drugs. A myriad of literature encompassing both in vitro and *in vivo* studies indicate apoptosis induction as a mechanism of action for a wide range of dietary phytochemicals.

   Eugenol (4-allyl (-2-methoxyphenol)), a phenolic compound, found abundantly in the essential oil of a commonly consumed spice, clove (*Syzgium aromaticum*), is one such phytochemical. It has been exploited for various medicinal applications such as antiseptic, analgesic, antioxidant, antiviral and antibacterial agent (Pramod et al., 2010). Furthermore, many recent studies have explored the anticancer potential of eugenol and its ability to induce apoptosis in diverse cancer cell

lines as well as in *in vivo* tumor models (Jaganathan and Supriyanto, 2012; Majeed et al., 2014).

   Capsaicin is yet another phytochemical found naturally as the pungent constituent of hot chilli peppers of the genus Capsicum (family Solanaceae). There are various reports which highlight the physiological and pharmacological properties of capsaicin including its anticancer property. The capacity of capsaicin to suppress the growth of cancer cells is considered to be primarily mediated through induction of apoptosis (Lin et al., 2013).

   Despite increasing evidences and molecular unmasking of apoptotic induction by the above mentioned phytochemicals, the involvement of p53 in the apoptotic cascade induced by eugenol and capsaicin in gastric cancer cells is not very clear. Although a fair number of studies have focused on the anticancer potential of capsaicin and eugenol, the influence of p53 status on induction of apoptosis by these two phytochemicals is not well characterized. Various mechanisms for the induction of apoptosis have been proposed for capsaicin and eugenol. While, several studies have reported that capsaicin induces apoptosis by upregulating p53, there are also few instances of p53 independent induction of apoptosis by capsaicin (Mori et al., 2006). Similarly, there are reports of both p53 dependent as well as independent induction of apoptosis

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by eugenol in diverse cellular systems (Al-Sharif et al., 2013).

The purpose of this study is to elucidate the mechanism of apoptotic induction by eugenol and capsaicin in gastric cancer cells and to investigate the outcome of treatment by these phytochemicals in the presence and absence of p53.

Materials and Methods

Chemicals

Annexin V-assay Kit was purchased from (Abcam, USA). Cycle TEST PLUS DNA reagent kit procured from Becton Dickinson Immunocytometry system (San Jose, CA). p53 siRNA transfection kit (Dharmacon ON-TARGET Plus siRNA) was purchased from GE Healthcare. Anti-mouse anti-bodies against p53, p21, Bax, Bcl-2, Cyt c, caspase-3, caspase-8, procaspase-9 and PCNA, were procured from Santa Cruz (USA), bacitracin, leupeptin, pepstatin A, PMSF, phosphatase inhibitor cocktails. A-Sepharose beads, RNase, NAC and NBT were purchased from Sigma (St. Louis, MO). Nitrocellulose membrane, and filter papers were obtained from Pall Corporation, USA. Acetonitrile, methanol and ethanol were HPLC grade and were purchased from Merck. The remaining chemicals and materials were purchased from local firms (India) and were of highest grade.

Cell culture

AGS cells were routinely maintained in RPMI 1640 supplemented with 10% fetal bovine serum, insulin (0.1units/mL), L-glutamine (2 mM), sodium pyruvate (100μg/mL), non-essential amino acids (100μM), streptomycin (100μg/mL), penicillin (50unit/mL) and tetracycline (1μg/mL) (Sigma Chemical Co.) at 37°C in a humidified incubator containing 5% CO_2. Cells were incubated with or without various concentrations of capsaicin and eugenol for 12 hrs. The cells were then processed for the analysis of cell count and cell cycle, detection of apoptosis and Western blotting of many pro- and anti-apoptotic proteins as described in the following sections.

Hemolytic assay

Fresh human blood was centrifuged at 4000Xg for 10 min and the cell pellet was washed thrice and re-suspended in 10mM PBS at pH 7.4 to obtain a final concentration of $1.6x10^9$ erythrocytes/ml. Equal volumes of erythrocytes were incubated with varying concentrations of capsaicin and eugenol and with shaking at 37°C for 1hr. Samples were then subjected to centrifugation at 3500Xg for 10 min at 4°C. RBC lysis was measured at different peptide concentrations by taking absorbance at an OD of 540 nm. Complete hemolysis (100%) was determined using 1% Triton X 100 as a control. Hemolytic activity of the spice active components was calculated in percentage using the following Equation:

$$H=100 \; X \; (Op-Ob)/(Om-Ob)$$

where, Op is the optical density of given peptide concentration, Ob is the optical density of buffer and Om is the optical density of Triton X 100.

Cell viability assay

The effect of capsaicin and eugenol on the viability of AGS cells was determined by Trypan blue exclusion test. Cells were treated with different doses of these two phytochemicals and at definite time point (12hrs) the cells that could exclude the Trypan blue dye were counted in haemocytometer as viable cells. Vehicle-treated cells were considered as control and viability of these cells was taken as 100%. IC_{50}, the dose which induced 50% cell-killing, was determined for the compounds.

MTT assay

The cytotoxicity of the spice principles was tested on AGS cells by the MTT-assay. Briefly, cells were seeded in a 96-well microtitre plate ($2 x 10^4$ cells/well in 100 μL of complete medium) and then incubated with different concentration of these two phytochemicals. After 12 h of exposure to the phytochemicals, 50 μl of MTT (5 mg/5 mL) was added to each well, and the cells incubated in the dark at 37°C for an additional 4 h. Thereafter, the medium was removed, the formazan crystals dissolved in 200 μL of dimethyl sulfoxide, and the absorbance measured at 570 nm.

siRNA transfection

Gene Silencing of p53 was done using siRNA in accordance with the manufacturer's instructions. For siRNA transfection experiments, AGS cells were plated and transfected after 24 h at ~70% confluency by using DharmaFECT 1 siRNA transfection reagent, according to the manufacturer's instructions. After 60 h of transfection, cells were exposed to capsaicin and eugenol for 12 h prior to analysis of experimental parameters. A non-targeting control siRNA ("scramble" siRNA) was used as negative control. Siglo incorporation was used to analyze the extent of transfection, which in this case was approximately 90%. West blot of protein expression was tallied with the result.

Cell cycle distribution analysis

For the determination of cell cycle phase distribution of nuclear DNA, *In vitro* AGS cells (1x106 cells) were harvested. Then, cells were fixed with 3% p-formaldehyde, permeabilized with 0.5% Triton X-100, and nuclear DNA was labeled with propidium iodide (PI, 125 μg/mL) after RNase treatment using Cycle TEST PLUS DNA reagent kit. Cell cycle phase distribution of nuclear DNA was determined on FACS Calibur using Cell Quest Software (Becton-Dickinson Histogram display of DNA content (x-axis, PI fluorescence) versus counts (y-axis) has been displayed. Cell Quest statistics was employed to quantitate the data at different phases of the cell cycle.

Annexin V assay

Apoptosis assays were carried out based on the instruction from the Annexin V Apoptosis Kit (Biovision). Briefly, PI and Annexin V were added directly to AGS cells. The mixture was incubated for 15 min at 37°C. Cells were fixed and then analyzed on FACS Calibur (equipped with 488 nm Argon laser light source; 515 nm band pass filter, FL1-H, and 623 nm band pass filter, FL2-H) (Becton Dickinson). Electronic compensation of the instrument

was done to exclude overlapping of the emission spectra. Total 10,000 events were acquired, the cells were properly gated and dual parameter dot plot of FL1-H (x-axis; Fluos-fluorescence) versus FL2-H (y-axis; PI-fluorescence) shown in logarithmic fluorescence intensity.

Western blot analysis

AGS cell lysates were obtained and equal amounts of protein from each sample were diluted with loading buffer, denatured, and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by protein transfer to polyvinylidene fluoride membranes (PVDF). The effect of the two spice principles on the expression of certain cell cycle proteins such as p53, p21 and PCNA and on apoptotic proteins such as Bax/Bcl-2 ratio, caspase-3 and caspase-8, was determined. Proteins were detected by incubation with corresponding primary antibodies (anti p53, anti-p21, anti-PCNA, anti-Bax, anti-Bcl-2 and anti-caspase-3, and anti-caspase-8) antibodies followed by blotting with HRP-conjugated secondary antibody. The blots were then detected by using a chemiluminescent kit from Thermofisher. This analysis was performed three times.

Co-immunoprecipitation

For the determination of direct interaction between cyt-c and procaspase-9, co-immunoprecipitation technique was applied. For this purpose AGS cells were harvested and lysed in IP buffer (50 mM Hepes, pH 7.6, 200 mM NaCl, 1 mM EDTA, 0.5% Noniodet P-40) containing protease (10 µg/mL each of benzamidine, trypsin inhibitor, bacitracin, 5g/mL each of leupeptin, pepstatin A and PMSF) and phosphatase inhibitor (5µM each of o-phosphoserine, o-phosphotyrosine, o-phosphothreonine,

β–glycerophosphate, p-nitrophenylphos-phate and sodium vanadate) cocktails. The lysate (200 µg protein) was incubated for 4 h in rocking condition at 4°C with 2 µg antibody of a cyt c and the immunecomplex was then incubated with protein A-Sepharose beads. The immunoprecipitate was centrifuged at 12,000 rpm for 2 min at 4°C and the pellet was washed with ice-cold PBS containing phosphatase inhibitor. The immunopurified protein was then used to detect the presence of associated protein, procaspase-9, by Western blot analysis using specific antibody of protein of interest as described above.

Statistical analysis

The experiments were repeated three times and the data were analyzed statically. Values have been shown as standard error of mean, except where otherwise indicated. Data were analyzed and one-way ANOVA were used to evaluate the statistical differences. Statistical significance was considered when $P<0.05$ or $P<0.01$.

Results

Selection of a sub-lethal concentration of capsaicin and eugenol

Trypan blue exclusion test and MTT assay revealed that more than 40% cell killing was observed for capsaicin and eugenol at and above a concentration of 250µM and 1mM respectively. Hemolytic data also suggests that capsaicin above 250 µM and eugenol above 1mM is very toxic. Hence, we selected a concentration of 200 µM for capsaicin and 0.7mM for eugenol for our experiments (Figure 1).

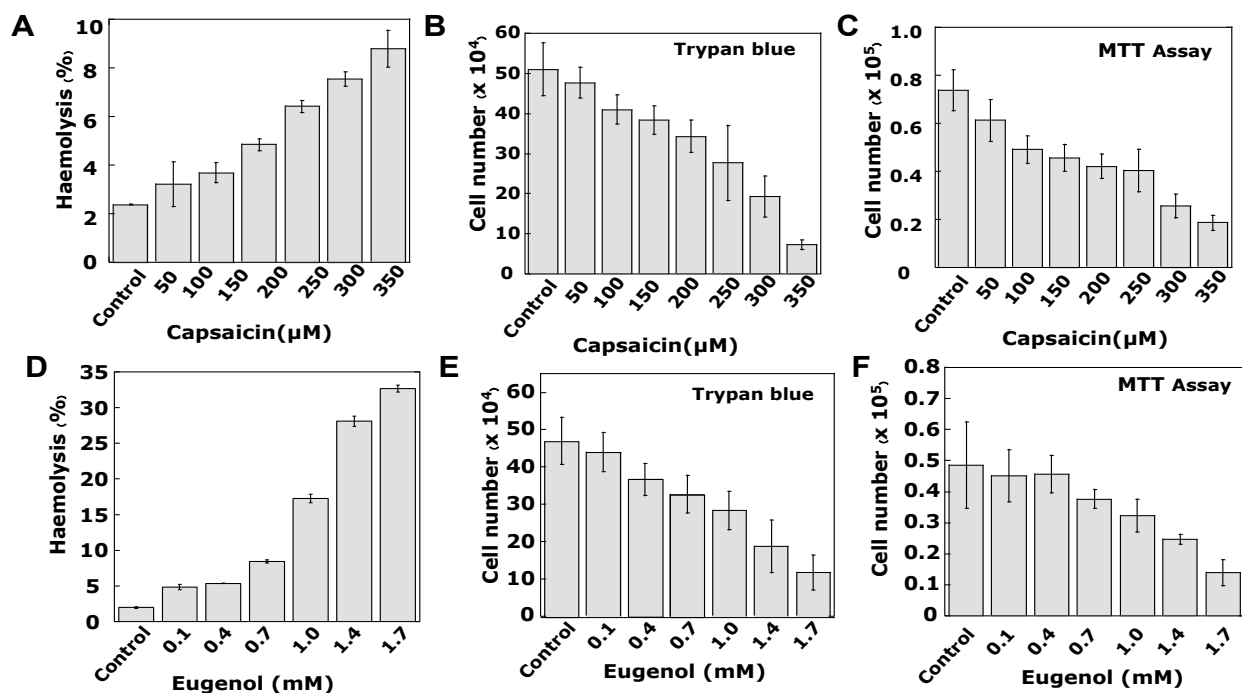*Modulation of Cell cycle phase distribution of AGS cells*

Figure 1. Effect of Treatment with Capsaicin and Eugenol on AGS Cell Viability. Percentage hemolytic activity of capsaicin (A) and eugenol (D) on human red blood cells. B) Cell count at various concentration of capsaicin (B) and eugenol (E) using Trypan Blue exclusion method. Percentage cell viability at various concentration of capsaicin (C) and eugenol (F) measured by MTT assay

by capsaicin and eugenol

In order to analyze the effect of the spice principles on AGS cells flowcytometric analysis of the cell cycle phase distribution was performed (Figure 2).

Treatment with capsaicin resulted in a significant increase in the sub-G0 region (hypolpoidy population); Figure 2 shows representative data of the various experiments. As compared to 5.33%±0.058% cells in the hypoploidy region of the AGS control group, capsaicin treatment recorded 19.86%±0.78% cells. Eugenol treatment at 0.7 mM, also caused an increase in the hypolpoidy peak (7.92%±0.06%) although to a much lesser extent than capsaicin. However, the synthetic phase or S-phase, showed distinctive depression in the eugenol treated group as compared to the untreated counterparts.

Effect of capsaicin and eugenol on AGS apoptosis

In order to confirm the increase in apoptotic induction by the phytochemicals, we performed annexin V/PI assay in all the groups by staining the AGS cells with FITC-tagged annexin V and PI and measuring the fluorescence intensity in a flowcytometer. Results suggest a significant increase in the percentage of annexin positive cells from 5.18%±0.04% in the AGS control group to 8.48%± 0.19% and 23.9%±0.2% respectively in eugenol and capsaicin treated groups. Percentage of PI positive cells also increased significantly by both the phytochemicals (9.03% by eugenol and 17.15% by capsaicin) (Figure 3).

Validating the intrinsic pathway of apoptosis

The increase in the percentage of apoptotic cells in the capsaicin treated group was further confirmed by co-immunoprecipitating caspase-9 with cyt c, two proteins which participate in apoptosome formation. Capsaicin as well as eugenol treatment increased the interaction of the two proteins as compared to the AGS control group (Figure 4)

Western Blot analysis of protein expressions

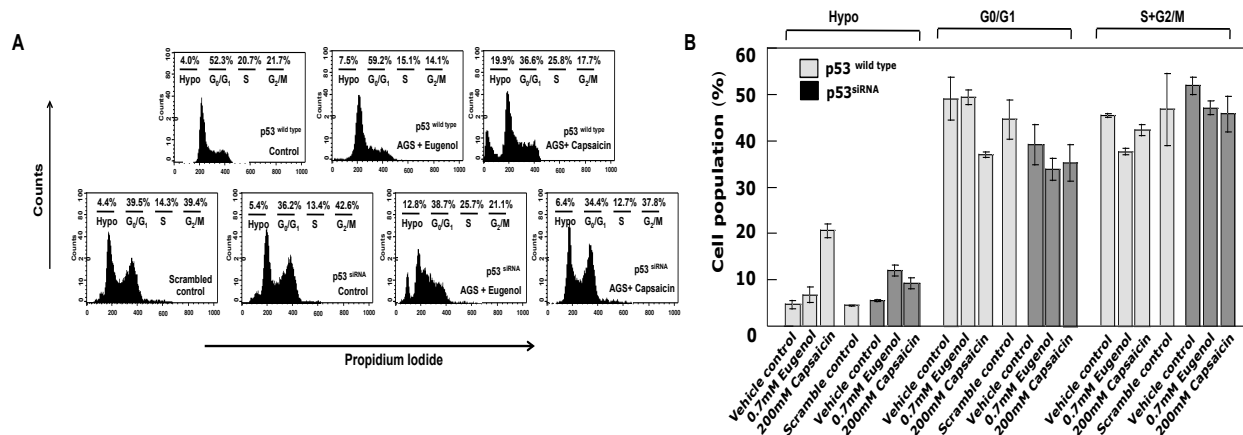Capsaicin and eugenol treatment was found to

Figure 2. Cell Cycle Analysis of Treated and Untreated AGS Cells by Flowcytometer using Propidium Iodide (PI) as DNA-binding Fluorochrome. A) Histogram display of DNA content (x-axis, PI-fluorescence) versus counts (y-axis) has been shown. The upper and lower panels display cell cycle phase distribution of treated and untreated AGS cells with wild type p53 and silenced p53 respectively. B) Bar diagram representation of cell cycle phase distribution of AGS (with wild type and p53 knock out cells) from different experimental groups. The data represented as mean+S.D. for the three different experiments performed in triplicate
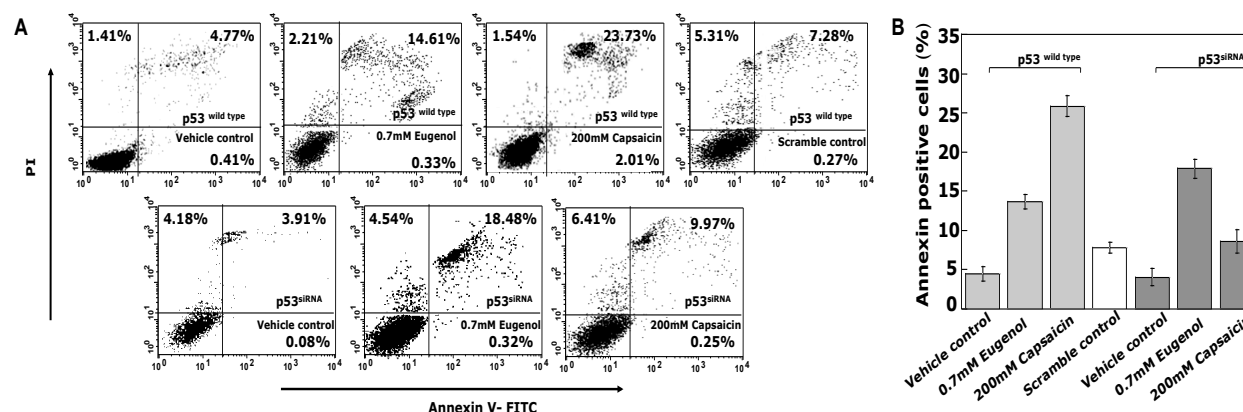

Figure 3. Annexin V Assay in AGS cells A) In a double Label System, Unfixed AGS Cells were Labeled with PI and Annexin V and then fixed and Analyzed on a Flowcytometer. Dual parameter dot plot of FITC-fluorescence (x-axis) versus PI-fluorescence (y-axis) has been shown in logarithmic fluorescence intensity. Quadrants: lower left, live cells; lower right, apoptotic cells; upper right, necrotic cells. The upper and lower panels demonstrated apoptosis induction in treated and untreated AGS cells with wild type p53 and silenced p53 respectively. B) Bar diagram representation of percent Annexin V positive AGS cells from different experimental groups are also given. The data represented as mean+S.D. for the three different experiments performed in triplicate

substantially increase the expressions of pro-apoptotic proteins such as caspase-3, Bax and caspase-8 with a simultaneous decrease in Bcl-2 and PCNA expression as compared to AGS control cells (Figure 4). The extent of decrease in PCNA expression was much more in the eugenol treated group than capsaicin treatment which corroborates the decrease in the S-phase cell population as mentioned above. Interestingly, the phytochemicals differed remarkably in their ability to induce p53 and p21 proteins. Whereas, there was a significant induction of these proteins by capsaicin, eugenol treatment didn't cause any significant change in the expressions of p53 and p21 as compared to AGS control.

p53 is essential for induction of apoptosis by capsaicin

but not for eugenol

Silencing p53 caused a significant decrease in the ability of capsaicin to induce apoptosis in AGS. As shown in Figure 2 lower panel. the percentage of hypoploid cells by capsaicin treatment was brought down to 8.62%±0.32% in p53-/- AGS as compared to 19.86%±0.78% in AGS with wild type p53 expression. Annexin V/PI assay also reflected a similar same trend with a significant reduction in the percentage of annexin positive cells by capsaicin treatment in p53-/- AGS cells.

However, p53 knock down did not influence the apoptotic activity of eugenol in AGS as evident from both the hypoploidy peak and percentage of annexin positive cells induced by eugenol treatment (Figure 3).
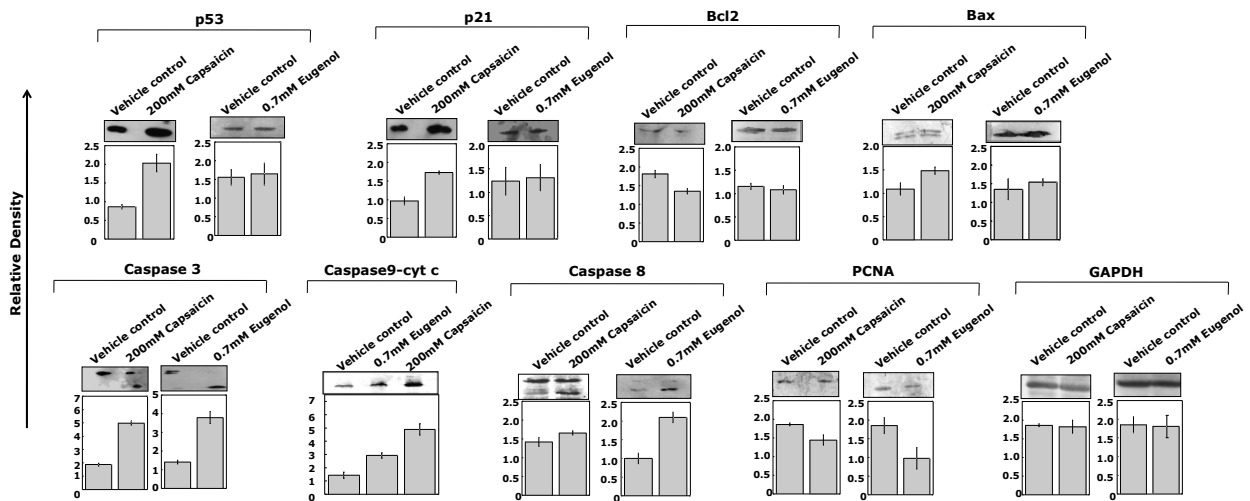

Figure 4. Western Blot detection of Pro-apoptotic and Proliferative Proteins in Treated and Untreated AGS with wild type p53. Detection of p53, p21, Bcl-2, Bax (upper panel) caspase-3, caspase-8 and PCNA (lower panel) in vehicle control, capsaicin and eugenol treated AGS. Co-IP of pro-caspase 9 with cyt c suggesting formation of apoptosome. Equal loading of protein in the lanes was confirmed by GAPDH. Indicated proteins are represented as bar diagrams of mean + SD of their relative densities as measured from three independent experiments. Each test was performed 3 times and images presented were typical of 3 independent experiments. The data were presented as mean ± SD
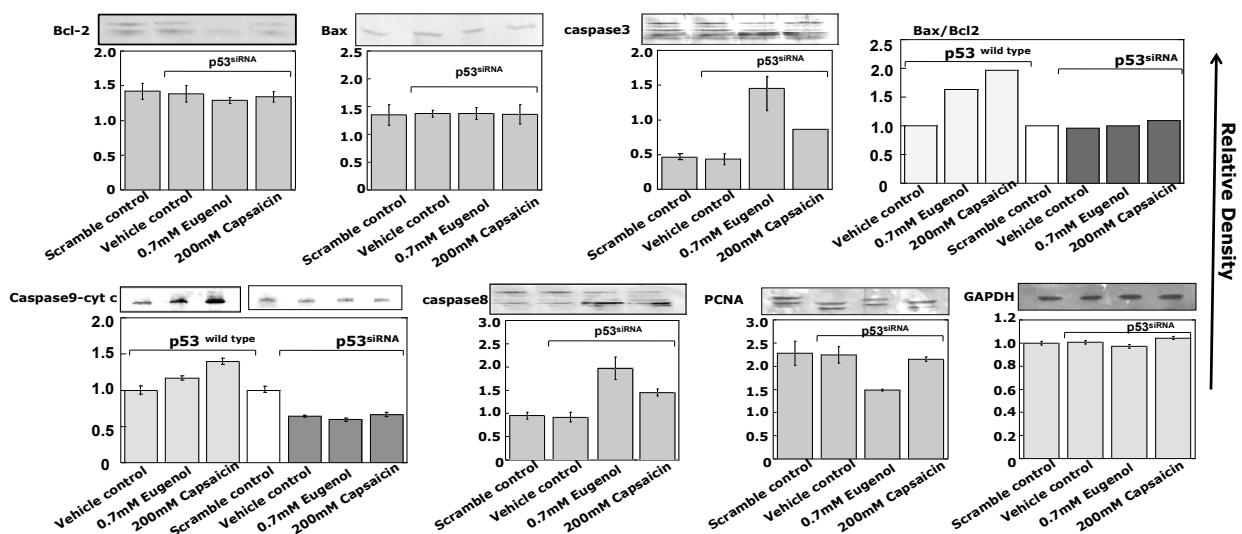

Figure 5. Western Blot detection of Pro-apoptotic and Proliferative Proteins in Treated and Untreated p53 Knock Down AGS. Detection of Bcl-2, Bax caspase-3 (upper panel), caspase-8 and PCNA (lower panel) in vehicle control, capsaicin and eugenol treated AGS. Equal loading of protein in the lanes was confirmed by GAPDH. Indicated proteins are represented as bar diagrams of mean + SD of their relative densities as measured from three independent experiments. Comparison of Bax/Bcl-2 ratio (upper panel) and pro-caspase 9-cyt c association (lower panel) in AGS cells with wild type and silenced p53. Each test was performed 3 times and images presented were typical of 3 independent experiments. The data were presented as mean ± SD

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Quite interestingly, silencing p53 significantly affected the capacity of eugenol to reduce the percentage of S-phase cell population.

Study of protein expressions in p53-/- AGS cells treated with spice principles

The significant elevation in the expression of pro-apoptotic proteins such as caspase-3, caspase-8 and bax, decrease in that of anti-apoptotic protein bcl-2 and proliferative marker PCNA by capsaicin in AGS with wild type p53 was completely offset by silencing p53 in AGS (Figure 5) suggesting a decreased ability of capsaicin to induce apoptosis in the absence of p53. In eugenol treated cells, silencing p53, didn't cause much alteration in the expression of either caspase 3 and 8 as compared to eugenol treated cells with wild type p53. Eugenol, however, was unable to modulate the Bax/bcl-2 ratio and cyt c-capsapse 9 association in p53-/- cells. Moreover, extent of reduction in PCNA expression was much lesser in eugenol treated p53-/- cells suggesting that eugenol modifies the expression of PCNA in a p53 dependent manner.

Discussion

p53 mutation is a common event in human cancers which causes defects in apoptosis and makes cancer cells resistant to chemotherapeutic agents (Do et al., 2012). Alterations in p53 occurs in gastric carcinoma and it increases in frequency during the course of gastric carcinoma developments (Fenoglio-Preiser et al., 2003; Busuttil et al., 2014). Chemosensitivity of gastric cancer towards chemotherapy has been shown to be abrogated in the absence of p53 (Osaki et al., 1997). Therefore, identification of agents that can kill cancer cells with mutated/deleted p53 is a promising anticancer strategy.

In the present study we showed that capsaicin induces apoptosis in AGS cells through upregulation of p53 and that the apoptotic activity of capsaicin is p53-dependent. In contrast, eugenol was found to induce apoptosis independent of p53. Moreover, eugenol was also found to be a potent inhibitor of cellular proliferation as evident from a significant decrease in the population of S-phase cells and a corresponding decrease in PCNA expression by eugenol treatment. This antiproliferative activity of eugenol was, however, dependent on p53.

Mechanistically, we found that the ability of capsaicin to induce the expression of proapoptotic proteins such as Bax, caspase-3 and caspase-8 was almost completely obliterated by knocking down p53. Moreover, the cyt-c-caspase-9 association, which is considered to be important in caspase-9 mediated apoptosis, didn't change significantly in capsaicin treated p53-/- AGS as compared to untreated controls. Similarly, eugenol too couldn't modulate the levels of either Bax/Bcl-2 ratio or cyt c-caspase-9 interaction in p53-/- AGS cells suggesting inhibition of the intrinsic pathway of apoptosis in the absence of p53. Interestingly, however, eugenol was able to enhance the expression of both caspase 8 and caspase 3 even in the absence of p53. As it is known that apoptosis can be induced by the extrinsic pathway via sequential activation of caspase-8 and capsase-3 (Kim et al., 2007), it can be hypothesized that while the intrinsic pathway of apoptotic induction by eugenol is dependent on p53, the extrinsic pathway is not. In case of capsaicin, on the other hands, both the intrinsic and extrinsic pathways were dependent on cellular p53 status. In line with our study, Al-Sharif et al. (2013) had previously demonstrated the p53-independent mechanism of apoptosis induction by eugenol in breast cancer cells. The dependence of capsaicin on p53 for the induction of apoptosis has also been reported in an earlier study (Jin et al., 2014).

This study also showed eugenol to be a potent inhibitor of cell proliferation and the antiproliferative activity of eugenol was compromised in the absence of p53. P53 is known to directly control DNA replication and repair by modulating the levels of PCNA and some studies have found that higher levels of p53 represses PCNA promoter (Xu and Morris, 1999). The decrease in the percentage of S-phase cells in eugenol treated AGS was matched by a similar reduction in PCNA expression. However, in the absence of p53, the repression on the PCNA promoter couldn't be affected by eugenol leading to obliteration of its antiproliferative effect. Capsaicin, on the other hand, was not found to exert any anti-proliferative on AGS cells either in the presence or absence of p53.

In conclusion, we demonstrated that both eugenol and capsaicin induced apoptosis in AGS by the intrinsic as well as extrinsic apoptotic pathways. Between the two phytochemicals, capsaicin caused a more potent apoptotic induction than eugenol in AGS cells in the presence of p53. In the absence of p53, however, eugenol was a better apoptotic agent than capsaicin because of its ability to induce the extrinsic pathway of apoptosis in a p53 independent manner. Loss of function of p53 tumor suppressor gene is implicated in defective apoptotic response of tumors to chemotherapy. Thus agents which can induce apoptosis irrespective of the cellular p53 status have immense scope for development as potential anticancer agents. Therefore, eugenol warrants further investigation for its potential use as anticancer agent against p53 defective or null tumors with poor prognosis.

Acknowledgements

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