

Strategy to enhance efficacy of doxorubicin by curcumin as a potent Pgp inhibitor in gastric cancer.

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Abstract

Gastric cancer which usually affects older people is the third leading cancer responsible for cancer related deaths. Chemotherapy used to treat gastric cancer usually fails due to the development of multidrug resistance. The aim of our work is to develop a strategy that overcomes the chemo resistance in gastric cancer cells which enhance the efficacy of doxorubicin by curcumin as a potent PGP inhibitor. In this study we generated a doxo resistant gastric cancer cell line (Doxo-SNU-5) with IC₅₀ 22 µM as compared to its wild type with IC₅₀ 2.2 µM. The resistance developed was due to overexpression of P-glycoprotein, MRP1 and BCRP confirmed by RT-PCR. Doxo-SNU-5 cells when treated with non-toxic dose of curcumin (PGP inhibitor) in combination with doxorubicin IC₅₀ was reduced up to 5 µM and decreased the cologenetic property. Morphological analysis by DAPI staining showed that the death was due to apoptosis that was further confirmed by annexin V FITC. The biochemical changes of apoptosis was confirmed by increased expression of pro-apoptotic proteins which was due to increase in the expression of CHEK2, BAX, γH2AX and decrease in the expression of anti-apoptotic gene (BCL2) in Doxo-SNU-5 as shown by RT-PCR. These results strongly supports the idea that Curcumin as a Pgp inhibitor enhances the sensitivity of doxorubicin in chemo resistant gastric cancer cell line.

Keywords: Chemo resistance, Doxorubicin, Doxo-SNU-5, Curcumin, PGP inhibitor, Apoptosis.

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Introduction

Gastric cancer also known as stomach cancer develops from the lining of the stomach and is still third leading cause of cancer mortality in China and one of the leading cause of cancer deaths worldwide with 750000 deaths reported worldwide in 2012 [1-3]. One of the significant treatment options for gastric cancer is chemotherapy, but the adequacy of chemotherapy for gastric cancer impoverished due to development of Multidrug Resistance (MDR) [4]. It's this development of MDR that makes the determination of second-line therapy way more difficult [5]. Moreover, no definitive findings are there as such that will indicate the best strategy for choosing the suitable substitutes for chemo-resistant tumours [6]. Doxorubicin is used in chemotherapy for gastric cancer since ages and is usually given in co-administration with 5-fluorouracil and mitomycin-C with an aim to block Multidrug Resistance (MDR) [7]. The use of these anticancer drugs has improved the effect of chemotherapy, however most tumours acquire MDR after undergoing consecutive treatments [8]. As a result, it is important to look for new agents that may be used to enhance the anti-cancer effects of mostly existing

chemotherapeutic drugs used for gastric cancer treatment [9,10]. Because of the best history of natural product compounds regarding the production of anticancer compounds, as over two-thirds of anti-cancer drugs that are novel have been discovered are of natural origin and have attracted the attention of researchers [11]. Natural compound like curcumin, a Chinese traditional medicine have shown potent anti-cancer activities in previous studies [12]. Curcumin (diferuloylmethane) is isolated from rhizome of *Curcuma longa* having wide range of biological activities of curcumin is thought to be anti-diabetic effects, anti-inflammatory, anti-oxidant, and anti-angiogenic [13]. Previous studies have revealed that the expression of oncogenes, cytokines, transcription factors and growth factors was modulated in the anti-cancer effects of curcumin [14-16]. CHEK2, a tumour suppressor gene whose functions are pivotal to the induction of apoptosis and cell cycle arrest following DNA damage is usually mutated in gastric cancer [17]. However mutations in CHEK2 have been linked with many cancers such as breast and prostate cancers, but the relationship between CHEK2 and gastric cancer has not been studied exclusively [18].

Generation of resistant cell lines can be used as effective models to understand the mechanism underlying MDR. However there have been only a few reports regarding this so far [19]. The aim of this study was to develop a doxo resistant gastric cancer cell line (Doxo-SNU-5) in which the breakage of chemo-resistance was the prime focus. In present study curcumin as a Pgp inhibitor was used to break the chemo-resistance in Doxo-SNU-5 cells and it was found that Curcumin potentiates doxorubicin induced cytotoxicity in Doxo-SNU-5 by apoptosis.

Material and Methods

Reagents, chemicals and drugs

Rose well park Memorial Institute medium (RPMI 1640), doxorubicin, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide), Hanks buffer, RIPA (Radioimmunoprecipitation assay buffer), protease inhibitor cocktail, curcumin with >99% purity and Rhodamin-123 were purchased from Sigma (St. Louis, MO, USA). Foetal bovine serum was purchased from GIBCO. Annexin V FITC and RNase were purchased from Santa Cruz biotechnology. RT PCR kit, Trizol reagent, primer and probe were purchased from Invitrogen.

Culture conditions and cell line

Gastric cancer cell line SNU-5 was obtained from ATCC (American Type Culture Collection). Increasing concentration of doxorubicin in parental SNU-5 cells developed MDR sub line. Parental SNU-5 was exposed to increasing concentration of anticancer drug doxorubicin for the development of MDR cell line (Doxo-SNU-5) over a period of 5 months. Cells were grown in Rose well park Memorial Institute medium (RPMI 1640) with 10% FBS and incubated in 5% CO₂ incubator with 37°C temperature. The medium for Doxo-SNU-5 was further supplemented with Doxorubicin (1 µM). Before two days of experiments, Doxo-SNU-5 cells were cultured in a drug free medium.

Cytotoxicity assay by MTT

At a density of 1.5×10^4 and 1×10^4 per well, Doxo-SNU-5 and SNU-5 were plated in 96 well plates respectively and kept overnight in CO₂ incubator with 5% CO₂ at 37°C temperature. Then after 24 hr cells were treated for 48 hr with curcumin and doxorubicin containing normal cell media at desired concentration. After 48 hr 20 µl of MTT was added into each well at a concentration of 2.5 mg/ml and cells were incubated as such for 4 hr. Finally after removing media from the desired wells 150 µl of DMSO was added and absorbance was taken at 570 nm using synergy MX plate reader and cell viability was calculated.

P-Glycoprotein inhibitory activity

In 96 well plates Doxo-SNU-5 was seeded at a same density per well as in cytotoxicity assay and cells were allowed to

grow for 24 hr. In Hanks' buffer containing 20 µM of Rh-123 as a PGP substrate cells were incubated with 30 µM of curcumin for 120 min. Cells were washed four times with cold PBS after removal of test compound followed by 150 µL of cell lysis buffer (RIPA buffer). Fluorescence of Rh-123 from 100 µL of lysate was taken at 485/529 nm by synergy MX plate reader. Florescence of sample were normalised by dividing total protein present in the lysate.

Intracellular accumulation of doxorubicin by flow cytometer

1×10^6 cells were plated in 6 well plates and treated with desirable concentration of doxorubicin in combination with curcumin for 48 hr. After 48 hr cells were harvested and washed 4 times with cold PBS. Cell suspension was performed to measure the intrinsic florescence of doxorubicin at an excitation of 488 and emission at 550 by using FACS BD Calibur flow cytometer. Fluorescence intensity was measured by histogram at 20,000 cells per event. Cell Quest Pro software was used to analyse the data.

Nuclear morphology

1×10^6 cells were plated in 6 well plates and treated with doxorubicin both in presence as well as absence of curcumin for 48 hr. in brief cells were harvested and fixed in methanol and acetic acid (3:1 ratio respectively) and kept overnight at 4°C. After overnight incubation cells were centrifuged, resuspended in methanol and acetic acid and then plated on chilled glass slides. After drying the slides, cells were stained with DAPI (1 µg/ml) in dark for 30 min and then slides were washed. Finally images were taken at fluorescence microscope.

Clonogenic assay

Cells were seeded in 6 well plates, treated with doxorubicin alone and in combination with curcumin for 48 hr. After 48 hr cells were trypsinized and replated at a density of 500/well for 21 days. Cells were washed with cold PBS and allowed to fix in 4% paraformaldehyde for 10 min. The living cells were stained with 0.06% crystal violet and images were taken by Olympus Tokyo, Japan camera.

Flow cytometer for apoptosis

Cells were plated in 6 well at a density of 0.8×10^6 for 48 hr followed by treatment with doxorubicin in presence and absence of curcumin. After 48 hr treatment, cells were harvested by centrifugation at 400 G for 5 min and washed with cold PBS. These cells were incubated with binding buffer containing Annexin V-FITC for 20 min in dark. Cells were incurred by flow cytometer (FACS Calibur) for 20000 events as to analysing apoptotic and non-apoptotic population.

Real time-PCR

The BCRP, MRP1, PGP, CHEK2, BAX, BCL2, γH2AX and P53 mRNA was examined by real time PCR. Doxo-SNU-5 cells were seeded at a density 1×10^6 cells/well into 6 well plates

and treated with 30 μM and 1 μM of curcumin and doxorubicin (combined treatment). Trizol reagent from invitrogen was used for total RNA isolation following manufacturer's protocol. RNA was reverse-transcribed using oligo (dT), Deoxynucleoside Triphosphate (dNTP) and Moloney murine leukaemia virus reverse transcriptase (invitrogen) as described by the standard methods [20,21]. Briefly the following forward and reverse respectively primers used were PGP F; 5'-GACTATGCCAAAGCCAAAATAT-3' and 5'-CTTCCAATGTGTTTCGGCATTAG-3', CHK2; 5'-GCGTCTGTGATGTCTCGG and 5'-TTCGTGTTCAAACCACGGA-3', p53; 5'-ATT-TGA-TGC-TGT-CCC-CGG-ACG-ATA-TTGAA(S)C-3' and 5'-ACC-CTT-TTT-GGA-CTT-CAG-GTG-GCTGGA-GT(S)G-3', MRP; 5'-TCTGGGACTGGAATGTCACG-3' and 5'-CAGGAATATGCCCCGACTTC-3' BCRP; 5'-CAGGTCTGTTGGTCAATCTCACA-3', and 5'-TCCATATCGTGGGAATGCTGAAG-3', H2AX; 5'-TGGAAAGGGTCAGGGAACG-3' and 5'-GACTTGTGCTGGTATCTGGGTG-3', BCL-2; 5'-TCCGATCAGGAAGGCTAGAGTT and 5'-TCGGTCTCCTAA-AAGCAGGC-3', BAX; 5'-CCGCCGTGGACACAGAC and 5'-CAGAAAACATGTCAGCTGCCA-3', GAPDH; PGP5'-GAAGGTGAAGGTCGGAGTCA-3' and 5'-GAAGATGGTGTGGGATTTC-3'. Each reaction was performed in triplicates. Briefly samples were heated for 2 min at 50°C and for 10 min at 95°C and then at 95°C, 40 cycles of amplifications were done for 15 sec and for 1 min at 60°C. Fluorescent signal was determined by Using sequence detector TM software and CT values i.e. threshold Cycle Number (CT) was determined. Finally for all genes the CT value was calculated by calculating the differences in their respective GAPDH mRNA and CT values.

Statistical analysis

Values of three independent experiments are shown as mean \pm SD of three independent experiments. Students test were used for comparison of three experiments that was performed. Treated and untreated samples were compared. $P < 0.05$ was considered to be a statistically significant. Graph pad prism versions 5 were used to perform Statistical analyses.

Result

Development of chemo-resistant cell line Doxo-SNU-5

In order to overcome chemo resistance in gastric cancer we have selected SNU-5 cell line for development of resistance over a period of four months. During this period the cells were treated with doxorubicin from lower concentration (0.5 μM) up to high concentration (30 μM). After four months the IC_{50} was again calculated in both cell lines. In wild type (SNU-5), IC_{50} was calculated as 2.2 μM , which got increased in Doxo-SNU-5 up to 22 μM as shown in Figure 1. It was seen that the resistance was due to up regulation of efflux pumps as was observed by overexpression of genes of major efflux pumps

like BCRP, MRP1 and PGP as shown by RT-PCR in Doxo-SNU-5 as compared to wild type SNU-5 as shown in Figure 2.

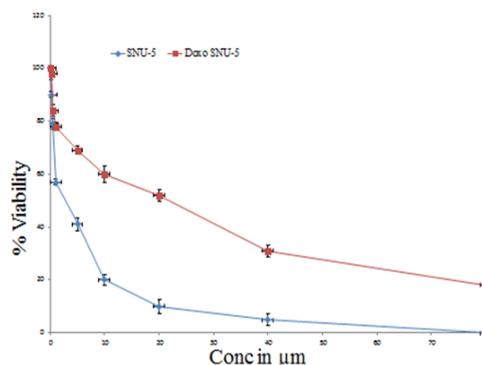


Figure 1. MTT assay of doxorubicin against SNU-5 and Doxo SNU-5: Both SNU-5 and Doxo SNU-5 were treated with different concentrations of doxorubicin (0.25-80 μM) for 48 hrs, wherein viability of Doxo SNU-5 was clearly less inhibited as compared to its wild type SNU-5 implying the effect of drug resistance.

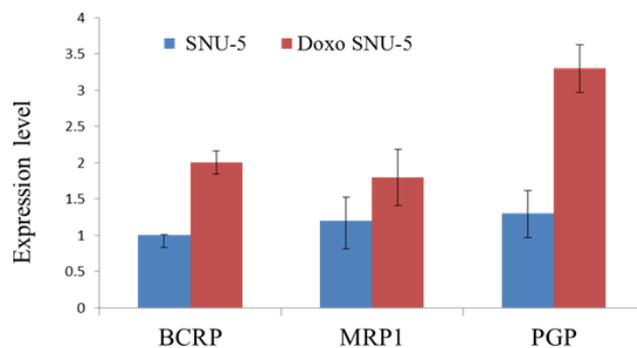


Figure 2. Doxorubicin enhanced PGP, MRP1 and BCRP levels: Both SNU-5 and Doxo SNU-5 cells were taken, RNA was isolated and expression of PGP, MRP1 and BCRP was determined by RT PCR. The results clearly indicated that Doxorubicin increases the expression of PGP, MRP1 and BCRP.

Effect of curcumin on PGP function in Doxo-SNU-5 cells

To investigate curcumin as a potent PGP inhibitor, rhodamin123 based assay was done. The intracellular accumulation of rhodamin 123 increased by 60% in curcumin treated Doxo-SNU-5 cells as compared to untreated control at a concentration 30 μM , elacridar was taken as a positive control as shown in Figure 3. Curcumin was non-toxic on 30 μM for 48 hr which was confirmed by cell viability assay (MTT assay) as shown in Figure 4. Effective PGP inhibition by curcumin was shown at 30 μM , thus this concentration were carried for further study.

Intracellular accumulation of doxorubicin enhanced by curcumin

To investigate the role of curcumin in an intracellular accumulation of doxorubicin in Doxo-SNU-5 cells, cells were seeded in 6 well plates and treated with doxo in presence and

absence of curcumin. Treated cells were acquired through flow cytometry in which there is an evident that fluorescence produced by doxorubicin is directly proportional to the amount of doxo accumulated inside the cells as doxorubicin is an auto-fluorescent drug. Interestingly curcumin enhanced the cellular uptake of doxorubicin which is by PGP inhibition when compared to untreated and doxo treated cells alone as evident by flow cytometry histogram as shown in Figure 5.

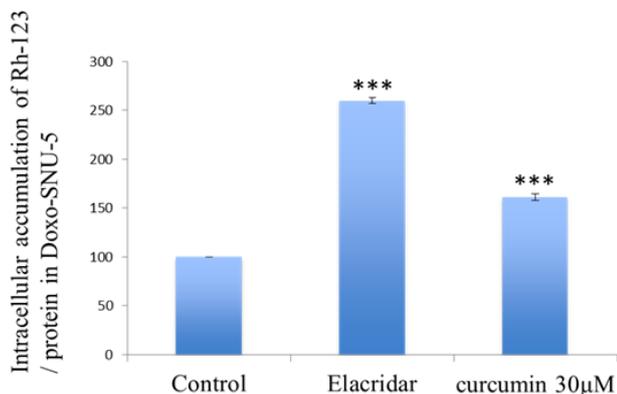


Figure 3. PGP inhibition by curcumin in Doxo-SNU-5: curcumin treatment of Doxo SNU-5 results in the decrease in the activity of PGP as was seen by intracellular accumulation of Rh-123/protein. Statistical comparisons were made between control vs. inhibitors by using bonferroni method. The p value<0.05 was considered to be significant. P-value *<0.5, **<0.01, ***<0.001.

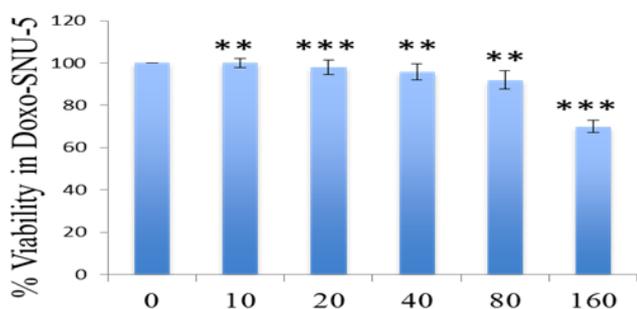


Figure 4. Effect of curcumin on the Viability of Doxo-SNU-5: Doxo-SNU-5 seeded treated with increasing concentrations of curcumin for 48 hours and cell viability was assessed by MTT.

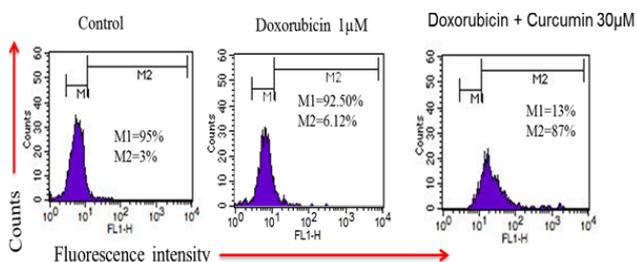


Figure 5. Curcumin enhances intracellular uptake of doxorubicin in Doxo-SNU-5 cells: Doxo-SNU-5 cells were treated with doxorubicin (1 µM) together with indicated concentration of curcumin in which intracellular accumulation of doxorubicin have been increased as represented by flow cytometric histogram.

Curcumin potentiates doxorubicin induced cytotoxicity in Doxo-SNU-5 cells

To study adverse effect of curcumin on Doxo-SNU-5 cells, preliminary experiments were done for combination treatment as to obtain a dose showing no toxicity. Curcumin does not affect cell survival as measured by MTT assay up to 100 µM as shown in Figure 4. Additionally, the use of doxo at marginal dose for combination treatment (curcumin+doxo) in Doxo-SNU-5 cells was calculated to be 1 µM. We assessed the effect of curcumin by combining with doxorubicin on gastric resistant carcinoma (Doxo-SNU-5) cells *in vitro*. Cells were treated with curcumin (30 µM) in combination with various doses of doxo for 48 h. As observed, at various doses of doxorubicin, viability of cells reduced from 22 µM to 5 µM as shown in Figure 6. Enhanced cytotoxic effect by doxorubicin in combination with curcumin was also confirmed by decrease in colony formation potential as shown in Figure 7. Thus the above result indicates that effect of doxo in combination with curcumin was enhanced as confirmed by inhibition of cell proliferation.

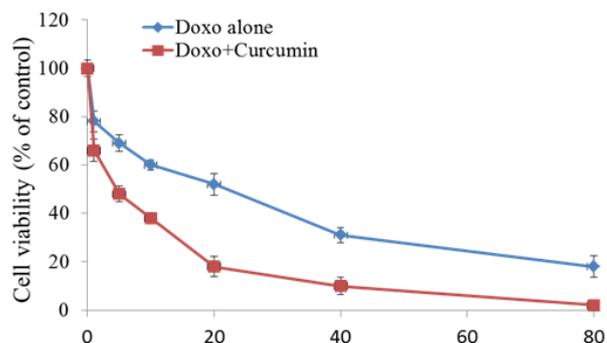


Figure 6. Curcumin potentiates effect of doxorubicin in Doxo-SNU-5 cells: cells were treated with indicated concentration of doxorubicin together with curcumin for 48 hr in which cytotoxic effect was enhanced with curcumin compared with doxorubicin alone as shown by MTT assay.

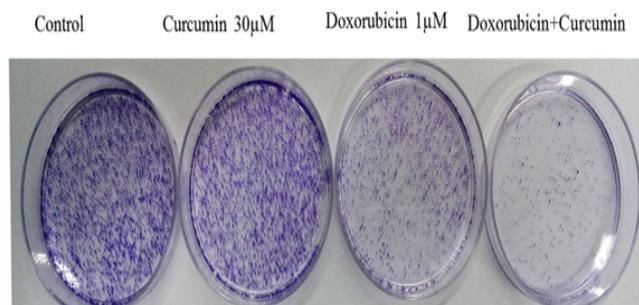


Figure 7. Curcumin in combination with doxorubicin inhibits colony formation in Doxo-SNU-5: Doxo-SNU-5 cells were treated with curcumin along with doxorubicin for 48 hours and then cells were allowed to grow for 21 days in drug free media in which curcumin along with doxorubicin inhibits the colony formation in Doxo-SNU-5 cells.

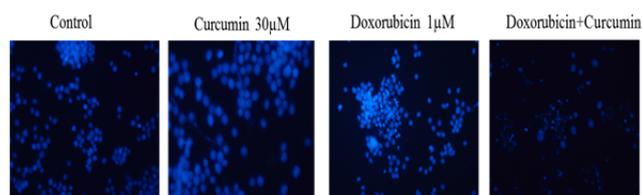


Figure 8. Change in nuclear morphology by treatment with doxorubicin together with curcumin: Doxo-SNU-5 were treated with doxorubicin in presence and absence of curcumin for 48 h stained with DAPI for 30 min. Apoptotic fragments was observed in combined treatment shown by fluorescence microscope.

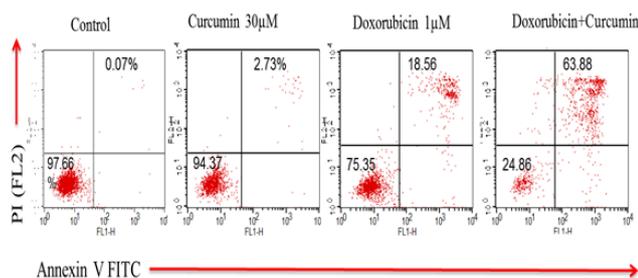


Figure 9. Combination treatment of doxorubicin and curcumin induces apoptosis in Dox-SNU-5 cells. Cells were treated with doxorubicin in presence and absence of curcumin. Annexin V-FITC was done as described in materials and methods. The result showed that there is a significant apoptotic population in Dox-SNU-5 cells when treated doxorubicin together with curcumin.

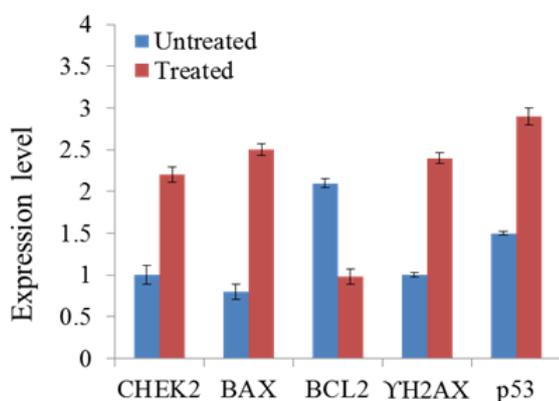


Figure 10. Gene expression profile by doxorubicin along with curcumin: Dox-SNU-5 cells were treated with doxorubicin in presence and absence of curcumin for 48 h. RT PCR was done as described in materials and methods. These results indicate that there is up regulation of CHEK2, BAX, γ H2AX, P53 and down regulates BCL2 genes.

Curcumin potentiates doxo induced apoptosis in Doxo-SNU-5 cells

PGP inhibitory effect of curcumin potentiated DOX induced effect was due to induction of apoptosis in Doxo-SNU-5 cells as evident by nuclear morphology (DAPI staining) in which there was numerous apoptotic fragments in combined treated cells (Doxo+curcumin) when compared to alone as shown in fluorescence microscopy as shown in Figure 8. Results were

confirmed by annexin V FITC in which the apoptotic population was 63.88% in combined treatment (Doxo+curcumin) as compared to individual treatment of doxorubicin which is only 18.56% as shown in Figure 9 and with real time PCR as shown in Figure 10 in which gene expression of anti-apoptotic gene Bcl-2 and apoptotic gene chek2, BAX, γ H2AX and P53 increased as compared to doxo alone. All the above results demonstrate that curcumin potentiates DOX induced cytotoxicity in gastric carcinoma (Doxo-SNU-5) by apoptosis.

Discussion

Chemo-resistance is one of the major challenges that everyone is facing nowadays as every cancer initially responds well to drugs but then develops resistance over a period of time. Chemo resistance is also one of the major hurdles in the treatment of gastric cancer, 2nd leading cause of cancer related deaths worldwide after lung cancer [1]. MDR has been attributed to the presence of molecular pumps in tumour cells that vigorously expel chemotherapy drugs from the cells. These pumps help the cells to avoid the toxic effect of the chemotherapy drugs and continue the molecular processes going on within the nucleus and cytoplasm. The three main pumps commonly found responsible for chemo resistance are P-glycoprotein, BCRP and multidrug resistance associated proteins. In this study we generated a resistant cell line Doxo-SNU-5 so as to look for the genes involved in resistance. It was seen that there was a particular set of genes which got up regulated due to resistance; the genes involved are BCRP, MRP1 and PGP. Curcumin a natural product inhibited the expression of PGP in Doxo-SNU-5 cell line so that intracellular accumulation of doxo have been increased as shown by flow cytometer histogram in which viability and colony formation had decreased. Increased cytotoxic effect of doxo by curcumin was due to induction of apoptosis in Doxo-SNU-5 cells, as was shown by nuclear morphological changes and annexin V FITC. These results were further confirmed by Real time PCR in which gene expression of antiapoptotic gene Bcl-2 decreased and apoptotic gene chek2, BAX, γ H2AX and P53 increased as compared to doxorubicin alone. In MDR studies, it was shown that curcumin is the most active Pgp inhibitor and increases vinblastine cytotoxicity in Pgp-expressing MDR cancer cells. Therefore, this continued research has been focussed more on the MDR reversing properties of curcuminoids using many kinds of chemotherapeutic drugs such as paclitaxel, doxorubicin, etoposide, mitoxantrone and topotecan. In addition, the effects of formulated curcuminoids, curcumin, demethoxycurcumin and bisdemethoxycurcumin on these drug accumulation and efflux in high and low Pgp expressing on MDR phenotypes or the changes in drug sensitivity of MDR cells were also investigated. Therefore our study confirms Curcumin enhanced the efficacy of doxorubicin in Doxo-SNU-5 through inhibition of Pgp and thus helped in breaking the chemo resistance. Thus curcumin can be used as a potential drug candidate to overcome chemo resistance in gastric cancer.

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