Dose Dependent Synergism from Combination of Platinum Drugs with Curcumin against Colorectal Cancer Cell Lines

Hana Bali[1], Jun Qing Yu[1], Philip Beale[2], and Fazlul Huq[3]

Abstract

Introduction: Colorectal cancer is the fourth most common cause of cancer mortality. More than 41265 new cases of colorectal cancer were detected and around 15903 colorectal cancer deaths occurred in year 2014 worldwide according to the statistical study from the cancer research. Chemotherapy is still in the main stream of the management of colorectal cancer along with surgery and radiotherapy. **Aim:** The objective of the present study was to investigate the activity of curcumin in combination with platinum drugs against colorectal cancer models (HT-29, Caco-2, LIM-1215 and LIM-2405). **Methods:** IC_{50} values of cisplatin (Cs), oxaliplatin (Ox), and curcumin (Cur) were determined against four human colorectal cancer cell lines using MTT reduction assay. Combined drug activity was determined as a factor of sequence of administration (0/0, 0/4 and 4/0 h) and added concentrations. DNA binding and proteomics were carried out to obtain insight into molecular mechanisms of drug action. **Results:** Oxaliplatin in combination with curcumin produced strong synergism in the tested cell lines. Cellular accumulation study, platinum-DNA binding study and DNA damage study revealed the mechanism of combined drug affects. Upregulation of K1C18, GRP78, IDHC and Cofilin-1 proteins was considered to be associated with the synergistic combined effects of Oxaliplatin with curcumin. **Conclusion:** Ox in combination Cur demonstrated very high synergism against HT-29 and Caco-2 cell lines. Synergism from Ox with Cur may be associated with greater platinum DNA binding. Proteomics revealed that the elevated expressions of K1C18, GRP78, IDHC and Cofilin1 may be responsible for the synergistic activity obtained from the combination of Ox with Cur.

Key Words: Colorectal cancer; platinum drugs; drug resistance, synergism; proteomics

Significance | This study showed synergistic drug combination and mechanistic information related to changes in protein expression.

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Introduction

Cancer is the major health concern all around the world and considered as the most devastating disease of our time. Among 200 different types of diagnosed cancers, colorectal cancer is the fourth most leading cause of death among all reported cancer mortality. The incidence of colorectal cancer is higher in developed countries, comprising 75% of all cases compared to lower income regions of the world (Ferlay, Soerjomataram et al. 2015). Primary treatment methods of colorectal cancer are: surgery, cryosurgery, stereotactic body radiation therapy, radiofrequency ablation and chemotherapy. Although surgery is

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is the gold standard for the treatment of localized colorectal cancer, chemotherapy is the treatment of choice to treat metastasized colorectal cancer patients. Currently, clinicians are use combination chemotherapeutic regimen (e.g. FOLFOX, FOLFIRI) for the treatment of advanced colorectal cancer (Holch, Ricard et al. 2017).

Epidemiological studies (more than 200) suggest that higher consumptions of fruits and vegetables which are the major sources of antioxidant phytochemicals can serve to reduce incidence of cancer (Willett and Trichopoulos 1996). Phytochemicals in combination with other chemotherapeutic drugs have been investigated against various cancer models and entered into clinical trials. Our group has published a significant number of research articles showing the combined effect of platinum drugs and phytochemicals against ovarian cancer (Yunos, Beale et al. 2011; Mazumder, Beale et al. 2012; Nessa, Beale et al. 2012; Al-Eisawi, Beale et al. 2013; Huq, Yu et al. 2014; Huq 2015; Arzuman, Beale et al. 2016; Alam, Yu et al. 2020). Now we have extended our research interest towards colorectal cancer models as well. In this study a well-known phytochemical, curcumin has been investigated in combination with platinum drugs (cisplatin and oxaliplatin) against colorectal cancer cell lines for the combined drug effects. Studies on DNA damage, cellular accumulation of platinum, platinum–DNA binding and proteomic study were carried out to identify the underlying mechanisms for the combined drug action.

Materials and methods

Chemicals: Cisplatin (Cs) was synthesized in the host laboratory using modified Dhara method (Dhara 1970). Oxaliplatin (Ox) was purchased from Sigma Aldrich, USA. Curcumin (Cur) was obtained from Sapphire Bioscience, Pty. Ltd., Australia.

Preparation of stock solutions for single drug cytotoxicity and combination study: 1 mM solutions of Cs and Ox were prepared by dissolving .0015 g and 0.0019 g of the respective compounds in 1 mL of DMF first, followed by the addition of 4 mL of H_2O. To prepare 10 mM solution of Cur, 0.018 g was dissolved in 5 mL of ethanol. The drug solutions were serially diluted from the stock solutions with freshly prepared RPMI-1640 medium to produce a range of concentrations.

Cell culture: Human colorectal cancer cell lines HT-29, Caco-2, LIM-1215 and LIM-2405 were seeded in 25 cm^2 cell culture flasks in an incubator at 37˚C in a humidified atmosphere consisting of 5% CO_2 in air. HT-29 and Caco-2 colorectal cancer cell lines were got as gifts from Dr. Mu Yao (Department of Endocrinology, The University of Sydney, Australia). LIM-1215 and LIM-2405 cell lines were purchased from Cell Bank Australia. All the collected cell lines were maintained in logarithmic growth phase in a complete medium consisting of RPMI-1640, 10% heat-inactivated foetal calf serum, 20 mM Hepes, 0.11% bicarbonate, and 2 mM glutamine.

MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] reduction assay:

Antitumour activity of the platinum drugs (Cs and Ox) and phytochemical Cur against four human colorectal cancer cells either alone or in combination was determined by following established method of MTT reduction assay (Mosmann 1983). Briefly, 3500 to 4500 cells per well in RPMI-1640 medium were seeded into flat-bottomed 96-well culture plate and allowed to attach overnight. When drugs (Cs/Ox/Cur) were added alone, four different concentrations of each drug were prepared from the stock solutions and 100 µl of drugs were added to equal volumes of cell culture in triplicate wells added to triplicate wells which were left in the incubator (37°C, 5% carbon dioxide in air, pH 7.4) for 72 h. During combination studies, cells were treated with increasing concentrations of compounds at constant ratios of their IC_{50} values using three different sequences of administration: bolus or 0/0 (platinum drug or phytochemical added at the same time), 0/4 (Cs/Ox was added first followed by Cur 4 h later) and 4/0 (Cur was added first followed by Cs/Ox 4 h later). The molar concentration ratios between Cs and Cur in HT-29, Caco-2, LIM-1215 and LIM-2405 cell lines were: 0.298, 0.814, 0.238 and 0.597 respectively. Whereas molar concentration ratios between Ox and Cur in in HT-29, Caco-2, LIM-1215 and LIM-2405 cell lines were 0.029, 0.128, 2.18 and 0.910 respectively. After 72 h of incubation of the drug treated cells and control in 5% CO_2 incubator, the medium was removed and 50 µl of the MTT solution were added to each well of 96-well plate. After completion of 4 h incubation period, 150 µl of DMSO were added to each well. The viable cells remained attached at the bottom stained with MTT as purple formazan product. The mean absorbance at 595 nm for each compound or drug treatment whether alone or in combination was expressed as a percentage of the untreated control well absorbance. The consequence of combined drug treatments was studied using a median effect analysis whereby a combination index (CI) was calculated from pooled data from 4 to 6 individual experiments each comprising at least three data points for each drug alone and for each drug combination. The combination index (CI) for two compounds or drugs was calculated from the following calculation based on Chou and Talalay median effect (Chou and Talalay 1984) equation:

$$CI = \frac{D_{pt}}{D_{ptz}} + \frac{D_p}{D_{pz}}$$

Where, Dpt refers to concentration of platinum drug required for z% cell kill while in combination; Dp refers to concentration of phytochemical required for z% cell kill in combination; Dpt refers to concentration of platinum drug required for z% cell kill while applied alone; Dp refers to concentration of phytochemical drug required for z% cell kill while applied alone. CI values of <1, =1 and >1 indicate respectively synergism, additiveness and antagonism in combined drug action. The CI, D_m and r values were obtained automatically using Calcusyn software (V2) (Biosoft, UK). The D_m sometimes reflect the values of IC_{50} value. The linear correlation coefficient, r (where r=1 indicates perfect fit), of the median effect plot should be reasonably good; for the cell culture system, r should be greater than 0.95 (r>0.95).

Cellular accumulation of Platinum: For cellular accumulation study, stock solutions of the compounds were again prepared (Cs: 1 mM; Oxa: 0.63 mM; Cur: 0.71 mM). Exponentially growing HT-29 and CACO-2 colorectal cancer cells in 4.75 mL 10% FCS/RPMI medium (cell density = 50 x 10^4 cells mL-1) were seeded into cell culture dishes and allowed to attach overnight. While drugs were added in combination, 125 µL of Cs/Ox and 125 µL of Cur was added to the cells. But in case of Cs and Ox alone treatment 125 µL of drug and 125 µL of each medium were added. The above treatments were conducted in two sets, one for cellular accumulation studies and another one for platinum and platinum–DNA binding studies. The cells containing the drugs were incubated for 24 h, at the end of which cell monolayers were trypsinized and cell suspension (10 mL) was transferred to a centrifuge tube and spun at 3,500 rpm for 2 min at 4˚C. The cells were washed twice with ice-cold phosphate-buffered saline. At least three independent experiments were performed. Following incubation with compounds singly and in binary combination, cell pellets were suspended in 0.5 mL of 1% Triton-X, held on ice then sonicated for 30 min. Total intracellular platinum contents were determined by graphite furnace (AAS) using a Varian SpectrAA-20 with a GTA 96 atomic absorption spectrophotometer.

Platinum–DNA binding study: Drug treatment and cell collection procedure was same as described for cellular platinum accumulation study. To extract pure genomic DNA, the protocol described for cultured animal cell in the KIT was exactly followed. Briefly, the pellet was resuspended with ACL solution buffer and mixed with protenase K and then incubated at 55°C for 10 min. RNase A was added and vortexed; incubated at room temperature for 5 minutes and then centrifuged for 5 minutes at 12,000 rpm for 5 minutes. Supernatant was taken and mixed with AB solution for 2 minutes. After discarding flow through, wash solution was added and spun at 10,000 rpm for 1 minute. The columns were put into Eppendorf tubes and elution buffer was added and incubated at 50° C for 2 minutes. The tubes were centrifuged 10,000 rpm for 2 minutes to elute the DNA and then quantified UV absorption spectrophotometer at 260 nm (Varian Cary 1E UV-Visible with Varian Cary Temperature Controller). A260/A280 ratio between 1.75 and 1.8 for all samples ensures high purity of DNA. The DNA concentration was calculated according to the following equation: concentration=absorbance at 260 nm × 50 ng/µl. Platinum content was determined using graphite furnace AAS.

DNA damage study using agarose gel electrophoresis: The protocol for extracting DNA was same as mentioned for Platinum–DNA binding study. During gel electrophoresis, 1% agarose gel was prepared and gently transferred into the tray with comb placed in position, left at room temperature for 45 min to solidify (Stellwagen 1998). 250 µL of ethidium bromide was added in both side of electrophoresis chamber. Entire gel was dipped into the electrophoresis chamber using TAE working buffer. Estimated volume of DNA sample (measured as corresponding to 0.2 µg of DNA) was mixed with required volume of mQ water to make the total of 18 µl, followed by mixing with 2 µl of bromophenol blue chromatogram. Electrophoresis was accomplished at 120 V for 2 hours. UV lamp was used to visualize the bands and the images were taken by Kodak Gel Logic 100 imaging system (GL 100).

Proteomic study: Proteomic studies were carried out to identify the proteins that were responsible for the drug actions either alone or in combination in Caco-2 cell line. The cells were cultured in 50 cm^2 petri dishes to produce the concentration of 10^6 cells/dish. Caco-2 cell line was treated with Ox alone, Cur alone and Ox+Cur (0/0) at IC_{50} concentrations. Control cells were treated will the medium only. Following 24 h of incubation period after drug treatment, cell pellets were collected through washing with PBS and centrifugation. The pellets were lysed using cell lysis buffer, 1st dimensional electrophoresis was done using non-linear ReadyStrip™ IPG Strip in Protean i12 IEF (Isoelectric focusing) cell unit. 2nd dimensional gel electrophoresis (SDS-PAGE) was conducted by using 4–20 % SDS Criterion™ TGX™ pre-cast gels in a Criterion Dodeca™ cell separation unit (BIO-RAD, Australia) at constant 200 V for 100 min in a Trisglycine- HCl buffer system. The detailed method has been described elsewhere (Al-Eisawi, Beale et al. 2016; Alam, Yu et al. 2020). The gel images were taken by ChemiDoc™ MP Imaging system (BIO-RAD, Australia) and spots were analysed by using Melanie version 7.0 software (GeneBio, Switzerland). A 1.5-fold change in the expression of a protein across the matched groups was used as the indicative point for significant expression. Analysis of variance (ANOVA), a statistical tool used to detect differences between experimental group means, was performed using a target significance level of 0.05.

Mass spectral characterization of differentially expressed proteins: Bio-Safe Coomassie Stain was used for staining the spots before

excision from 2-D gels. Destaining of the spots was done by using 120 µl of (50 % acetonitrile/50 mM NH_4HCO_3) solution and digestion of spots was performed by trypsin. The obtained peptides were extracted with 0.1 % trifluoroacetic acid (TFA) then extracted and concentrated by C18 zip-tips (Millipore, µ-C18, P10 size) on Xcise (Proteome Systems). Matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS) was performed with 4800 plus MALDI TOF/TOF Analyser (AB Sciex). Detailed procedure has been described in our earlier article (Al-Eisawi, Beale et al. 2016). The data on peptides masses were analysed using database search program Mascot (Matrix Science Ltd, London, UK). The peak lists were searched against Homo sapiens entries in the SwissProt database.

Results

Anticancer activity of single drugs: The results of the antitumour activity of tested compounds (Cs, Ox and Cur) against four human colorectal cancer cell lines obtained from MTT reduction assay are presented Figure 1. It is evident from Figure 1 that, Ox produced highest anticancer activity among the tested compounds against all tested colorectal cell lines except LIM-2405 cell line where Cis showed the greatest cell kill. Although Cur showed the lowest antitumour activity among the investigated compounds, the phytochemical still presented significant anticancer activity against the tested cell lines.

Anticancer activity of the drugs in combination: Combined drug effect as a function of sequence of administration and added concentrations from selected combinations was determined using dose response curves and combination indices (CI). Dose response curves provide qualitative measure of combined drug actions while CI values represent the same in a quantitative manner. Figure 2a, Figure 2b, Figure 2c and Figure 2d present the dose response curves obtained from combinations of Cs with Cur against HT-29, Caco-2, LIM-1215 and LIM-2405 cell lines respectively. Figure 3a, Figure 3b, Figure 3c and Figure 3d present the dose response curves obtained from combinations of Ox with Cur against HT-29, Caco-2, LIM-1215 and LIM-2405 cell lines respectively. Table 1 and Table 2 give dose–effect parameters in terms of median-effect dose, shape (sigmoidicity), conformity (linear correlation coefficient), represented as Dm, m and r respectively. ED_{50}, ED_{75} and ED_{90} represents the combined drug concentration required for 50%, 75% and 90% cell kill, respectively.

It can be seen from Figure 2a-2d and Table 1 that, when Cs was administered in combination with Cur against the colorectal tumour models, strong synergism was found in LIM-1215 cell line and LIM-2405 cell lines depending on concentrations and sequence of administrations. In LIM-1215 cell line, stronger synergism was observed at lower concentrations than at higher concentrations. But only 0/4 and 4/0 sequences showed synergism against LIM-2405 cell line, with greater synergism being observed at lower concentrations. On the contrary, antagonism was predominant in HT-29 and CACO-2 cell lines except at ED_{90} level in HT-29 model where moderate synergism was evident.

Figure 3a-3d and Table 2 show that when Ox was administered in combination with Cur, synergism was found against all tested colorectal tumour models at all added concentrations and sequences of administrations. A general trend of increasing synergistic effect was evidenced with the increase in added concentration for all sequences of administrations against the colorectal cancer models. Stronger synergism was demonstrated against Caco-2 tumour model compared to other cell lines.

Cellular accumulation and Platinum–DNA binding study study: The study was conducted with selected combinations of platinum drugs (Cs/Ox) and the phytochemical (Cur) to find out the relationship between combined drug action and cellular accumulation of platinum and platinum–DNA binding using two human colorectal cell lines (HT-29 and Caco-2). The results obtained from the study are given in Table 3. It can be seen that synergistic combinations produced higher platinum-DNA binding compared to that of single administration of Cs or Ox in both tested colorectal cell lines. However, cellular accumulation study did not show any correlation with the accumulation of platinum and synergism from combination.

DNA damage study: The study was also conducted using drugs administered alone and in selected combinations to understand the correlation between DNA damage and drug effects (alone/combination). The results of the study are given in Table 4. Increase in mobility or decrease in intensity in the cellular DNA band of the drug treated cell compared to untreated blank indicates the qualitative damage to DNA. It can be seen from the study that synergistic combination (Ox+Cur 4/0) caused the highest damage to DNA in both HT-29 and Caco-2 cells. Whereas additive to antagonistic combinations caused less damage to DNA. The results indicate that death of cancer cell was due to interactios of the compounds with DNA so that synergism obtained from the combination of platinums (Cs/Ox) with Cur was positively correlated with DNA damage.

Proteomics: The study was conducted to get mechanistic information concerning changes in expression of proteins observed in Caco-2 colorectal cancer cell lines after treatment with Ox alone, Cur alone and combination of Ox with Cur (0/0). Among 195 protein spots identified in reference (untreated) Caco-2 gels, 80 spots underwent significant changes in expression in Cur alone treated gel. The number of spots experiencing significant changes in expression with Ox alone treatments were 148. After treatment with Ox with Cur (bolus) 86 protein spots underwent significant altered expression. Finally, 24 spots (which met the

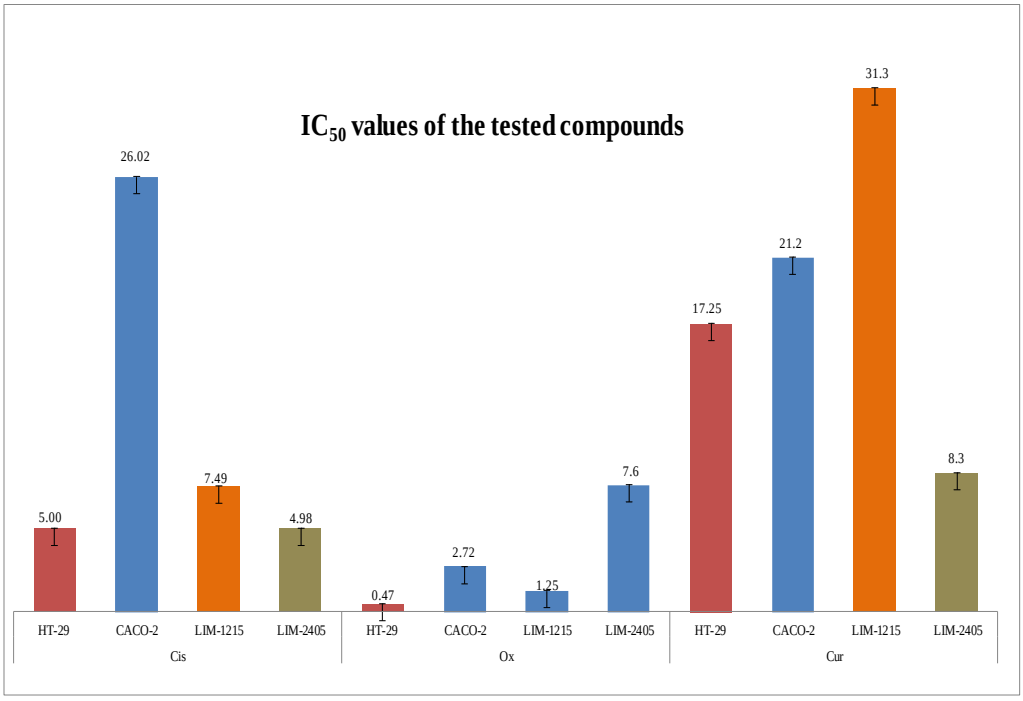
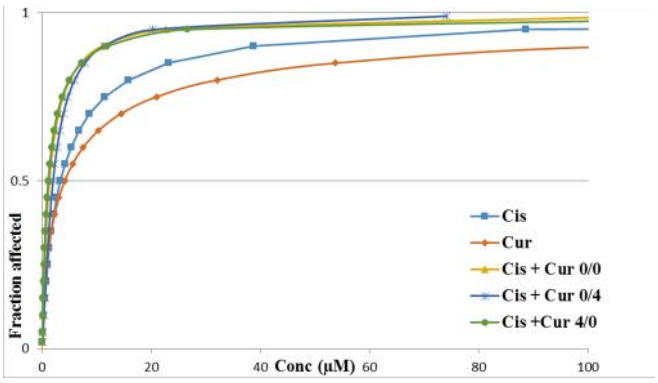
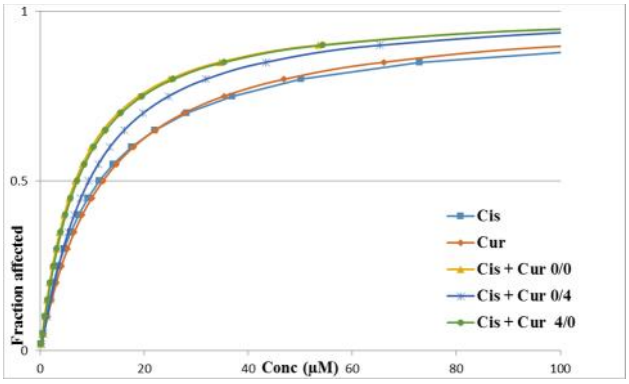


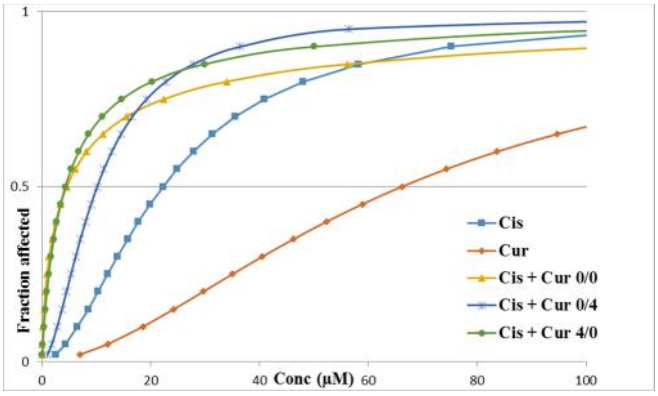
Figure 1 | Cytotoxicity of the single drugs against colorectal cancer cell lines (bars on top indicate errors in measurements) prevention.



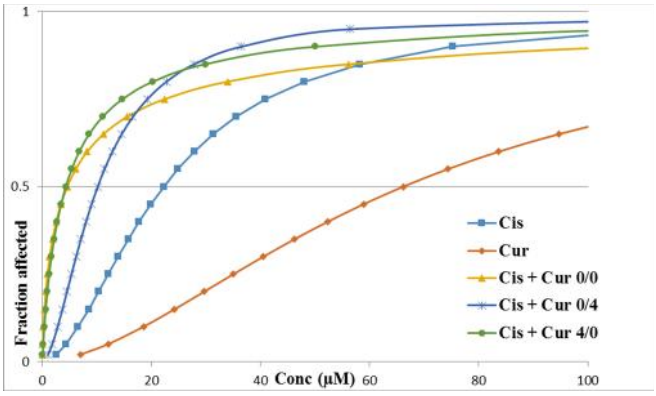
2-A



2-B

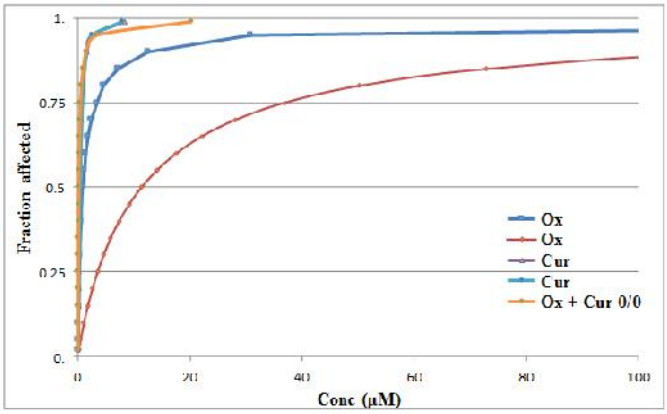


2-C

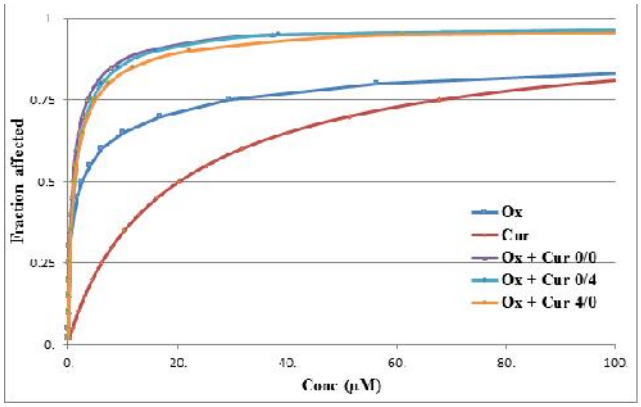


2-D

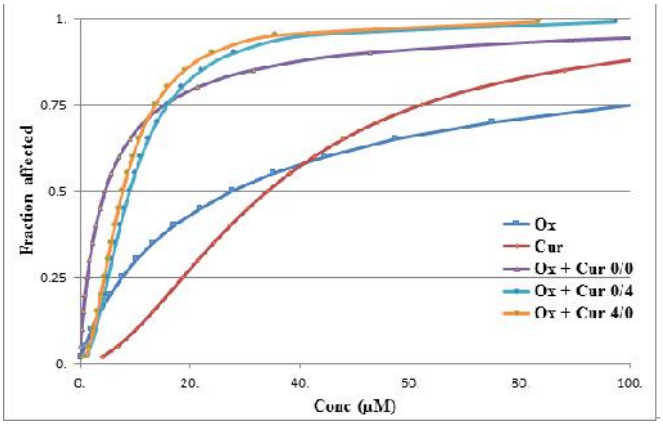
Figure 2 | Dose response curves obtained from combination of Cs with Cur. 2a: HT-29 cell line; 2b: Caco-2 cell line; 2c: LIM-1215 cell line; 2d: LIM-2405 cell line.



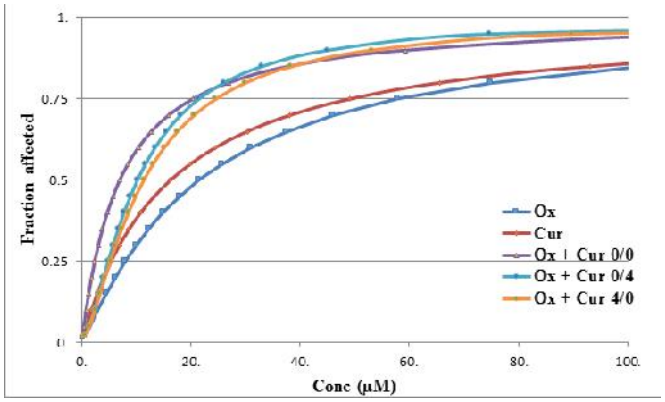
3-A



3-B



3-C



3-D

Figure 3 | Dose response curves obtained from combination of Ox with Cur. 3a: HT-29 cell line; 3b: Caco-2 cell line; 3c: LIM-1215 cell line; 3d: LIM-2405 cell line

Table 1 | Dose-effect parameters applying to combinations of cisplatin with curcumin in the colorectal cancer cell lines (results are based on at least triplicate independent experiments)

Cell line	Drug or drug combination	Sequence (h)	CI values at					
			ED_{50}	ED_{75}	ED_{90}	D_m	m	r
HT-29	Cs		NA	NA	NA	3.38	0.90	0.99
	Cur		NA	NA	NA	4.15	0.67	0.96
	Cs+Cur	0/0	1.51	0.97	0.65	1.33	1.04	0.98
	Cs+Cur	0/4	2.26	1.20	0.665	2.00	1.27	0.99
	Cs+Cur	4/0	1.27	0.92	0.69	1.12	0.93	0.98
Caco-2	Cs		N/A	N/A	N/A	11.34	0.93	0.98
	Cur		N/A	N/A	N/A	12.05	1.02	0.99
	Cs+Cur	0/0	1.05	0.95	0.86	6.74	1.06	1.00
	Cs+Cur	0/4	1.45	1.24	1.06	9.34	1.13	1.00
	Cs+Cur	4/0	1.11	0.99	0.88	7.10	1.08	1.00
LIM-1215	Cs		N/A	N/A	N/A	22.23	1.80	0.99
	Cur		N/A	N/A	N/A	66.17	1.73	0.93
	Cs+Cur	0/0	0.22	0.59	1.57	4.57	0.69	0.95
	Cs+Cur	0/4	0.49	0.51	0.52	10.14	1.72	0.99
	Cs+Cur	4/0	0.21	0.39	0.72	4.30	0.89	1.00
LIM-2405	Cs		N/A	N/A	N/A	12.94	1.73	0.93
	Cur		N/A	N/A	N/A	29.32	0.69	0.96
	Cs+Cur	0/0	0.74	1.66	4.00	7.62	0.70	0.97
	Cs+Cur	0/4	0.30	0.38	0.51	3.08	1.10	0.98
	Cs+Cur	4/0	0.28	0.41	0.65	2.89	0.96	0.98

Table 2 | Dose-effect parameters applying to combinations of oxaliplatin with curcumin in the colorectal cell lines (results are based on at least triplicate independent experiments)

Cell line	Drug or drug combination	Sequence (h)	CI values at					
			ED_{50}	ED_{75}	ED_{90}	D_m	m	r
HT-29	Ox		NA	NA	NA	0.86	0.82	0.98
	Cur		NA	NA	NA	0.31	1.39	1.00
	Ox +Cur	0/0	0.35	0.21	0.12	0.31	1.39	1.00
	Ox +Cur	0/4	0.44	0.24	0.13	0.38	1.52	1.00
	Ox +Cur	4/0	0.16	0.14	0.12	0.14	0.92	1.00
Caco-2	Ox		N/A	N/A	N/A	2.49	0.44	1.00
	Cur		N/A	N/A	N/A	20.28	0.91	1.00
	Ox +Cur	0/0	0.35	0.13	0.05	0.86	0.78	0.98
	Ox +Cur	0/4	0.49	0.16	0.05	1.21	0.85	0.98
	Ox +Cur	4/0	0.46	0.18	0.08	1.13	0.74	1.00
LIM-1215	Ox		N/A	N/A	N/A	27.70	0.85	0.92
	Cur		N/A	N/A	N/A	34.12	1.83	0.93
	Ox +Cur	0/0	0.45	0.70	1.16	4.55	0.90	0.96
	Ox +Cur	0/4	0.90	0.72	0.62	9.04	1.93	0.99
	Ox +Cur	4/0	0.78	0.62	0.53	7.79	1.94	0.97
LIM-2405	Ox		N/A	N/A	N/A	21.45	1.11	0.95
	Cur		N/A	N/A	N/A	16.26	0.99	0.92
	Ox +Cur	0/0	0.72	0.73	0.74	6.98	1.03	0.95
	Ox +Cur	0/4	1.05	0.77	0.57	10.22	1.48	0.99

Table 3 | Cellular accumulation of platinum and platinum-DNA binding (based on triplicate measurements).

Cell line	Sample	Combined Effect at ED_{50}	Pt (nmol/5x10^6cell)	Pt (nmol)/DNA(mg)
HT-29	(alone)	Not applicable	4.27±0.04	1.00±0.08
	Cs with Cur (4/0)	Additive to antagonistic	4.90±0.26	0.93±0.15
	Ox (alone)	Not applicable	0.09±0.28	0.20±0.03
	Ox with Cur (0/0)	Synergistic	0.08±0.01	0.26±0.15
	Ox with Cur (4/0)	Synergistic	0.09±0.22	0.24±0.04
Caco-2	Cs (alone)	Not applicable	2.02±0.03	1.28±0.14
	Cs with Cur (4/0)	Additive to antagonistic	3.52±0.03	1.55±0.27
	Ox (alone)	Not applicable	0.27±0.3	0.19±0.50
	Ox with Cur (0/0)	Synergistic	0.09±0.19	0.17±0.12

Table 4 | DNA mobility and fluorescence obtained from interactions of the compounds with DNA (Based on triplicate measurements).

Bands	Combined drug action	Mobility (mm)	Net Intensity
HT-Blank	N/A	3.96	49801.49
HT-Cs	N/A	3.7	45927.49
HT-Cs + Cur (4/0)	Additive to antagonistic	3.79	39748.15
HT-Ox	N/A	3.79	32815.53
HT-Ox + Cur (4/0)	Synergistic	4.13	6273.96
HT-Cur	N/A	4.46	10979.88
CA-Blank	N/A	5.8	16623
CA-Cs	N/A	5.21	13041.2
CA-Cs + Cur (4/0)	Additive to antagonistic	5.29	9644
CA-Ox	N/A	5.04	23210.5
CA-Ox + Cur (4/0)	Synergistic	5.13	11958.9
CA- Cur	N/A	5.13	16467.5

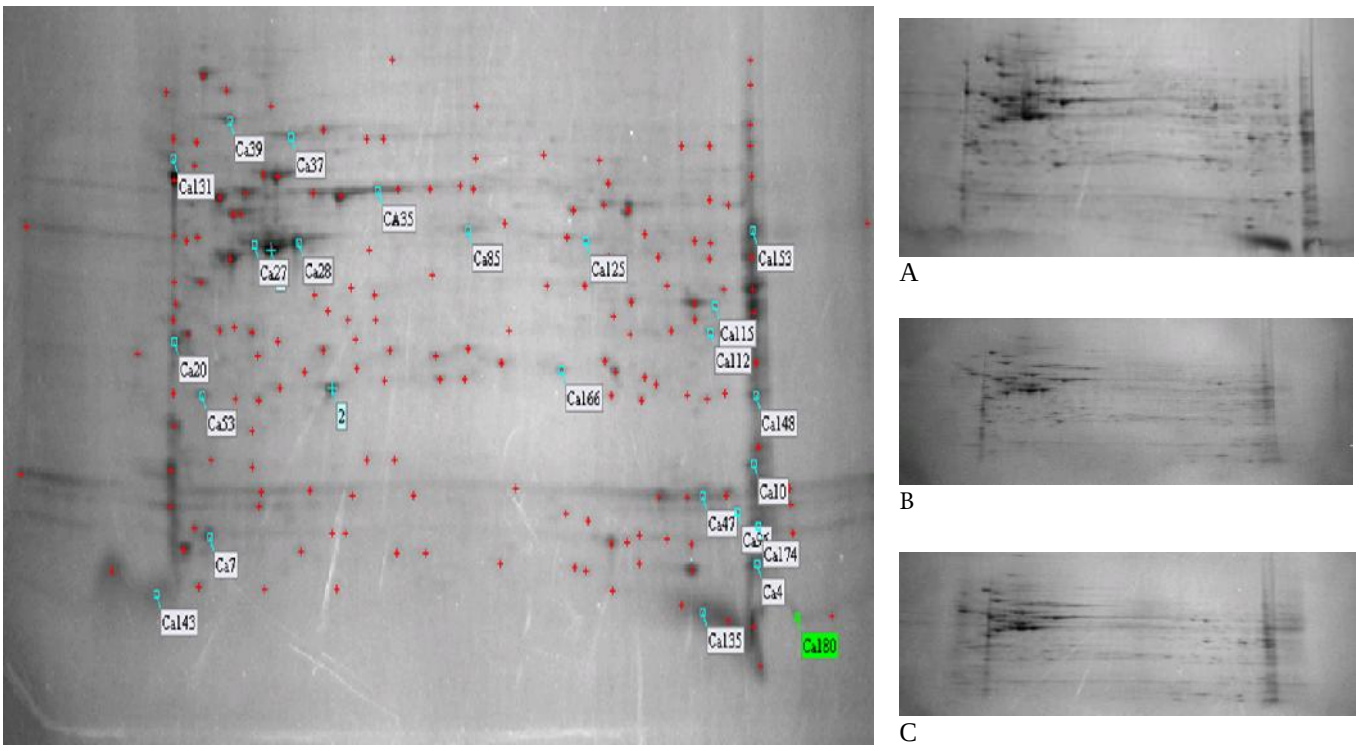


Figure 4 | Two dimensional gel images (a-c) of treated Caco-2 gels (A) Ox alone treated Caco-2 gel; (B) Cur alone treated CACO-2 gel; (C) Ox with Cur (bolus) treated Caco-2 gel

Table 5 | Selected protein spots displaying changes in expression as applied to CACO-2 cell

Match ID	Ox alone	Cur alone	Ox with Cur (0/0)
Ca35	SDR	Up-regulated	Up-regulated
Ca37	SUR	Up-regulated	Up-regulated
Ca39	Up-regulated	Up-regulated	Up-regulated
Ca53	Down-regulated	Up-regulated	Not changed
Ca85	SUR	Down-regulated	Up-regulated
Ca125	Up-regulated	SUR	Up-regulated
Ca166	SUR	Up-regulated	Up-regulated

SDR denotes slightly downregulated and SUR denotes slightly upregulated

Table 6 | Proteins from CACO-2 cell lines characterized (MALDI-MASS analysis)

Match ID	Short name	Full Name	Mass (Da)/pI	Mascot score and Sequence coverage (%)
Ca35	K2CB	Keratin, type II cytoskeletal 8	53671/4.95	256 and 19
Ca37	HSP7C	Heat shock cognate 71 kDa protein	70854/5.37	536 and 24
Ca39	GRP78	78 kDa glucose-regulated protein	72288/5.07	588 and 27
Ca53	PSB6	Proteasome subunit beta type-6	25341/4.80	62 and 10
Ca85	COF1	Cofilin-1	18491/8.22	138 and 31
Ca125	IDHC	Isocitrate dehydrogenase [NADP] cytoplasmic	46630/6.53	130 and 16
Ca166	K1C18	Keratin, type I cytoskeletal 18	48029/6.36	345 and 4

Discussion

Combination therapy can have significant advantage over monotherapy by improving the efficacy and reducing the side effects. However, most of the combination therapy currently being used (e.g. chemotherapy in combination with monoclonal antibody, chemotherapy in combination with mRNA, two or more chemotherapeutics in combination) suffers from increase in treatment cost (Lu, Lu et al. 2013). Combining phytochemicals having anticancer potential with chemotherapeutic drug might provide cost-effective solution of drug resistance in cancer. Of note, sequence of administered chemotherapeutic drugs has been implicated in combined drug actions (Levis, Pham et al. 2004). In this study binary combination of platinum drugs (Cs/Ox) and phytochemical Cur have been investigated using three different sequences of administration and concentrations against colorectal cancer models. The results of the combination study have been given in Table 1 and Table 2. It can be concluded from the results that combination of Ox with Cur is better in cell kill than that of Cs with Cur against studied four colorectal cancer models. Higher concentrations have shown greater synergism in the combination of Ox with Cur but the effect is converse in case of Cs with Cur. Previous studies from our group against ovarian cancer models (including cisplatin and picoplatin resistant A2780 cell lines) also revealed synergism from the combination of cisplatin and curcumin (Yunos, Beale et al. 2011; Nessa, Beale et al. 2012).

Younos *et al.* also reported that the observed synergism from the combination of cisplatin and curcumin is stronger at ED_{50} levels compared to that of ED_{75} and ED_{90} levels. Similarly, oxaliplatin in combination with curcumin also produced sequenced dependent synergism against ovarian tumour models. Sequenced combination of curcumin and platinum drugs with curcumin administered first and platinum drug 2 h later was found to show more pronounced synergistic effect against three ovarian cancer cells (Nessa, Beale et al. 2012). Another group also noticed significant synergism from combination of curcumin with cisplatin and oxaliplatin against 2008 and C13 ovarian cancer cell lines (Montopoli, Ragazzi et al. 2009). Moreover, curcumin and carboplatin in combination synergistically inhibited apoptosis and metastasis against lung cancer (Kang, Kang et al. 2015).

A number of studies against CRC models also demonstrated synergism from combinations of curcumin with platinum drugs. Oxaliplatin in combination with liposomal curcumin showed significant synergism during *in vitro* and *in vivo* xenograft model study using Lovo and Colo-205 colorectal cancer cells (Li, Ahmed et al. 2007). Another *in vitro* and *in vivo* model study using HCT-116 cell lines reported that curcumin in combination with oxaliplatin reduces the chemoresistance towards oxaliplatin (Howells, Sale et al. 2011). Curcumin in combination with camptothecin also exhibited strong synergism against a colorectal cancer model (Xiao, Si et al. 2015). In an animal model study using

FOLFOX resistant HT-29 and HCT-116 cancer cells, curcumin in combination with dasatinib showed inhibition of tumour growth, metastasis and colonosphere formation. The combination drug therapy significantly decreased the number of cancer stem cells by reducing CD133, CD44, CD166 and ALDH (Nautiyal, Kanwar et al. 2011). The mechanism behind the synergistic effects from combination of curcumin with platinum drugs was reported to be associated with the down regulation of matrix metalloproteinases (MMP-2 and MMP-9), BCL-2, NF-κB as well as upregulation of caspases (caspase-3 and caspase-9) and p53 (Kang, Kang et al. 2015).

To reach into the targets to impart antitumour activity, drugs must enter the cell while one of the key mechanisms for the development of resistance against platinum based anticancer drugs is to decrease the influx of the drugs into the cell. Another important mechanism is to increase the efflux of platinums from the cell (Zhu, Shanbhag et al. 2017). In both cases, outcome is reduced accumulation of platinums to reach into the target DNA to show its antitumour activity (Yu, Yang et al. 2015). Cellular accumulation study was conducted with the idea that: synergistic and additive treatments would cause increased cellular accumulation of platinum or at least would not reduce the accumulation; and antagonistic treatment might cause lower cellular accumulation of platinum. But in the present study, synergistic combinations of Ox with Cur (Bolus and 4/0) against HT-29 and Caco-2 cell line did not show increase in cellular platinum accumulation. However, synergistic combined treatments of Ox with Cur using bolus and 4/0 administration demonstrated 1.2 to1.3 times greater extent of platinum⊠DNA binding than Ox alone treatment in HT-29 cell line. The results suggest that synergism attained from the combination of Ox with Cur in this study is directly relate to the extent of platinum⊠DNA binding.

Proteomics revealed seven proteins e.g. Keratin, type II cytoskeletal 8 (K2CB); Keratin, type I cytoskeletal 18 (K1C18); Heat shock cognate 71 kDa protein (HSP7C); 78 kDa glucose regulated protein (GRP78); Proteasome subunit beta type-6 (PSB6); Isocitrate dehydrogenase [NADP] cytoplasmic (IDHC) and Cofilin1 which underwent significant altered expression after drug treatments and considered to be associated with drug actions either alone or in combination.

Keratin, type II cytoskeletal 8 (K2CB) is a type II cytoskeletal 8 keratin (K8) and usually coexpressed with K18 in normal epithelial cells. K2CB protein is the oldest keratin among all identified and play significant role in regulation of cell cycle (Magin, Vijayaraj et al. 2007), protecting cells from stress (Ku, Soetikno et al. 2003), injury and apoptosis (Caulin, Ware et al. 2000). Altered expression of K2CB protein is evidenced in lung (Hmmier, O'Brien et al. 2017), pancreatic, breast, renal, colorectal (Yamamoto, Kudo et al. 2016), liver (Takegoshi, Okada et al. 2016), endometrial, ovarian and gastric cancer (Moll, Divo et al. 2008). K2CB protein was reported to be upregulated in breast cancer (Wu, Hancock et al. 2003; Hamler, Zhu et al. 2004), skin carcinoma (Larcher, Bauluz et al. 1992), bladder cancer cells (Lei, Zhao et al. 2013) compared to non cancerous counterparts. However downregulation of the protein in cisplatin resistant esophageal cell line and lung cancer has also been reported (Lai, Chan et al. 2016; Hmmier, O'Brien et al. 2017). In the present study, the protein was upregulated following the treatments of Cur alone and synergistic combined treatment of Ox with using bolus administration but downregulated after the treatment of Ox alone. The role of the protein remained unclear from this study due to inconsistency in the expression of K2CB protein following different treatments. However, literature suggests that K2CB protein contributes significantly in promoting colorectal cancer. Natural tumour active compound sulforaphene has been reported to give anticancer activity by supressing K2CB protein; subsequently increased Fas concentration, decreased cFLIP activity and induced apoptosis (Yang, Ren et al. 2016). In a study on 25 patients of prostate cancer while receiving chemotherapy, level of K18 was monitored which is usually coexpressed with K2CB protein. It was observed that circulatory K18 level was increased or decreased following adminstration of different drugs. The authors commented that cell death induced by chemotherapy does not inevitably depend on apoptosis, rather multiple mechanisms are involved (Ueno, Toi et al. 2005). Further study is required to ascertain the role of K2CB protein in colorectal cancer.

K1C18 protein is a type I cytoskeletal 18 keratin (K18) protein which is highly conserved from teleosts to mammals. In 1950s the association of keratins including K1C18 protein was reported (Björklund and Björklund 1957; Björklund 1978). Later on it was discovered that K1C18 protein is cleaved during apoptosis of normal and malignant cells at two sites into three fragments (Ueno, Toi et al. 2005). Upregulation of K1C18 protein in ovarian carcinoma (Wang, Kachman et al. 2004), cholangiocarcinoma (Srisomsap, Sawangareetrakul et al. 2004) and breast carcinoma (Wu, Hancock et al. 2003; Vergara, Simeone et al. 2013) has been observed in different studies. On the contrary, other studies showed downregulation of K1C18 protein in several cancers including: prostate (O'Connell, Prencipe et al. 2012), cervical (Buddaseth, Göttmann et al. 2013) and colon carcinoma (Roblick, Hirschberg et al. 2004). One earlier studies suggested upregulation of K1C18 protein is the good prognostic factor in breast cancer (Schaller, Fuchs et al. 1996). In the present study, the protein has been identified from Caco-2 cell line. K1C18 protein was upregulated following all the treatments indicating that the protein might act as apoptotic protein. The highest upregulation of the protein was caused with Cur alone treatment by a factor of 7.26.

Whereas, synergistic treatment of Ox with Cur (bolus) caused 6.26 folds upregulation of K1C18 protein and Ox alone produced 2.6 times upregulation of the same protein. Similar to this study, oxaliplatin has been reported to cause upregulation of the protein in three different colorectal cancer cell lines (Yao, Jia et al. 2009). Moreover, the result of the present study is in accordance with earlier findings where maslinic acid (antitumour compound) downregulated the expression of K1C18 protein significantly in HT-29 colon cancer cell line (Rufino-Palomares, Reyes-Zurita et al. 2013). Another *in vivo* study also revealed that elevated expression of the protein leads to suppression of malignancy in breast cancer cell (Bühler and Schaller 2005). It can be concluded that K1C18 protein is an apoptotic protein and could be targeted to design newer anticancer drugs.

Heat shock cognate 71 kDa protein (HSP7C) belongs to HSP70s class of molecular chaperones. In an earlier study, expression of HSP7C protein was found to be higher in cancerous cell lines compared to non-malignant cells e.g. lung, gastric, pancreatic, breast, cervical and endometrial cancer (Maeda, Ohguro et al. 2000). The level of HSP7C protein was found to be significantly increased in 95% samples collected from the patients with colon cancer compared to the control group (Kubota, Yamamoto et al. 2010). The protein has been suggested as potential biomarker in neuroblastoma (Sandoval, Hoelz et al. 2006). In the present study, the protein was upregulated following all the treatments. Synergistic combined treatment of Ox with using bolus administration produced highest upregulation by a factor of 4.53, followed by Cur alone treatment by the factor of 2. It can be assumed that observed upregulation of HSP7C protein (following the treatments) and cell death might have a positive link. In both colorectal cell lines, synergistic combined treatments caused the highest amount of cell death. It could be the role of HSP7C protein as inducer of autophagy, promoting cytotoxicity by upregulating HSP7C. The protein has been reported to induced all three forms of autophagy: chaperone mediated autophagy, macroautoghagy and microautophagy (Stricher, Macri et al. 2013).

78 kDa glucose regulated protein or GRP78 acts a master regulator during endoplasmic stress. The primary responsibility of GRP78 protein is to translocate the proteins, controlling the folding and assembly of proteins, identification and deletion of misfolded proteins (Macias, Williamson et al. 2011). However, exposure of stress caused detachment of GRP78 protein from the mentioned transmembrane sensor proteins of endoplasmic reticulum and switches on the protective mechanism to avoid cell death (Lee 2007). Since cancer cells are continually subjected towards endoplasmic reticulum stress, GRP78 protein plays an integral role in survival of cancer cells and promote carcinogenesis. Literature suggests that GRP78 protein can also mediate chemotherapy resistance and inhibit apoptosis (Wang, Wey et al. 2009). Elevated expression of GRP78 protein has been documented in many cancers: liver (Shuda, Kondoh et al. 2003), breast (Lee, Nichols et al. 2006), lung (Fu and Lee 2006) and prostate (Miyake, Hara et al. 2000; Daneshmand, Quek et al. 2007). In the present study, the protein was upregulated following all the treatments in CACO-2 cell line. Highest upregulation was evidenced after the treatment with Cur alone by the factor of 2.2 whereas Ox alone did upregulation of the protein by 1.61 times. Synergistic combined treatment of Ox with Cur using bolus administration upregulated GRP78 protein by a factor of 1.56. However, it was difficult to understand the relationship between observed upregulation of GRP78 protein following drug treatments and anticancer activity.

PSB6 or proteasome subunit beta type-6 is part of 20S catalytic core of the proteasome. PSB6 protein modulates the cell cycle and many other processes through the breakdown of regulatory components and transcription factors (Frankland-Searby and Bhaumik 2012). Upregulation of PSB6 protein has been observed in lung cancer (Lu, Song et al. 2014), breast cancer (Canelle, Bousquet et al. 2006), thyroid cancer (Onda, Emi et al. 2004), hypoxia (Wang, Xu et al. 2013) and prostate cancer (Davalieva, Kostovska et al. 2015). In a study of HCT-116 colon cancer cell line, elevated expression of the protein also detected. In the present study, PSB6 protein was downregulated after the treatment with Ox alone in CACO-2 cell line. In contrast, upregulation of the protein was observed following the treatment with Cur alone in the same cell line. But PSB6 protein did not show significant changes in expression following synergistic combined treatment of Ox with Cur using bolus administration in CACO-2 cell line. This might be due to counterbalancing effect between Ox and Cur. In accordance to this study, PSB6 protein reported to be upregulated following treatment with curcumin in a breast cancer model (Fang, Chen et al. 2011). Further study is warranted to target PSB6 protein as anticancer drug target. Upregulation of PSB6 protein has been observed in lung cancer (Lu, Song et al. 2014), breast cancer (Canelle, Bousquet et al. 2006), thyroid cancer (Onda, Emi et al. 2004), hypoxia (Wang, Xu et al. 2013) and prostate cancer (Davalieva, Kostovska et al. 2015). In a study of HCT-116 colon cancer cell line, elevated expression of the protein also detected. In the present study, PSB6 protein was downregulated after the treatment with Ox alone in Caco-2 cell line. In contrast, upregulation of the protein was observed following the treatment with Cur alone in the same cell line. But PSB6 protein did not show significant changes in expression following synergistic combined treatment of Ox with Cur using bolus administration in Caco-2 cell line. This might be due to counterbalancing effect between Ox and Cur. In accordance to this study, PSB6 protein reported to be upregulated following treatment with curcumin in a breast cancer model (Fang, Chen et

al. 2011). Further study is warranted to target PSB6 protein as anticancer drug target.

IDHC or Isocitrate dehydrogenase [NADP] cytoplasmic is a protein belongs to oxidoreductase enzyme class. These enzymes are responsible for transfering electrons from oxidants to reductants which could be oxidases or dehydrogenases. Mutation of IDHC is evident in many cancers specially in glioma, which cause the enzyme to act in faster rate and more efficiently. However, controversy still exists regarding the role of IDHC in cancer, whether it provides oncogenic effects or tumour suppressive effects (Reitman and Yan 2010). In the present study, IDHC protein was identified from CACO-2 cell line. Following drug treatments either alone or in combination, the protein displayed uprgulation. Ox alone treatment caused the protein to be upregulated by 3 folds whereas Cur alone treatment did 1.8 folds upregulation. Synergistic combined treatment of Ox with Cur using bolus administration caused highest upregulation of IDHC by a factor of 5.75. It can be assumed from this study that IDHC protein might have proapoptotic action. To the best of my knowledge, this is the first report showing the changes in expression of IDHC protein in colorectal cancer cell following treatments with tumour active compounds. However, upregulation of IDHC protein has been documented in lung (Tan, Jiang et al. 2012), breast (Russell Hilt, Wittliff et al. 1973; Xu, Yan et al. 2010) and esophageal cancer cells (Qi, Chiu et al. 2005) compared to their noncancerous counterparts. A recent study has proved that, elevated expression of IDHC protein leads towards aggravation of tumour and therapy resistance in glioblastoma. The author suggested that inhibition of IDHC protein could be a promising therapeutic strategy against glioblastoma (Calvert 2017).

Cofilin1 is one of the traditional cofilins along with cofilin2 (muscle isoform-skeletal or cardiac) and destrins (available various tissues) (Bernstein and Bamburg 2010). Cofilin1 is responsible for actin polymerization and depolymerization through severing of filaments. At lower concentration cofilin1 favours severing of actin filaments and facilitate depolymerization, whereas at higher concentration actin nucleation and polymerization takes place (Shishkin, Eremina et al. 2016). Cofilin1 facilitates the polymerization process of actin filaments by creating free pointed ends and providing actin monomers. Thus it is considered as essential regulator of cell motility and metastasis in malignant cells (Ghosh, Song et al. 2004). Moreover, cofilin1 plays vital role in restructuring of actin cytoskeleton when exposed towards variety of stimuli and stressed conditions. Other molecular level functions of cofilin1 includes: release of cytochrome C (Chua, Volbracht et al. 2003) and activation of phospholipase D1 (Han, Stope et al. 2007). Elevated expression of cofilin1 has been found in lung (Keshamouni, Michailidis et al. 2006), pancreatic (Sinha, Hütter et al. 1999), oral (Turhani, Krapfenbauer et al. 2006), kidney (Unwin, Craven et al. 2003), colorectal (Zhao, Liu et al. 2007) and ovarian cancer (Martoglio, Tom et al. 2000). However downregulation of cofilin1 is also evidenced in lymphoma, cervical, hepatic and colon cancer (Nebl, Meuer et al. 1996). In the present study, the protein was slightly upregulated after treatment with Ox alone and significantly upregulated following the treatment of synergistic combination of Ox with Cur using bolus administration in Caco-2 cell line. However, the protein was downregulated after the treatment with Cur alone in the same cell line. The variation in the expression of cofilin1 following drug treatments makes it difficult to ascertain the nature of the protein. The controversial nature of cofilin1 has been reported earlier as well (Tsai, Lin et al. 2015). The authors mention that, although strong evidence on upregulation of cofilin1 in multiple cancers is evident but overexpression of the protein may also cause suppression of cancer growth and invasion of cancer cells (Tsai, Lin et al. 2015). It was suggested that overexpression of cofilin1 cause cell cycle arrest but not induce apoptotic cell death. That is why strict control of cofilin1 expression is indispensable for normal functioning of cells (Tsai, Chiu et al. 2009).

Conclusion

In terms of synergistic outcomes, combination of Ox with Cur proved to be better than combination of Cs with Cur for all sequences of administration (0/0, 0/4 and 4/0) against the tested four colorectal cell lines (HT-29, Caco-2, LIM-1215 and LIM-2405). Ox in combination Cur demonstrated very high synergism against HT-29 and Caco-2 cell lines. Synergism from Ox with Cur was found at higher added concentrations (ED_{90} and ED_{75}) than lower added concentrations (ED_{50}) for all sequences of administration. Synergism from Ox with Cur may be associated with greater platinum⊠DNA binding. Proteomics revealed that the elevated expressions of K1C18, GRP78, IDHC and Cofilin1 may be responsible for the synergistic activity obtained from the combination of Ox with Cur.

Author Contributions

Hana Bali carried out the lab work, carried out data analyses and drafted the manuscript. Jun Qing Yu contributed to development of experimental methods and assisted in data analyses. Philip Beale contributed to the discussion and writing of the manuscript. Fazlul Huq conceptualized the project, provided overall supervision including development of methods, data analyses and drafting of the manuscript.

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Competing financial interests

The author(s) declare no competing financial interests.

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