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Original Article

Chou-Talalay Analysis: Exploring Synergistic Pharmacological Effects of 5-Fluorouracil and Curcumin for Dose Optimization

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ABSTRACT

Background: 5-Fluorouracil (5-FU) is a chemotherapy drug commonly used in the treatment of colon cancer. There is a significant drive to mitigate the limitations of 5-fluorouracil while maintaining its therapeutic potency by exploring the potential of using natural products such as curcumin in conjunction with 5-FU. Thus, the present study investigates the *in vitro* pharmacodynamic interaction between 5-FU and curcumin using the Chou-Talalay method in HT29 cell lines. **Materials and Methods:** Initially, the estimation of half-maximal concentration of the medications against HCT116 and HT29 cell lines was performed by 3-4,5-dimethylthiazol-2-yl-2,5 diphenyl tetrazolium bromide assay. Later, the combination effect was studied in HT29 cell lines by constant ratio experiment design. **Results and Discussion:** The research findings revealed that the 5-FU and curcumin exhibit dose-dependent anti-proliferative activity in HCT116 and HT29 cells. Moreover, it was observed that HT29 cell lines are less sensitive to 5-FU than HCT116 cell lines, rather than curcumin was found to inhibit cell proliferation in both cell lines with no significant statistical difference ($p<0.05$). The Combination Index (CI) values of less than 1 for all the combined experimental ratios indicated the synergistic effect of the two compounds. The generated Dose Reduction Index (DRI) values for the combination imply for the favourable dose reduction in the treatment of colorectal cancer. **Conclusion:** Significantly, the study indicates that 40:1 ratio of 5-FU to curcumin might represent an ideal proportion for co-administration using an appropriate delivery system.

Keywords: Chou-Talalay analysis, Colorectal cancer, Curcumin, 5-fluorouracil, Dose optimization, Synergistic effect.

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INTRODUCTION

Colorectal cancer shares its anatomical origin either in the colon or rectum. Though these malignancies are also known as colon cancer or rectal cancer, they display a number of identical characteristics that make them clubbed together. This malignancy typically starts as a tiny growth called a polyp, which can eventually progress to cancer. Later, metastasis of colon cancer befalls when these cancer cells from the colon invade other organs, the liver being the most common site of metastasis, although it exhibits a substantial impact on the lungs and bones.[1]

Global Cancer Statistics 2020, exemplified colorectal cancer as the third most frequently diagnosed cancer with a massive estimate of 1.9 million new cases in 2020. Colorectal cancer accounts for approximately 10.0% of all cancer cases and is one of the topmost leading malignancies contributing to about 935,000 (9.4%) cancer-related deaths globally in 2020.[2]

The multimodal treatment strategies of colorectal cancer typically encompass surgical interventions alongside conventional radiation therapy and chemotherapy, as well as the current era targeted therapies.[3] Nonetheless; these therapies carry certain demerits in terms of substantial adverse reactions due to non-selectivity[4] and drug resistance over time, resulting in relapses.[5]

Currently, combination chemotherapy is emerging as a key treatment strategy for colorectal cancer. This approach is anticipated to confer beneficial outcomes in terms of enhanced efficacy besides addressing chemoresistance. In a previous study, they compared the clinical outcomes of patients with advanced colorectal cancer receiving combination chemotherapy (FOLFOX

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regimen) versus fluorouracil therapy and demonstrated that the combination chemotherapy group had significantly longer median survival and higher response rates compared to the single drug group.[6]

Of late, experimental studies are demonstrating the combination of chemotherapy and phyto-constituents as promising choices to navigate the future roadmap of colorectal cancer therapy. One such phyto constituent is curcumin, a polyphenol derived from the plant *Curcuma longa*, which is extensively reported to prevent and alleviate cancer by suppressing tumour cell proliferation besides downregulating the oncogenic transcription factors. Curcumin is witnessed to play a remarkable role in inhibiting tumour development, progression and metastasis.[7] Besides synergistic activity, a number of studies reported that curcumin enhances the sensitivity of colon cancer stem cells to 5-fluorouracil, FOLFOX and irinotecan,[8-10] thereby, preventing the emergence of chemo-resistant colorectal cancer cells. Additionally, by down-regulating the drug-efflux transporters, curcumin demonstrates an increased intracellular accumulation of oxaliplatin and colorectal cells.[11,12]

The utilization of the co-delivery system provides a new insight in combination cancer chemotherapy.[13] Before designing delivery systems for combined drugs, comprehending their interaction dynamics is essential.[14] The Chou-Talalay method precisely does this by assessing the synergistic or antagonistic effects of drug combinations.[15-18] This analysis provides critical insights into the ideal ratios and concentrations of drugs that work optimally together.[19] Thus, in this study, towards designing more efficient therapeutic protocols, we intend to explore the appropriate doses of curcumin to be administered as an adjunct to 5-FU, in order to establish the former's synergistic cytotoxic potential towards the selected chemotherapeutic agent against HT29 cells.

MATERIALS AND METHODS

5-Fluorouracil (99%) was purchased from Otto Chemie, Mumbai; Curcumin was obtained as gift sample from Zeus-Hygia Life Sciences, Bangalore; HT29 (Human Colon Cancer Cell line, NCCS, Pune), HCT116 (Human colorectal carcinoma cell line, NCCS, Pune), DMEM High Glucose medium (#AL066A, Himedia), Fetal Bovine Serum (#RM10432, Himedia), MTT Reagent (#4060, Himedia), Dimethylsulfoxide (#PHR1309, Sigma), D-PBS (#TL1006, Himedia)

Cytotoxicity assay for single drug

Seeded 200 µL cell suspension in a 96-well plate at required cell density (10,000 cells per well), without the test agent and allowed the cells to grow for about overnight. Appropriate concentrations of 5-FU (1, 10, 20, 50 and 100 mM) and curcumin (20, 50, 100, 150 and 200 µM) were added to respective wells seeded with HT29 cells. The following concentration ranges (1, 10, 50, 100 and 1000 µM) of 5-FU and Curcumin were added to the corresponding wells seeded with HCT116 cells. Incubated the plate for 48 hr at 37°C in a 5% CO_2 atmosphere. After the incubation period (48 hr), spent media was removed from the wells and MTT reagent to a final concentration of 0.5mg/mL of total volume was added to each well and incubated for 3 hr. Removed the MTT reagent and then 100 µL of solubilisation solution (DMSO) was added to each well. Finally, read the absorbance on an ELISA reader at 570 nm. The percentage cell growth inhibition was estimated using Equation 1.[20,21]

$$\%\ Inhibition = \left(1 - \frac{OD\ of\ treated\ cells - OD\ of\ media\ blank}{OD\ of\ vehicle\ control - OD\ of\ media\ blank}\right) * 100 \quad \text{..... Eq. (1)}$$

Development of dose-response curve for individual drugs and identification of specific parameters

The CompuSyn software which performs based on the median effect equation was utilized to construct the dose-response curve of 5-FU and curcumin. The dose-effect parameters such as IC_{50} (the concentration required to inhibit 50% of the cell growth), the correlation co-efficient (R_2) and the slope value (m) are determined for the individual drugs. The co-efficient m gives the shape of the dose-effect curve. The median effect equation is given in Equation 2;

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m}\right)^2 \text{..... Eq. (2)}$$

in which f_a is the fraction of drug effect (inhibition of cell growth) and f_u ($1-f_a$) being the unaffected fraction. D_m is the median effect dose (IC_{50}) and D is the drug dose or concentration. The shape of the curve is defined by the m value where m>1, m<1 and m=1 indicate the sigmoidal, flat sigmoidal and hyperbolic dose response curves respectively. The R_2 values denote the extent of correlation of the data. The Chou-Talalay method can be applied provided the above-mentioned parameters for individual drugs are known.[15,22,23]

Cytotoxicity assay for combination of 5-FU with curcumin

The pharmacological interaction (synergism, additivity or antagonism) between 5-FU and curcumin was identified using the Chou-Talalay method. The method recommends applying the equipotent constant combination ratios of the chosen drugs. Thus, five different combination ratios as specified in the Table 1 are used and their cytotoxic effects are measured by MTT assay after incubation for 48 hr.

Combination Index value is a dimensionless quantity which explains the type of pharmacodynamic interaction of the drug combinations. Based on the method proposed by Chou-Talalay, CI<1, CI=1 and CI>1 indicate synergism, additive effect and antagonism respectively. The CI values can be computed directly from the CompuSyn software using the CI equation as written in Equation 3

$$CI = \frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} \quad ; \quad D = Dm \left[\frac{fa}{1-fa}\right]^{1/m} \quad \text{..... Eq. (3)}$$

Where $(D_x)_1$ and $(D_x)_2$ are the doses of the drug D_1 and D_2 respectively when they are present alone that inhibits the cell growth by x%, $(D)_1$ and $(D)_2$ are the doses of the same drugs in combination inhibiting the cell growth by x%.

The Dose Reduction İndex (DRI) is a parameter that measures the extent by which the dosage of a drug in a combination treatment can be reduced while maintaining the same level of efficacy compared to when the drug is used alone. It is calculated using the Equation 4.[22,23]

$$DRI = \frac{(D_x)_1}{D_1} \quad \text{..... Eq. (4)}$$

Statistical Analysis

All data are presented as the mean±SD from three independent experiments. GraphPad Prism v7.0 software was used for statistical analysis. The two-way ANOVA followed by Tukey's *post hoc* test was used for multiple comparisons. p-value<0.05 was considered statistically significant.

RESULTS

Cytotoxicity assay of individual drugs

Following the MTT experiment for each medication alone against HCT116 and HT29 cells, the CompuSyn software was used to produce the single-drug dose-effect curves as well as calculate the parameters (Dm), (m) and (R_2). The dose-response and median effect curves of 5-FU and curcumin against HCT116 cell lines are presented in Figure 1.

The study results showed that 5-FU and curcumin inhibit both HCT116 and HT29 cell proliferation in a concentration-dependent manner. From the dose-response curves, it is observed that the 5-FU and curcumin were able to inhibit the HCT116 cell proliferation with the IC_{50} value of 34.07±1.09 µM and 65.09±3.54 µM respectively. Also, 5-FU and Curcumin had Dm values (IC_{50}) of 2.2±1.35 mM and 51.5848±5.25 µM respectively against HT29 cell lines which are similar to those reported earlier.[24] The (R_2) correlation coefficient values of the curves were all more than 0.95, indicating acceptable compliance with the mass-action law.[25,26] The above findings demonstrate that the HCT116 cells are more sensitive to 5-FU than the HT29 cells wherein the natural bioactive compound curcumin effectively inhibits both the cell proliferation at lower concentrations. The anti-proliferative activity of the individual components, 5-FU and curcumin were significant from the untreated cells (p<0.0001) (Figure 2).

Assessing Combined Medication Effects in HT29 Cell Lines

The single-drug cytotoxicity assay results fulfilled the criteria required by the Chou-Talalay method to initiate *in vitro* pharmacodynamic drug interaction analysis. Consequently, potential binary drug interactions were examined using an equipotency constant ratio combination design. Based on the single drug study data on HCT116 and HT29 cells, it was observed that HT29 cells were less sensitive to 5-FU, hence the combination study between 5-FU and curcumin was further investigated in the HT29 cell line.[27]

Considering the IC_{50} values of both the compounds against HT29, the combination study was performed by treating the cells with 5-FU and curcumin in constant potency ratios for 48 hr with the concentrations as specified in Table 1. The findings demonstrated that 5-FU and curcumin were able to notably increase the inhibition of HT29 cell proliferation compared to the single-drug treatments.

The dose-effect curves and the median-effect plots were constructed by entering the data into CompuSyn software.[22] The range of Combination İndex (CI) values, falling between 0.14 to 0.2 for the experimental concentration suggests very

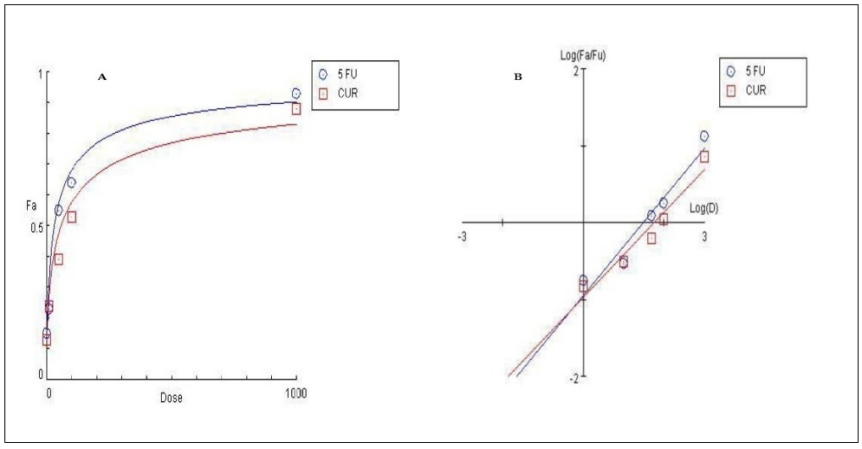

Figure 1: Results of cytotoxicity assay of 5-FU and curcumin as monotherapy against HCT116 cell lines. A) The dose-effect curves of 5-FU and curcumin with (m) slope of 0.638 and 0.556 respectively. B) Median-effect plot gives the half-maximal inhibitory concentration (D_m).

strong synergistic effects based on the Chou-Talalay method.[26] The Dose Reduction Index (DRI) for the actual experimental concentrations were above 1 signifying a beneficial reduction in dose for 5-FU and curcumin.[26] The IC_{50} values of 5-FU and curcumin were found to decrease by 13 and 9-fold respectively when combined in the specified ratio. The morphological changes observed during mono and combined therapy against HT29 cells are illustrated in Figure 3.

Employing CompuSyn software for computerized simulations: modeling synergistic effects

An algorithm was developed using the median-effect equation, CI equation and DRI equation, leveraging the automation features of CompuSyn software. This algorithm enables the simulation of CI and DRI values across various fa levels, beyond the specific experimental dose and effect values. Consequently, the software automatically generates simulated plots including fa-CI, fa-Log CI, fa-DRI and fa-Log DRI plots for drug combinations.[22]

The analysis of the median effect indicated that the combination indices were less than 1, spanning from fa=0.05 to 0.95. This implies a synergistic impact on inhibiting tumor growth between the two drugs, observed consistently across a wide spectrum of cell death rates ranging from 5% to 95%. The dose reduction index values were found to be greater than 1 for all the simulated combination ratios. The DRI values of 5-FU and curcumin at 50, 75 and 90 % cell growth inhibition levels are greater than 1. (Table 2).

The simulated plots for the combination assay are presented in Figure 4. The median-effect plot represents the linear form of the dose-effect curves of both single and combination treatments. The slope of the aforementioned plot was less than 1 which represents

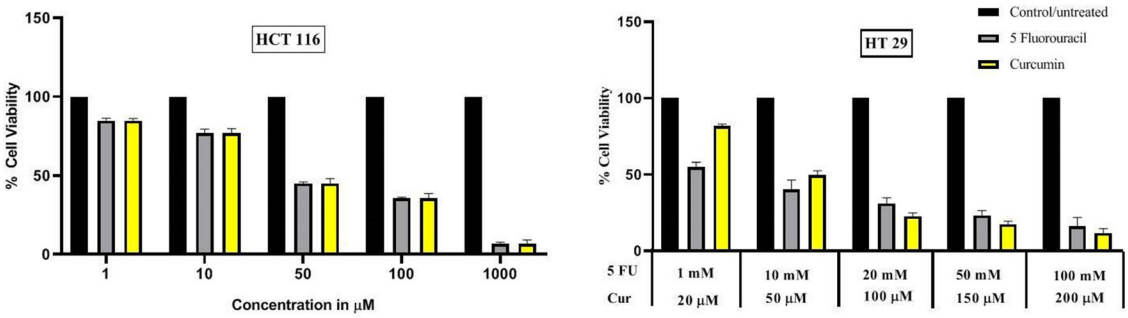

Figure 2: Comparison of the antiproliferative activity of 5-FU and curcumin in HCT116, HT29 cell lines with the untreated cells using two-way ANOVA followed by Tukey's *post hoc* test. Both the drugs demonstrated a statistically significant difference with a *p*-value less than 0.0001 in both cell lines.

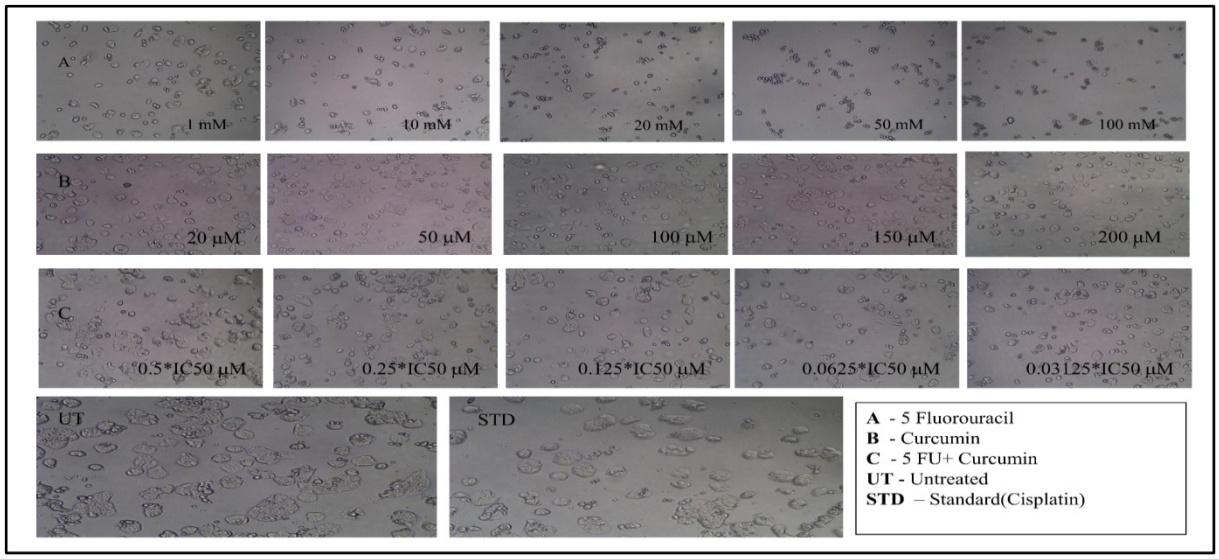

Figure 3: Morphological changes were observed in HT29 colorectal cancer cell lines at the end of 48 hr after treatment with 5-FU and curcumin as individual drugs and in combination. Antiproliferative study was performed using MTT assay. Cisplatin was used as standard which showed an IC_{50} value of 52 µM. A) 5-FU treated group; B) Curcumin treated group; C) Combined treatment group. All the groups exhibited statistically significant differences with the *p*-value<0.0001 when analysed using two-way ANOVA followed by Tukey's *post hoc* test.

Table 1: Study design and summary of pharmacodynamic parameters of 5-FU and curcumin combinations against HT29 colorectal cancer lines after 48 hr of treatment period generated using CompuSyn software.

Drug	Dose (μM)	Fraction inhibition(fa)	Parameters				
			M	Dm (μM)	r^2	CI*	DRI#
5-FU + Curcumin	0.5*IC_{50}	0.81	0.6808+/-0.0639	Total dose =160.02 (156.12+3.90)	0.9870	0.2176	5-FU=79.473 Cur=4.878
	0.25*IC_{50}	0.69				0.1896	5-FU=30.812 Cur=6.362
	0.125*IC_{50}	0.55				0.1903	5-FU=13.568 Cur=8.5774
	0.0625*IC_{50}	0.49				0.1359	5-FU=14.7807 Cur=14.6426
	0.03125*IC_{50}	0.38				0.1511	5-FU=9.5029 Cur=21.7875

*Range of CI: <0.1, very strong synergism; 0.1-0.3, strong synergism; 0.3-0.7, synergism; 0.7-0.85, moderate synergism; 0.85-0.90, slight synergism; 0.90-1.10, nearly additive; 1.10-1.20, slight antagonism; 1.20-1.45, moderate antagonism; 1.45-3.3, antagonism; 3.3-10, strong antagonism; >10, very strong antagonism. CI, combination index.# DRI- Dose reduction index greater than 1 indicate the favourable dose reduction for the drug.[22,28]

Table 2: Simulated analysis of CI and DRI values of 5-FU and curcumin combinations at 50%, 75% and 90% growth inhibition levels of HT29 cells.

Drug	CI values			Drug	DRI values		
	50%	75%	90%		50%	75%	90%
5-FU+Curcumin	0.1452	0.2062	0.4562	5-FU	13.361	45.850	146.24
				Cur	12.284	5.420	2.255

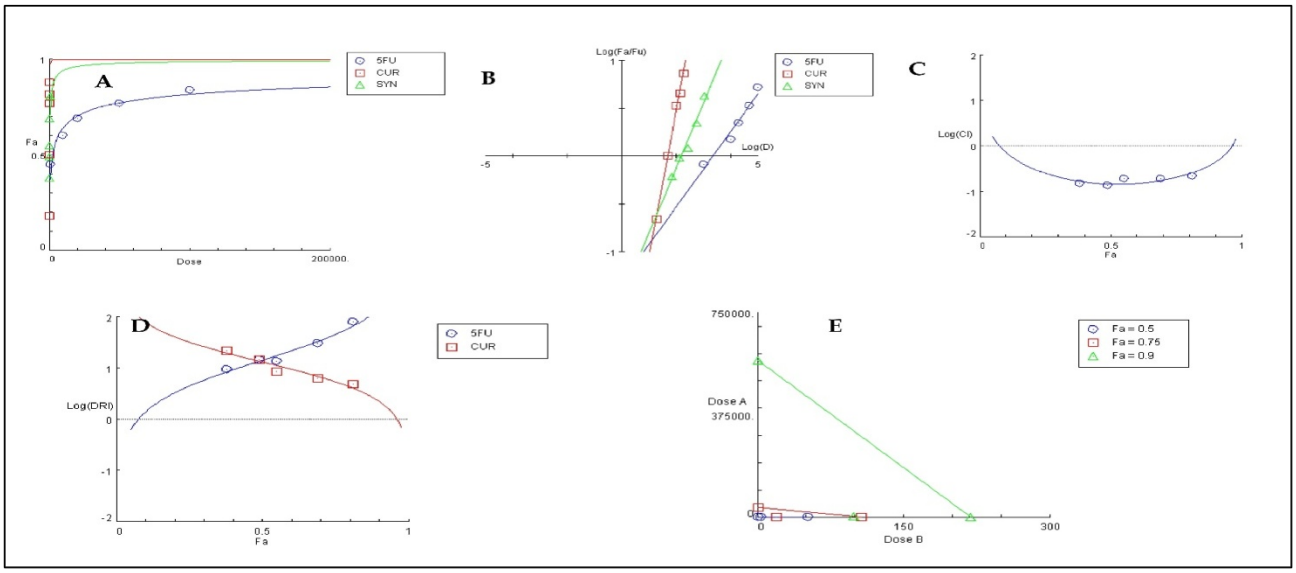

Figure 4: Simulated plots for analysis of pharmacodynamic interactions generated from CompuSyn software. A) Dose-effect curves of 5-FU, curcumin alone and in combination; B) Median-effect plots of single and combined drugs; C) fa-Log CI plot -straight lines show computersimulated values and circles represent experimental data points; D) fa-log DRI plot -Unbroken straight lines show computersimulated values and the shapes (circle and square) represent experimental data points; E) Isobolograms illustrated the nature of the drug interaction at constant ratios. Data points below the line denoted synergy, those on the line showed additivity and points above the line signified antagonistic effects. Fa stands for fraction affected, CI for combination index, DRI for dose reduction index, 5-FU for 5 fluorouracil and CUR for curcumin.

the shape of the curve as flat sigmoidal. An isobologram gives the sum of equipotent doses of combined drugs.

DISCUSSION

Combining chemotherapy agents indeed often proves more effective against many malignancies compared to single-agent treatments. Breakthroughs in high-throughput sequencing and the development of molecular targeted drugs have significantly contributed to advancements in cancer treatment. In recent years, there has been a significant number of research conducted to investigate the pharmacodynamic interactions between chemotherapeutic agents and various natural chemopreventive compounds.[29-34]

Despite its potential efficacy in treating cancer, 5-FU possess a short half-life, vulnerability to resistance to the drug and potential for serious side effects like myelosuppression, dermatitis, cardiotoxicity and neurotoxicity limit its clinical use. These issues stem from its widespread nonspecific distribution in the body *in vivo*. Due to its well-established antioxidant properties, curcumin is advised for anti-angiogenic, antimetastatic and chemopreventive uses.

Our findings indicate that the 5-FU and curcumin exhibited dose-dependent growth inhibition of HCT116 and HT29 cells with a single drug assay.[35] Also, it is evident from the study that the HT29 cells are less sensitive to 5-FU treatment at the end of 48 hr. The above can be corroborated by its time-dependent activity. Tawfik *et al* demonstrated that the IC_{50} values of 5-FU against HT29 cells were observed only after 5 days.[27] In addition, the IC_{50} values of 5-FU against HT29 cells from our work are consistent with the other studies.[24] The antiproliferative activity of curcumin against both the cell lines may be attributed to its potent apoptotic effects related to pAKT kinase reduction, as well as increased p-AMP protein Kinase (AMPK) signal as reported elsewhere.[36,37]

Moreover, considering chemotherapy as the primary treatment of colorectal cancer, we found it crucial to investigate whether curcumin enabled a reduction in the dosage of 5-FU while still preserving comparable antiproliferative effects *in vitro*. Median-drug effect analysis (Calcusyn) was employed to scrutinize the nature of drug interactions, aiming to ascertain whether there was antagonism (Combination İndex (CI)>1), additivity (CI=1), or synergism (CI<1) between 5-FU and curcumin. This analysis, along with isobologram and Dose Reduction İndex (DRI) assessments, was conducted across three levels of cell growth inhibition to delineate the combined effects of these compounds on HT29 cells.

The combination treatment in HT29 cells resulted in structural changes and decrease in the number of cells. The changes in cell morphology were identified as detached shrunken cells indicating a strong antiproliferative activity in combination. Furthermore, the quantitative and graphical outcomes suggest that the 5-FU and curcumin when given together behave as a third drug and exhibit synergistic effects in HT29 cells.[38] Numerous studies has demonstrated that curcumin with other chemo therapeutic agents like 5-FU, paclitaxel, doxorubicin, cisplatin shows synergism against various cancer cell lines. Various researchers have elucidated the anti-carcinogenic activities of curcumin via varying signaling pathways.[39,40]

The calculated CI values consistently being less than 1 across the tested concentrations and at three levels of fa signify a strong synergistic effect between 5-FU and curcumin. Therefore, this synergism often could stem from the distinct antineoplastic mechanisms exhibited by each compound. The tested concentrations in the combination study were less than the individual IC_{50} values of 5-FU and curcumin. Despite these low dose combinations they were able to produce significant cytotoxicity to the colorectal cancer cells compared to the monotherapy.[41]

In the context of our study involving 5-FU and curcumin against cell lines, the DRI values obtained hold significant implications for optimizing therapeutic strategies. The higher DRI values of 5-FU (146) at the highest fraction inhibition indicate a reduction in the overall drug dosage, which may contribute to mitigating potential side effects and improving patient tolerability. Additionally, lower drug doses may enhance patient compliance and adherence to the treatment regimen, positively impacting the overall success of the therapeutic intervention. Above all, it implies that there is more flexibility in adjusting drug dosages within a safer range while maintaining therapeutic efficacy. These findings provide valuable insights into the ongoing efforts to optimize combination therapies for more effective colorectal cancer treatment strategies.

In conclusion, our study demonstrates a compelling synergistic interaction between 5-FU and curcumin in the ratio of 40:1 against HT29 cell lines, as evidenced by low CI values, along with a remarkable dose reduction potential indicated by DRI values exceeding 1.

CONCLUSION

Finally, our study, utilizing the Chou-Talalay method, elucidated the interactions between 5-FU and curcumin when combined, revealing a robustly synergistic pharmacological effect. This finding paves the way for optimizing delivery systems, potentially allowing for a reduction in the doses of both compounds. Notably, our investigation suggests that a 40:1 proportion of 5-FU to curcumin could be deemed an optimal ratio for co-delivery via a suitable delivery system. This insight holds promise for advancing therapeutic approaches by harnessing the synergistic potential of these compounds while optimizing their combined delivery for enhanced efficacy and reduced individual doses.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

HT29: Human Colon Cancer Cell line; **HCT116:** Human colorectal carcinoma cell line; **MTT:** 3-4,5-dimethylthiazol-2-yl-2,5 diphenyl tetrazolium bromide; **DMSO:** Dimethyl sulfoxide; **CI:** Combination Index; **DRI:** Dose Reduction Index; **pAKT:** Phosphorylated Protein kinase B.

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