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Research article

Synergistic effect of 5-fluorouracil and the small molecule Wnt/β-catenin inhibitor iCRT3 on Caco-2 colorectal cancer cells *in vitro*

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ABSTRACT

Although 5-fluorouracil (5-FU) is a cornerstone of colorectal cancer (CRC) treatment, its efficacy is often limited by resistance. Wnt/β-catenin signalling plays a crucial role in CRC carcinogenesis and resistance, as Wnt expression is upregulated in 5-FU-resistant cells, protecting them from cell cycle arrest and apoptosis, thereby contributing to drug resistance. The small molecule inhibitor β-catenin responsive transcription inhibitor 3 (iCRT3) disrupts Wnt/β-catenin signalling and may enhance CRC sensitivity to 5-FU, overcoming resistance. In this study, the cytotoxic effects of 5-FU and iCRT3 were investigated using the Caco-2 colon adenocarcinoma cell line, marking the first investigation of their combined effects. To this end, the half-maximal inhibitory concentration (IC_{50}) values were determined using the MTT assay. Subsequently, the drugs were combined in different ways, and drug combination index (DCI) calculations were performed to evaluate their interaction. iCRT3 was found to be 2.45-fold more potent than 5-FU ($p = 0.1982$). Drug combination significantly increased the IC_{50} compared to 5-FU, with a 40.95-fold increase ($p = 0.0022$) when 5-FU was fixed (2.56 μM) and a 43.5-fold increase ($p = 0.0023$) when iCRT3 was fixed (2.41 μM). Two-way ANOVA showed significant impacts from both drug concentration (50.93 %) and treatment condition (25.31 %) on cell viability ($p < 0.0001$). DCI analysis confirmed strong synergism with fixed 5-FU (DCI = 0.154) and synergism with fixed iCRT3 (DCI = 0.618), indicating that combining 5-FU and iCRT3 could be a promising strategy for CRC treatment, warranting further investigation.

1. Introduction

Colorectal cancer (CRC) remains the third most common cancer worldwide, accounting for 9.6 % of all cases, with men being 1.5 times more affected than women [1–3]. While it predominantly affects older people, incidences in people younger than 50 are increasing steadily. Despite advances in early detection and treatment, CRC mortality remains high (9.3 %), ranking second among cancer-related deaths with a 5-year survival rate of around 60 % [1–4].

One of the cornerstone chemotherapeutic agents for CRC is the antimetabolic pyrimidine analogue 5-fluorouracil (5-FU), a derivative of the nucleic acid base uracil with a fluorine substitution at the C-5 position. 5-FU inhibits the enzyme thymidylate synthase and incorporates its metabolites into DNA and RNA, triggering apoptosis in rapidly dividing cells [5–9]. To exert its cytotoxic effects,

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5-FU must undergo enzymatic reactions with phosphorylated sugars to form one of its three active metabolites: 5-fluorouridine-5′--triphosphate (FUTP), 5-fluoro-2′-deoxyuridine-5′-triphosphate (FdUTP), and 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) shown in bold (Fig. 1) [6,7]. However, 5-FU efficacy is often undermined by the development of drug resistance, contributing to tumour recurrence, metastasis, and treatment failure [6,9].

Key signalling pathways involved in CRC development and progression include mutations in the canonical Wnt/β-catenin pathway, which are responsible for 80–90 % of all CRC cases, leading to hyperactivation, β-catenin accumulation, and abnormal gene expression that promote CRC development and progression [4,10–14]. Moreover, there is a strong correlation between Wnt/β-catenin signalling and chemotherapy resistance, as studies on oral squamous cell carcinoma have demonstrated that upregulated Wnt expression in 5-FU-resistant cells protects them from apoptosis, thereby contributing to drug resistance [6,9,13].

In the absence of Wnt, the constitutively synthesised transcriptional activator β-catenin is bound and regulated by the destruction complex (Fig. 2A) [11,14,15]. Binding of Wnt to its receptor Frizzled and the lipoprotein receptor-related protein 5/6 (LRP 5/6) co-receptor induces a conformational change and receptor dimerization, leading to the recruitment of the protein Dishevelled and other components of the destruction complex, separating it. In this way, β-catenin phosphorylation and degradation are prevented, increasing its half-life from approximately 20 min to 1–2 h, enabling its accumulation and translocation into the nucleus where it activates Wnt target genes (Fig. 2B) [11,14–16]. In adenomatous polyposis coli (APC)-mutated cells (Fig. 2C), the formation of the destruction complex is impaired, allowing β-catenin to escape phosphorylation and proteasomal degradation, leading to its accumulation and subsequent nuclear translocation, resulting in abnormal gene expression [10,14]. Small molecule β-catenin responsive transcription inhibitors −3, −5 and −14 specifically bind to β-catenin, blocking its interaction with T cell factor 4 (Fig. 2D). As a result,

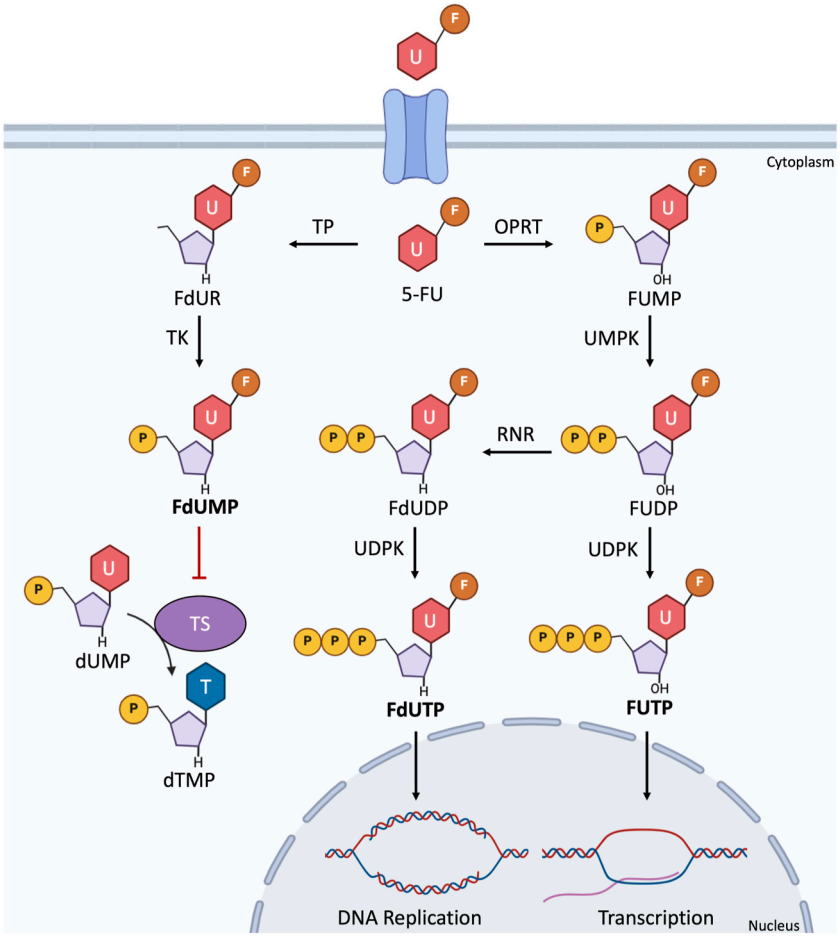

Fig. 1. 5-FU Metabolism and Actions. Metabolic conversion of 5-FU into its three active metabolites (shown in bold) and their mode of action. 5-FU = 5-fluorouracil; dTMP = Deoxythymidine-5′-monophosphate; dUMP = Deoxyuridine-5′-monophosphate; FdUDP = 5-fluoro-2′-deoxyuridine-5′-diphosphate; FdUMP = 5-fluoro-2′-deoxyuridine-5′-monophosphate; FdUR = 5-fluorodeoxyuridine; FdUTP = 5-fluoro-2′-deoxyuridine-5′-triphosphate; FUDP = 5-fluorouridine-5′-diphosphate; FUMP = 5-fluorouridine-5′-monophosphate; FUTP = 5-fluorouridine-5′-triphosphate; OPRT = Orotate phosphoribosyl transferase; RNR = Ribonucleotide reductase; TK = Thymidine kinase; TP = Thymidine phosphorylase; TS = Thymidylate synthase; UMPK = Uridine monophosphate kinase; UDPK = Uridine diphosphate kinase. (Made with BioRender.com).

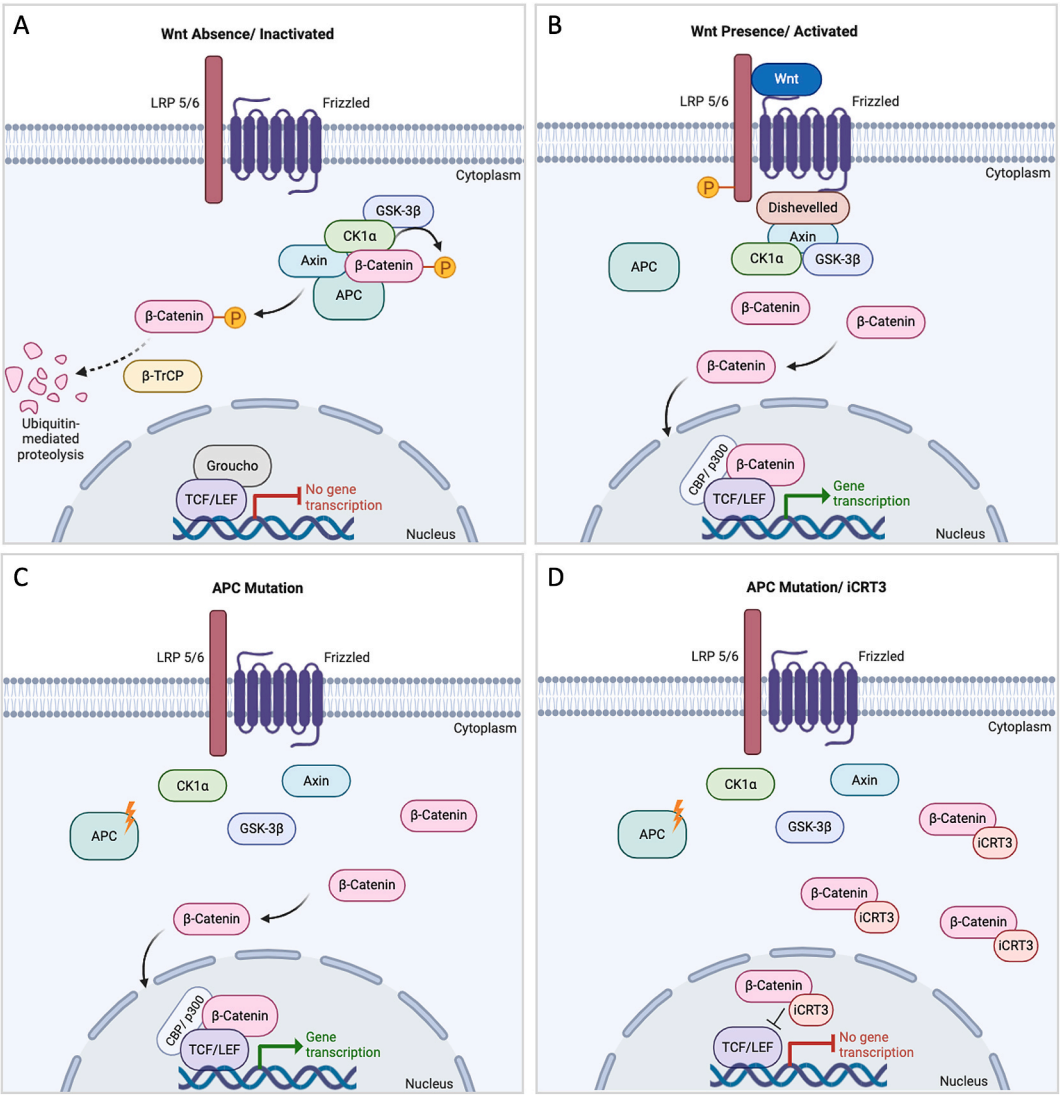

Fig. 2. Healthy and Mutant Wnt/β-catenin Pathway with iCRT3 Mechanism of Action. A: In the absence of Wnt, β-catenin is regulated by the destruction complex (Axin, APC, GSK-3β, CK1α), leading to its phosphorylation, ubiquitination by β-TrCP, and proteasomal degradation. **B:** Wnt binding to its receptor disrupts β-catenin degradation, allowing its nuclear accumulation and activation of Wnt target genes. **C:** APC mutations disrupt the destruction complex, allowing β-catenin to remain unphosphorylated. **D:** iCRT3 inhibits β-catenin by preventing its interaction with TCF/LEF, blocking transcription. APC = Adenomatous polyposis coli; Axin = AXIS inhibition protein; β-TrCP = Beta-transducin repeat-containing protein; CBP = CREB-binding protein; CK1α = Casein kinase 1 alpha; GSK-3β = Glycogen synthase kinase 3 beta; LRP 5/6 = Lipoprotein receptor-related protein 5/6; TCF/LEF = T cell factor/lymphoid enhancer factor; iCRT3 = β-catenin responsive transcription inhibitor 3. (Created with BioRender.com).

activation of Wnt target genes is inhibited, preventing uncontrolled cell proliferation [10,12,17,18]. Among these, β-catenin responsive transcription inhibitor 3 (iCRT3) has emerged as the most promising candidate [17,20,21]. By interfering with the Wnt/β-catenin signalling cascade, iCRT3 might increase sensitivity to 5-FU, overcoming treatment resistance [10,12,17–19].

In this study, the cytotoxic effects of 5-FU and iCRT3 were investigated, both individually and in combination, using the Caco-2 colon adenocarcinoma cell line, marking the first investigation of their combined effects. To this end, the half-maximal inhibitory concentration (IC_{50}) was determined using the MTT assay. Subsequently, the IC_{50} of 5-FU was combined with various concentrations of iCRT3 (fixed 5-FU) and the IC_{50} of iCRT3 was combined with different concentrations of 5-FU (fixed iCRT3). In addition, drug combination index (DCI) calculations were performed to assess the nature of their interaction.

It was hypothesised that 5-FU and iCRT3 modulate cancer cell growth and that drug combination has a synergistic effect.

2. Materials and methods

2.1. Ethics

This study was approved by the Research Ethics Board of London Metropolitan University, UK.

2.2. Reagents

5-FU was purchased from Sigma-Aldrich, Gillingham, UK. iCRT3 was ordered from Merck KGaA, Darmstadt, Germany. Dimethyl sulfoxide (DMSO) was acquired from MP Biomedicals, Eschwege, Germany. MTT and propanol were sourced from Thermo Fisher Scientific, Leicester, UK. RPMI 1640, PBS, foetal calf serum (FCS), trypsin-EDTA, and penicillin/streptomycin were procured from VWR International, Lutterworth, UK. Stock solutions were prepared as follows: 5-FU was prepared in PBS; iCRT3 was prepared in DMSO. A 5 mg/ml MTT stock solution was prepared in PBS and an MTT working solution (0.5 mg/ml) was prepared freshly in complete medium.

2.3. Cell culture

Human colon adenocarcinoma Caco-2 cells (London Metropolitan University, London, UK) were cultured in RPMI-1640 all-purpose growth medium containing 2 mM L-glutamine supplemented with 10 % FCS and 1 % penicillin/streptomycin. Cells were grown in T75 culture flasks in a humified incubator at 37 °C with 5 % CO_2. Every 72–96 h, cells were washed with PBS, dissociated with trypsin, spun at 203 RCF/1500 RPM for 4 min, and split 1:6. Confluent cell passages 2–16 were used for experiments.

2.4. MTT assay

Caco-2 cells were seeded in 24-well plates at a density of 5×10^4 cells per well, and allowed to adhere for 24 h at 37 °C with 5 % CO_2. After adherence, cells were treated for 48 h with different concentrations (0.1–100 μM) of 5-FU or iCRT3, individually or in combination, maintaining the same incubation conditions. Subsequently, cell viability was assessed using the MTT assay. In brief, the medium was aspirated from each well and 500 μl MTT working solution was added. The plates were then incubated for 2–3 h at 37 °C with 5 % CO_2 to allow formazan crystal formation. Following incubation, wells were gently washed with PBS to remove residual serum proteins. Subsequently, 1 ml propanol was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm with background subtraction at 650 nm using a microplate reader (FLUOstar Omega, BMG Labtech, Ortenberg, Germany). Data were analysed using Microsoft Excel Version 16.88 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism Version 10.3.1

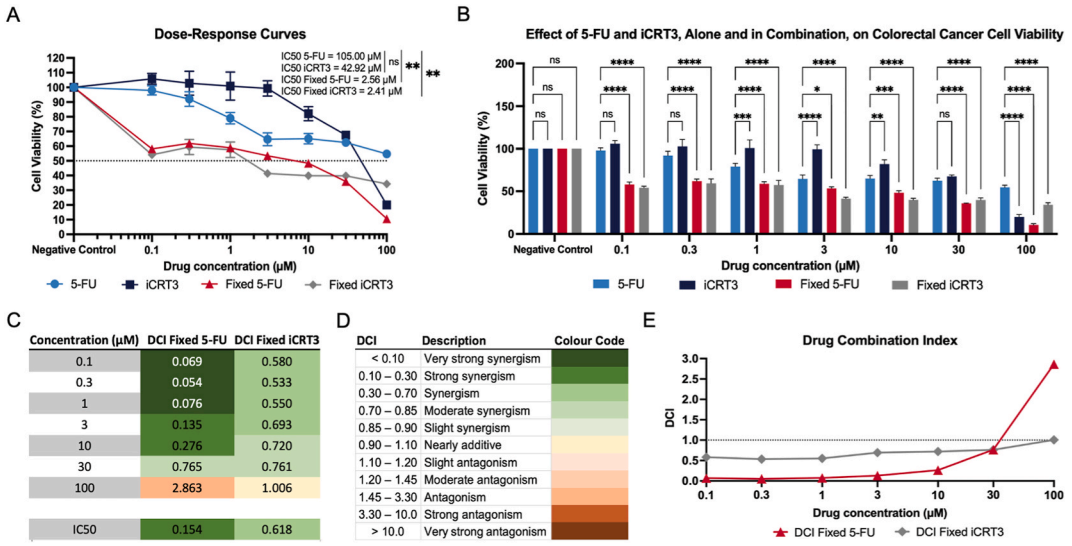

Fig. 3. Synergistic Effect of 5-FU and iCRT3. Caco-2 cells were treated under various conditions and incubated for 48 h. Cell viability was assessed using the MTT assay. Data are presented as percentage deviation from control and expressed as mean ± Standard Error of the Mean (n = 21; n = 9 for iCRT3). **** = $p < 0.0001$, *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$, ns = not significant. P values were calculated using ordinary one-way ANOVA followed by Tukey's multiple comparisons **(A)** or ordinary two-way ANOVA followed by Dunnett's multiple comparisons **(B). C:** The DCI was calculated for all non-constant drug concentrations and the IC_{50} values. **D:** Range of DCI values with corresponding descriptions and colour code, based on Chou [23]. **E:** Graphical presentation of the DCIs. 5-FU = 5-fluorouracil; DCI = Drug combination index; Fixed 5-FU = 105 μM 5-FU + iCRT3; Fixed iCRT3 = 42.9 μM iCRT3 + 5-FU. IC_{50} = Half-maximal inhibitory concentration; iCRT3 = β-catenin responsive transcription inhibitor 3.

(GraphPad Software, San Diego, CA, USA).

2.5. Statistical analysis

The IC_{50} for iCRT3 was determined based on three different experiments, while the IC_{50} values for 5-FU and the drug combinations were assessed based on seven different experiments each. Toxicity control using DMSO was performed once, and statistical differences were analysed using an unpaired *t*-test. Data were analysed using GraphPad Prism Version 10.3.1 (GraphPad Software, San Diego, CA, USA). IC_{50} values were determined using nonlinear regression analysis of dose-response inhibition curves. Statistical differences between IC_{50} values were assessed using ordinary one-way ANOVA followed by Tukey's multiple comparisons. The effects of drug concentration and treatment condition on cell viability were analysed using ordinary two-way ANOVA, followed by Dunnett's multiple comparisons. Statistical significance was defined as $p < 0.05$. The DCI was calculated for each combination using the median-effect equation as described by Chou and Talalay [22] and further refined by Chou [23].

3. Results

3.1. IC_{50} and cytotoxic effects of 5-FU and iCRT3

Before assessing the cytotoxic effects of combining 5-FU and iCRT3, the individual IC_{50} values were established. The IC_{50} of 5-FU was then combined with varying concentrations of iCRT3 (fixed 5-FU), and vice versa (fixed iCRT3). As shown in Fig. 3A, both drugs induced a dose-dependent reduction in cell viability. The IC_{50} values were determined to be 105 μM for 5-FU (95 % CI 55.66–254.00 μM) and 42.92 μM for iCRT3 (95 % CI 33.72–54.82 μM). Interestingly, minimal effects on cell viability were observed at low iCRT3

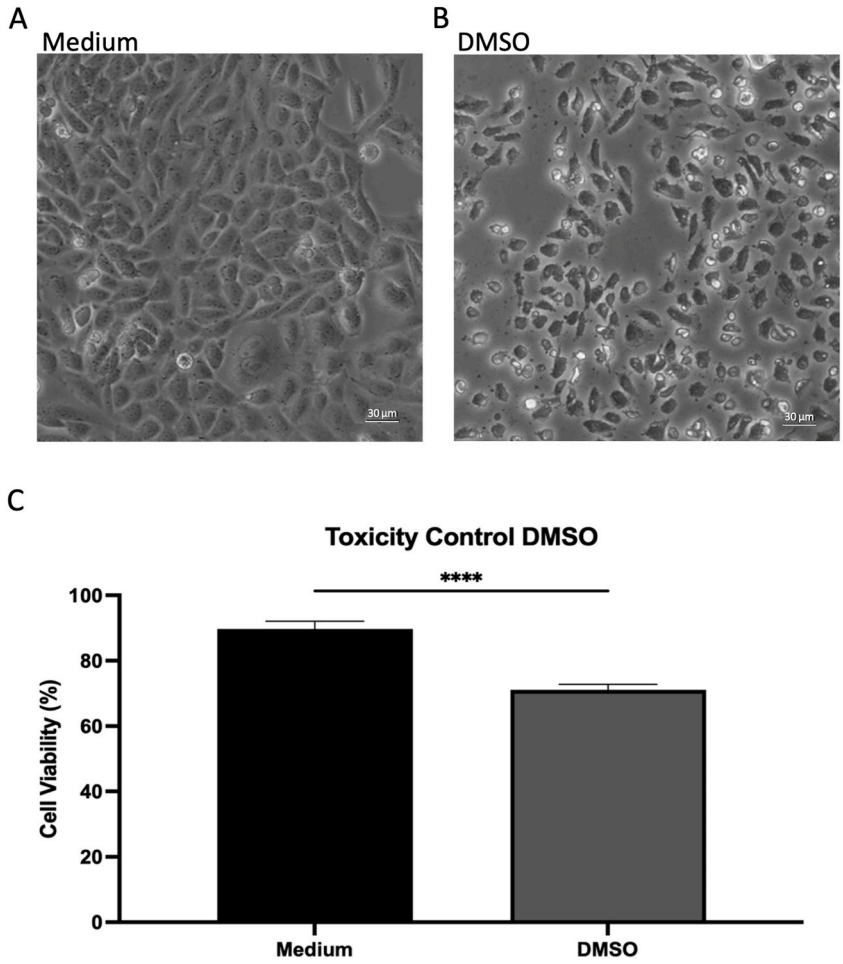

Fig. 4. Toxicity Control DMSO. A/B: Caco-2 cells were treated with either medium **(A)** or 1%v/v DMSO **(B)** and incubated for 48 h. Cell morphology was assessed using inverted light microscopy under phase contrast settings at 200x magnification. Scale bar = 30 μm. **C:** Cell viability was assessed using the MTT assay. Data are presented as percentage deviation from the maximum and expressed as mean ± Standard Error of the Mean (n = 12). **** = $p < 0.0001$. P values were calculated using an unpaired t-test. DMSO = Dimethyl sulfoxide.

concentrations, with a sharp decline above 3 μM. For the combinations, the IC_{50} was 2.56 μM (95 % CI 1.66–3.98 μM) for fixed 5-FU and 2.41 μM (95 % CI 1.33–4.56 μM) for fixed iCRT3. One-way ANOVA revealed significant differences between the treatment groups ($p = 0.0012$), with further details provided by Tukey's multiple comparisons. iCRT3 was found to be 2.45-fold more potent than 5-FU. However, this difference was not statistically significant ($p = 0.1982$). Drug combination, however, significantly increased the IC_{50} compared to that obtained for 5-FU. The IC_{50} increased 40.95-fold ($p = 0.0022$) when 5-FU was fixed (2.56 μM) and 43.5-fold ($p = 0.0023$) when iCRT3 was fixed (2.41 μM), indicating synergism.

As seen in Fig. 3B, two-way ANOVA with Dunnett's multiple comparisons showed that fixed 5-FU exhibited strong significance at lower concentrations but varied across the range. In contrast, fixed iCRT3 maintained a consistently high significance level across all concentrations. Overall, both drug concentration and treatment conditions had a significant effect on cell viability, accounting for 50.93 % ($p < 0.0001$) and 25.31 % ($p < 0.0001$) of the total variation, respectively. Their interaction explained 11.93 % ($p < 0.0001$), indicating that the effect of drug concentration was influenced by the treatment conditions used.

3.2. Drug combination index analysis

To further analyse the interactions, the DCI was calculated using the median-effect equation. Fig. 3C shows the DCI values across the concentration range when either 5-FU or iCRT3 was fixed, as well as for the IC_{50} values (2.56 μM for fixed 5-FU and 2.41 μM for fixed iCRT3), along with the corresponding interpretation and colour code (Fig. 3D). Fig. 3E provides a graphical representation of the DCIs across the various concentrations.

As demonstrated by the results, the DCI values for fixed 5-FU indicated very strong synergism at lower concentrations, but as the concentration of iCRT3 increased, the synergism decreased in a dose-dependent manner, approaching antagonism at the highest concentration tested (DCI = 2.863). In contrast, fixed iCRT3 demonstrated consistent synergism to moderate synergism across most concentrations, transitioning to a nearly additive effect at the highest concentration (DCI = 1.006).

For the IC_{50} values, the DCI was 0.154 for fixed 5-FU and 0.618 for fixed iCRT3, representing strong synergism and synergism, respectively. Notably, except for the highest concentration, all DCI values remained below 1, underscoring the overall synergistic effect of combining 5-FU with iCRT3.

3.3. DMSO toxicity control

During microscopic observation, cell damage was noticed in the negative control wells containing 1 % v/v DMSO. DMSO is an organic, amphipathic solvent frequently used in research, at a concentration of 0.1–1.5 % v/v. Generally, a DMSO concentration of 0.1 % v/v is considered safe while a concentration of 0.5 % v/v is widely used. However, concentrations above 1 % v/v may be cytotoxic in some cell lines [24–26]. Nevertheless, information on the maximum tolerated concentration varies considerably, depending on the incubation period and cell line used. To investigate the cytotoxic effects of DMSO, a toxicity control was performed using either medium or 1 % v/v DMSO.

Upon visual assessment using inverted light microscopy (Fig. 4A/B), cells treated with DMSO exhibited clear signs of cell damage and cell death, including cell shrinkage and irregular cell membranes, indicating a cytotoxic effect of DMSO. As shown in Fig. 4C, DMSO caused a significant decrease in cell viability, corroborating the visual results. Cell viability decreased by 18.6 % ± 2.893 % ($p < 0.0001$).

4. Discussion

To date, 5-FU remains the cornerstone chemotherapeutic agent used in CRC treatment. However, its efficacy is often undermined by the development of drug resistance [6,9]. Therefore, there is an urgent need for novel therapeutic agents and combination strategies to improve clinical outcomes.

In this study, the cytotoxic effects of 5-FU and iCRT3 were investigated, both individually and in combination, using the Caco-2 colon adenocarcinoma cell line, marking the first investigation of their combined effects. The IC_{50} was determined as 105 μM for 5-FU and 42.92 μM for iCRT3. Notably, at low iCRT3 concentrations, only minimal changes in cell viability were observed until a sharp decline occurred above 3 μM. One explanation for the delayed cytotoxicity could be that β-catenin must first be bound before iCRT3 can exert its full effect, leading to a slight delay. However, once this threshold is reached, iCRT3 exhibits a strong cytotoxic effect, as evidenced by the sharp decrease in cell viability.

Due to its recent discovery, literature regarding the cytotoxicity of iCRT3 is limited. The primary study by Gonsalves *et al.* [17] reported an average IC_{50} of 36 μg/mL (91.25 μM) in human primary culture samples. Further studies by Sogutlu *et al.* [27] revealed an IC_{50} of 70.68 μM in hypopharyngeal cancer cells and 130.32 μM in head and neck cancer stem cells after 72 h. These IC_{50} values are higher than those observed in this study, implying that iCRT3 exhibits a strong cytotoxic effect on Caco-2 cells. Moreover, iCRT3 exhibits similar cytotoxic effects as other small molecule Wnt/β-catenin inhibitors such as ICG-001. ICG-001 interacts with CREB-binding protein (CBP), thereby competing with β-catenin for its binding. For example, Lin *et al.* [24] reported an IC_{50} of 106.39 μM for ICG-001 in HCT-116 CRC cells after 24 h using the CCK-8 assay, while Choi *et al.* [25] found a significantly lower IC_{50} of 5.57 μM in the same cell line after 72 h using the MTT assay. Hence, it can be concluded that iCRT3 is very potent, achieving comparable results to other inhibitors, making it a potentially effective agent for targeting the Wnt/β-catenin pathway.

For the drug combination, the IC_{50} increased 40.95-fold with fixed 5-FU (2.56 μM) and 43.5-fold with fixed iCRT3 (2.41 μM). Tukey's multiple comparisons revealed significant differences between 5-FU and both fixed concentrations ($p < 0.01$), indicating

synergism. Two-way ANOVA with Dunnett's multiple comparisons showed that fixed 5-FU exhibited strong significance at lower concentrations but varied across the range. In contrast, fixed iCRT3 maintained a consistently high significance level across all concentrations. Overall, both drug concentration and treatment conditions significantly affected cell viability ($p < 0.0001$), with interaction effects indicating that drug efficacy depended on the treatment condition. Analysis of the IC_{50} values using the DCI revealed strong synergism for fixed 5-FU and synergism for fixed iCRT3. Subsequent analysis of the DCI for each concentration pair revealed a dose-dependent increase in the DCI for fixed 5-FU up to an antagonistic effect at the highest concentration, indicating a decrease in synergism with increasing iCRT3 concentrations. In contrast, the DCI for fixed iCRT3 remained more stable, with a nearly additive effect at the highest concentration. Nevertheless, except for the highest concentration, all DCIs remained below 1, confirming the general synergistic effect between 5-FU and iCRT3. These results indicate that maintaining a constant 5-FU concentration enhances the synergistic effect of the drug combination, especially at lower iCRT3 concentrations. In contrast, combination at a fixed iCRT3 concentration does not show the same degree of synergistic enhancement as with fixed 5-FU, suggesting that 5-FU may serve as the more effective anchor drug.

As this study was the first to evaluate the cytotoxic effect of 5-FU and iCRT3 in combination, no direct comparisons are available in the literature. Nevertheless, other drug combinations currently being investigated show equally pronounced synergism. For instance, De Castro E Gloria *et al.* [26] reported that the combination of 5-FU with the PARP inhibitor Olaparib showed synergistic effects in HCT-116 and HT-29 cancer cells, with average DCI values between 0.3 and 0.7. In another study, Lin *et al.* [24] reported a DCI of 0.3583 for the IC_{50} value when combining ICG-001 with Auranofin, a gold complex used in rheumatology treatment, in HCT-116 cells, also indicating synergism. Furthermore, Oncu *et al.* [28] reported strong synergism (DCI = 0.143) for the IC_{50} value of 5-FU in Caco-2 cells when combined with Berberine, a herbal alkaloid known to modulate various signalling pathways, including the Wnt/β-catenin pathway. A combination therapy of 5-FU with iCRT3 could therefore be an effective approach against CRC, warranting further investigation.

During this study, cytotoxicity was observed in the negative control wells when DMSO was present. According to Santos *et al.* [29], DMSO can have a variety of (side-) effects both *in vitro* and *in vivo*, leading to experimental artefacts and incorrect result interpretations that are often underestimated. Most importantly, Yuan *et al.* [30] reported that DMSO induced significant cytotoxicity in astrocytes by disrupting mitochondrial integrity and membrane potential. Since the MTT assay depends on mitochondrial activity to reduce MTT to formazan, mitochondrial dysfunction can significantly distort the results. Consequently, DMSO-induced mitochondrial toxicity could interfere with this process and skew the results. Consistently, our toxicity control revealed that 1 % v/v DMSO significantly reduced cell viability over 48 h ($p < 0.0001$), highlighting the potential cytotoxic effects of DMSO. Therefore, it is recommended to determine the cell line-specific tolerance limit by performing a toxicity control using various DMSO concentrations.

It was hypothesised that 5-FU and iCRT3, both individually and in combination, modulate cancer cell growth and that drug combination has a synergistic effect. Our results confirm this hypothesis, demonstrating that both drugs effectively modulate cancer cell growth, with their combination yielding a synergistic effect. Studies have shown that Wnt/β-catenin expression is upregulated in 5-FU-resistant cells, enabling them to evade cell cycle arrest or apoptosis, thus contributing to drug resistance [6,9,13]. Using Caco-2 cells, iCRT3 achieved comparable results to other inhibitors and combinations, highlighting its potential to interfere with the Wnt/β-catenin signalling pathway.

The cytotoxicity of 5-FU and iCRT3 is currently being evaluated in different cell lines, using apoptosis and luciferase assays to ascertain the mechanism of cell death, while providing direct insights into the impact of iCRT3 on β-catenin/TCF transcriptional activity. Going forward, 5-FU and iCRT3 will be tested *in vivo* in a Caco-2 xenograft model to see the effect on survival rate and occurrence of metastasis.

CRediT authorship contribution statement

Maren Smarslik: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Jameel M. Inal:** Funding acquisition, Project administration, Software, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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