Synergistic Cytotoxicity of 5-Fluorouracil and Epigallocatechin-3-Gallate on Colorectal Cancer Stem Cell

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INTRODUCTION

Colorectal cancer (CRC) stands as a formidable global health challenge, claiming a significant number of lives annually [1–3]. As research endeavors intensify to enhance therapeutic strategies, understanding the cytotoxic effects of both conventional chemotherapeutic agents and natural compounds becomes imperative [4–6]. In this context, our investigation delves into the cytotoxicity profiles of two key compounds—5-fluorouracil (5-FU) and epigallocatechin-3-gallate (EGCG)—in HCT-116 cells, a well-established human colon carcinoma cell line.

The 5-FU is a cornerstone in CRC chemotherapy. The anti-cancer activity of EGCG involves inhibition of proliferation and induction of apoptosis thereby reducing recurrence by as much as 51.6% in patients with colorectal adenoma after polypectomy. The significance lies in optimizing treatment strategies by understanding the potential synergies between conventional chemotherapeutic agents and natural compounds. Given 5-FU's status as a cornerstone in CR-CSCs chemotherapy and EGCG's emergence as a promising natural compound, the study delves into their individual and combined cytotoxicity profiles.

METHODS

The single and combination assay aimed to determine the cytotoxicity of EGCG and 5-FU, including establishing the half inhibitory concentration (IC50) and combination index (CI) values. CR-CSCs colonies were disassociated, counted, and cultured in 96-well plates. Test solutions of varying concentrations were applied, and subsequent steps involved incubation, media removal, washing, MTT reagent addition, and absorbance measurement.

RESULTS

The single cytotoxicity tests established individual IC50 values, revealing 141.26 µM for 5-FU and 464.56 µM for EGCG. Subsequent combination cytotoxicity tests demonstrated a synergistic effect at specific doses, indicated by CI values below 1.

CONCLUSIONS

These findings highlight the potential for increased cytotoxicity against CR-CSCs when treated with the combination of 5-FU and EGCG.
This exploration provides insights into the potential of 5-FU as monotherapy and its role in shaping future treatment modalities [9,10]. While, EGCG unveils the Anticancer Potential of Green Tea Polyphenol Epigallocatechin-3-gallate, a polyphenol derived from green tea, has emerged as a promising natural compound with anticancer properties [11–13]. Our study seeks to elucidate the cytotoxic effects of EGCG on HCT-116 cells, exploring its potential as a standalone therapeutic agent.

Toward optimizing treatment strategies understanding the cytotoxicity profiles of 5-FU and EGCG is pivotal in optimizing treatment strategies for CRC. This research contributes valuable data and highlights potential synergistic effects when combining conventional chemotherapeutic agents with natural compounds. Such insights hold the promise of refining therapeutic approaches, potentially improving treatment outcomes while minimizing adverse effects. Toward enhanced CRC treatment our study focuses on three primary objectives: determining the individual cytotoxicity of 5-FU and EGCG on HCT-116 cells, assessing their combined cytotoxic effects, and analyzing the potential synergistic benefits of the combined treatment. Through these objectives, we aspire to unveil new avenues for CRC therapy, contributing to the evolving landscape of cancer treatment.

By assessing its cytotoxicity in HCT-116 cells, we aim to unravel the impact of this conventional chemotherapeutic agent on CRC cells. Additionally, we investigate whether EGCG, combined with 5-FU, augments the cytotoxicity, offering a novel approach to colorectal cancer treatment. In summary, our investigation into the cytotoxicity of 5-FU and EGCG in HCT-116 cells seeks to bridge the gap between conventional and natural therapeutic approaches, offering a comprehensive understanding of their potential in colorectal cancer treatment.

METHODS

A methodological framework was meticulously crafted, employing sophisticated tools and substances. Critical equipment was used, including a low-adherent 6-well plate, precise micropipettes, specialized culture vessels, and state-of-the-art analytical instruments such as a BD C6 Plus flow cytometer and Western blot apparatus. The study utilized CR-CSC HCT116 cells, procured from ATCC, USA, and sorted with CD44+/CD133+ microbeads. The pure isolate of EGCG was sourced from Sigma Aldrich, USA. This study is included in an in vitro laboratory experimental study using a post-test-only control group design. This design was used to analyze the effect of EGCG and 5-FU extracts on CR-CSC by comparing the results of the study between treatment and control. To calculate the viability of cells subjected to trypan blue exclusion assay to count viable cells as previously described. Preliminary research was conducted to determine the dose of IC50 through treatment with EGCG doses: 10, 25, 50, 100, 200, 300, 500 μmol. Treatment with a dose of 5-FU: 10, 25, 50, 100, 200, 300, 500 μmol.

CR-CSCs isolation and validation

Intricate processes involving MACS sorting and flow cytometry were employed to isolate and validate CR-CSCs from the HCT-116 cancer cell population. Phenotypic validation was achieved through the expression of CD44+/CD133+ [6]. These validated CR-CSCs exhibited the distinctive ability to form mammosphere morphology, a hallmark feature of cancer stem cells [7]. Further characterization included protein surface marker analysis, affirming the expression of CD133, CD44, CD90, CD166, and CD326 at an impressive rate of 68.97%.

Cytotoxicity assay

This preliminary research uses the MTT assay method, to measure the metabolic activity of cells, in this case CS-CSCs as a marker of cytotoxicity, viability, and proliferation. This MTT assay uses the principle of colorimetric reduction of yellow salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT), which will change color to purple formazan crystals [8]. The single and combination assay aimed to determine the cytotoxicity of EGCG and 5-FU, including establishing the IC50 and CI values. CR-CSCs colonies were dissociated, counted, and cultured in 96-well plates. Test solutions of varying concentrations were applied, and subsequent steps involved incubation, media removal, washing, MTT reagent addition, and absorbance measurement. Subsequently, a combination cytotoxicity test of 5-FU and EGCG was conducted to determine whether the interaction between the two compounds could provide a synergistic or even additive effect on CR-CSCs. The concentration series of both test compounds used were 1/8, ¼, ½, and 1 IC50. Each concentration series was then combined to obtain the Combination Index (CI), which would indicate the interaction relationship between 5-FU and EGCG. A CI value of less than <1 indicates a synergistic effect, equal to one is additive, and more than one is antagonistic.

Statistical analysis

Statistical analysis involved ANOVA to assess mean differences among dose combinations. Assumptions of normality and homogeneity were tested, and appropriate post hoc tests, such as Duncan or Games-Howell, were applied accordingly. These analyses ensured robust insights into the experimental outcomes [9].
RESULTS

Isolation and validation of colorectal cancer stem cells [CR-CSCs]

CR-CSC clones were isolated from the HCT-116 colorectal cancer cell population expressing CD44+/CD133+ using MACS CD44/CD133 microbeads [10]. The isolated CR-CSC clones were then cultured in T75 flasks using a specialized colorectal cancer stem cell medium (DMEM low glucose: mamo cults medium, 3:1). After the third passage, the cultured CR-CSCs exhibited a floating, non-adherent morphology with a round shape.

IC50 value with single cytotoxicity test of 5-FU on CR-CSCs

Cytotoxicity tests using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagents were conducted to analyze the cytotoxicity of agents such as 5-FU and EGCG on colorectal cancer stem cells. The results of the 5-FU cytotoxicity test on CR-CSCs indicated an IC50 value of 141.26 µM, signifying moderate cytotoxicity as the IC50 falls between 100–200 µM.

Table 1. Viability of colorectal cancer stem cells (CR-CSCs) with 5-Fluorouracil (5-FU) using MTT assay

<table>
<thead>
<tr>
<th>Concentration [µM]</th>
<th>Mean Viability [%] ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±0.045</td>
</tr>
<tr>
<td>10</td>
<td>53.018±1.142</td>
</tr>
<tr>
<td>25</td>
<td>54.486±0.908</td>
</tr>
<tr>
<td>50</td>
<td>52.855±2.990</td>
</tr>
<tr>
<td>100</td>
<td>52.039±1.274</td>
</tr>
<tr>
<td>200</td>
<td>47.961±0.748</td>
</tr>
<tr>
<td>400</td>
<td>35.726±2.936</td>
</tr>
<tr>
<td>600</td>
<td>30.832±0.748</td>
</tr>
</tbody>
</table>

Figure 3. Cytotoxicity test graph 5-Fluorouracil (5-FU) on colorectal cancer stem cells (CR-CSCs)
IC50 Value with single cytotoxicity test of EGCG on CR-CSCs

The results of the EGCG cytotoxicity test on CR-CSCs as shown in Figure 5 indicated an IC50 value of 464.56 µM, suggesting weak cytotoxicity as the IC50 falls between 400–500 µM.

Table 2. Viability of colorectal cancer stem cells (CR-CSCs) with epigallocatechin gallate (EGCG)

<table>
<thead>
<tr>
<th>Concentration [µM]</th>
<th>Mean Viability [%] ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±0.009</td>
</tr>
<tr>
<td>50</td>
<td>77.886±7.942</td>
</tr>
<tr>
<td>100</td>
<td>61.644±6.676</td>
</tr>
<tr>
<td>200</td>
<td>62.427±6.029</td>
</tr>
<tr>
<td>400</td>
<td>53.033±3.810</td>
</tr>
<tr>
<td>600</td>
<td>44.814±1.190</td>
</tr>
<tr>
<td>1000</td>
<td>39.726±3.233</td>
</tr>
</tbody>
</table>

Table 3. Combination index values for 5-Fluorouracil and epigallocatechin gallate

<table>
<thead>
<tr>
<th>5-FU [µM]</th>
<th>EGCG [µM]</th>
<th>CI Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>62.5</td>
<td>125</td>
<td>0.08*; -0.41; 0.38*; -0.66</td>
</tr>
<tr>
<td>125</td>
<td>250</td>
<td>-0.25; -0.02; 1.20; 0.65*</td>
</tr>
<tr>
<td>250</td>
<td>500</td>
<td>-0.60; 1.57; -1.14; 4.90</td>
</tr>
<tr>
<td>500</td>
<td>250</td>
<td>-0.63; -2.26; 0.51*; 0.81*</td>
</tr>
</tbody>
</table>

Noted: *synergistic
Isolation and validation of CR-CSCs revealed unique morphology and surface marker expressions. Single cytotoxicity tests indicated an IC50 of 141.26 µM for 5-FU and 464.56 µM for EGCG. Combination cytotoxicity tests unveiled synergistic effects at specific doses. The CI values demonstrated synergy in several combinations, emphasizing the potential for enhanced cytotoxicity against CR-CSCs [14].

In this combination test, 5-FU doses were (18.75; 37.5; 75; 150), and EGCG doses were (62.5; 125; 250; 500), showing a synergistic effect (CI<1) in the combination 18.75 5-FU±62.5 EGCG (CI = 0.08); 18.75 5-FU±250 EGCG (CI = 0.38); 150 5-FU±250 EGCG (CI = 0.51); 37.5 5-FU±500 EGCG (CI = 0.65); 150 5-FU±500 EGCG (CI = 0.81) as shown in Table 3.

DISCUSSION

The research utilized colorectal cancer stem cells derived from sorting human colorectal cancer cell line HCT-116 and validated with CD44+/CD133+ (Figure 2) [15–17]. These validated cells formed mammosphere morphology, referred to as CSCs (Figure 1). Based on morphology, CSCs were characterized by a round and floating shape in non-adherent culture flasks. Protein surface markers analysis showed that CSCs expressed CD133, CD44, CD90, CD166, and CD326 [10,18,19].

The study comprised single-treatment groups of 5-FU, EGCG, and their combination at various concentrations (5-FU-EGCG). The untreated control group received a complete medium without any drug compounds. 5-FU was chosen as the basis for therapy, following National Comprehensive Cancer Network standards [20,21]. EGCG was obtained from Sigma-Aldrich.

Single cytotoxicity tests were employed to determine the cytotoxicity and doses of EGCG and 5-FU used to assess the synergy of these compounds using combination cytotoxicity, proliferation, apoptosis, stemness, and chemoresistance tests using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [8,22,23]. Based on MTT results, the single IC50 value for 5-FU was obtained at a concentration of 141.26 µM, while the IC50 value for EGCG was 464.56 µM. These data indicate that a single dose of 464.56 µM EGCG can inhibit 50% of CR-CSC growth, while 5-FU can inhibit 50% of CR-CSC growth at a single dose of 141.26 µM. The results prove that 5-FU exhibits a greater cytotoxic effect on CR-CSCs compared to EGCG. Consequently, there was a decrease in cell viability with increasing doses of both single 5-FU (Figure 3) and EGCG (Figure 4).

EGCG at doses of 50–400 µM did not reduce 50% viability of CR-CSCs. The 50% cell death effect of EGCG was shown at doses above 400 µM, with a noticeable decrease in 50% viability at doses ranging from 400–1000 µM. Thus, EGCG at doses above 400 µM can provide a stronger cytotoxic effect compared to concentrations below 400 µM in reducing 50% cell viability. Treatment with 5-FU on CR-CSCs showed dose-dependent cytotoxicity, as evidenced by decreased cell viability at higher doses. Compared to EGCG, 5-FU is more cytotoxic, but EGCG has also been proven to inhibit CR-CSC growth. Therefore, to determine the interaction between the two compounds, a combination cytotoxicity test was performed.

The obtained CI values (Figure 6) indicate that the synergy of the two compounds in producing a combination cytotoxic effect is greater than the single cytotoxic effect (24). This synergistic combination effect can also be seen from the decreasing cell viability along with the addition of doses (25,26). The decrease in cell viability indicates that the combination of 5-FU and EGCG can enhance cytotoxicity in CR-CSCs. This synergistic effect suggests that this combination could be developed for the treatment of CR-CSCs. The cytotoxicity test results were then followed by exploring the proliferation, apoptosis, stemness, and chemoresistance pathways in CR-CSCs (27–30).

This experimental study bridges conventional and natural therapeutic approaches, offering a comprehensive understanding of 5-FU and EGCG’s potential in CRC treatment. While 5-FU exhibited superior cytotoxicity,
the combination demonstrated synergistic effects, emphasizing the promise of combined treatments. Insights into proliferation, apoptosis, stemness, and chemoresistance pathways further support the potential of this combined strategy in CRC therapy.

CONCLUSIONS

The study suggests that the combined use of EGCG and 5-FU holds promise for enhancing therapeutic outcomes against CR-CSCs. While 5-FU showed greater individual cytotoxicity, the synergy observed in combination tests indicates a potential avenue for refining CRC treatment. These findings contribute to the evolving landscape of cancer treatment, emphasizing the importance of understanding interactions between conventional and natural compounds for optimized therapeutic strategies.

DECLARATIONS

Competing interest:
The authors report no conflicts of interest. The authors are responsible for the content and writing of this article.

Ethics approval and consent to participate
This research approved by Health Research Ethics Committee, Faculty of Medicine, Universitas Diponegoro on November 22nd 2022 with reference number 411/EC/KEPK/FK UNDIP/XI/2022

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