Synergistic anti-cancer activity of combined 5-fluorouracil and gallic acid-stearylamine conjugate in A431 human squamous carcinoma cell line

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INTRODUCTION

Skin cancer is a tumour formed from the uncontrolled growth of abnormal skin cells. It has a multifactorial aetiology involving genetic alterations, environmental factors, and lifestyle factors. 5-Fluorouracil (5-FU) is an anticancer drug that suppresses the activity of thymidylate synthetase. However, the use of 5-FU has many disadvantages. One of these drawbacks is that 5-FU leads to inactivation of dihydropyrimidine dehydrogenase, thereby reducing its absorption through the gastrointestinal tract. Other disadvantages include its short half-life and toxic effects on the bone marrow and normal cells. Scientists have attempted to improve the efficacy of this drug by increasing its circulation period and minimising its side effects by targeting it to the affected cells through targeted approaches [1,2].
Gallic acid (GA; 3, 4, 5-trihydroxy benzoic acid) is a naturally occurring polyphenolic group found in many plants either as free GA or gallotannins, which are the glucose-esterified products of GA. Gallic acid (GA) possesses remarkable antioxidant [3], anti-inflammatory and anti-carcinogenic [4], and antifungal properties [5]. These properties may be enhanced by the conjugation of GA to stearylamine (SA), forming GA–SA amide conjugate. This conjugation also initially increases the solubility of GA within a mixture of solvents. Thus, in future, the conjugate is most likely to be used in the formulation of lipid-based carriers for the vesicular system: greater entrapment of GA induces higher GA uptake by cells [6]. The present study investigates the synergistic cytotoxic activity of the combination of GA–SA conjugate with 5-FU in A431 human squamous carcinoma cell line.

**EXPERIMENTAL**

**Chemicals**

Gallic acid (GA), Stearylamine (SA), Tris-(2,2,2-trifluoroethyl)borate, Amberlyst A-26(OH), Amberlyst 15, Amberlite IRA743, and 5-FU were procured from Sigma Aldrich Pvt Ltd, Bengaluru, India. A431 human squamous carcinoma cell line and normal HaCaT human immortalised keratinocyte cell line were obtained from the National Centre for Cell Science (NCCS), Pune, India. Dulbecco’s modified Eagle’s medium (DMEM), streptomycin, penicillin and foetal bovine serum (FBS) were brought from Himedia Laboratories Pvt Ltd, Mumbai, India. Ethylene diamine tetra acetic acid (EDTA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and trypsin were procured from Sigma Aldrich Pvt Ltd, Bengaluru, India.

**Preparation of gallic acid–stearylamine conjugate**

In the present study all reactions were achieved on a 1-mmol scale. Tris-(2,2,2-trifluoroethyl) borate (chemical formula: B(OCH$_2$CF$_3$)$_3$; 2 mmol, 2 equiv) was added to a solution of GA (1 mmol, 1 equiv) whereas SA (1 mmol, 1 equiv) was prepared in acetonitrile (2 mL, 0.5 M). Then the reaction mixture was stirred at 80 °C in a properly sealed carousel tube for 5 h. Figure 1 shows the chemical reaction of gallic acid (GA)–stearylamine (SA) conjugate.

**Solid stage workup**

At the end of the reaction (5 h), the mixture was diluted with CH$_2$Cl$_2$ (3 mL) and water (0.5 mL). Amberlyst 15 (150 mg), Amberlyst A-26(OH) (150 mg), and Amberlite IRA743 (150 mg) were added to the mixture and stirred for 30 min. Then, MgSO$_4$ was added and thereafter, the mixture was filtered. To produce the amide product, the solids obtained were separated from CH$_2$Cl$_2$ three times through concentration *in vacuo* [7].

![Figure 1: Chemical reactions involved in the preparation of gallic acid–stearylamine conjugate](image-url)
Fourier transform infrared spectrometry (FT-IR)

FT-IR spectra of the test compounds were generated using a Shimadzu Prestige 21 FT-IR spectrometer through the KBr approach. The spectra were determined between 4000 and 400 cm⁻¹.

Nuclear magnetic resonance (NMR)

Proton nuclear magnetic resonance (¹H NMR) spectra were obtained using a Bruker Ultra shield (400 MHz) spectrometer.

Mass spectrometry (MS)

The MASS spectra of the test compounds were generated in ESI-MS mode on a MicroTOF-Q-II instrument (Bruker Daltonics).

Determination of cytotoxic concentrations of test compounds

The cytotoxic activities of the test compounds (5-FU and the GA–SA conjugate) were evaluated against A431 human squamous carcinoma cell line. The cells were trypsinized and counted using Trypan blue method within Neubauer chamber, and they were plated in a flat bottom 96-well plate at a density of 8 × 10⁴ cells/well/180 µL media. Following overnight incubation, the cells were treated with the test compounds (20 µL/well) at the ratios of 5:1, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, and 1:5, in a total volume of 200 µL in each well. Untreated cells served as negative control. Cells treated with DMSO (0.1 – 0.5 %) were considered as vehicle group. After treatment, the cells were placed in a 5 % CO₂ incubator for 48 h [9].

Determination of toxicity of test compounds

The safety of the test compounds (5-FU and the GA–SA conjugate) was screened using HaCaT human immortalised keratinocyte cell line. The toxicity of the test compounds was investigated by MTT assay [8].

Statistical analysis

MTT assay results are expressed as mean ± standard error of mean (SEM) of three replicates. The results were evaluated using Graph Pad Prism 5.0. All statistical analysis were done with Statistical Package for Social Sciences (SPSS) version 16.0, using one-way analysis of variance (ANOVA) and post hoc Tukey’s test to determine differences between means. P < 0.05 was considered statistically significant.

RESULTS

Fourier transform infrared spectra

Successful conjugation of GA with stearylamine was confirmed using FTIR spectra, as shown in Figure 2 (KBr pellet, cm⁻¹): 3365 -NH stretching, 1708 -C=O stretching.

Nuclear magnetic resonance spectra

Figure 3 shows the ¹H NMR spectra of the GA–SA conjugate. The ¹H NMR (d₂-DMSO) results were as follows:

¹H NMR 400 MHz (d₂-DMSO, δ ppm): 12.16 (OH, 2H, br), 9.14 (NH of conjugate, 1H, S), 8.74–8.9 (OH, 1H, br), 7.6–7.84 (aromatic H, S, 2H, m), 2.64–2.79 (methylene units of conjugate,
17H, m), 1.42–1.55 (methylene units of conjugate, 17 H, m), 0.80–0.83 (3H, m, Me Hs of the conjugate).

Cytotoxic concentration of 5-FU and GA–SA conjugate

The cytotoxic activities of the test compounds (5-FU and GA–SA conjugate) determined based on IC₃₀ values, were to be 1 and 10 µg/mL respectively. The cytotoxicity data for the test compounds (5-FU and GA–SA conjugate) in A431 cell line are shown in Table 1, Table 2 and Figure 5.

Anticancer efficacy of a combination of 5-FU/GA–SA conjugate in A431 cell line

The combination ratios were selected based on the IC₃₀ values obtained from the cytotoxic data of individual test compounds (5-FU and GA–SA conjugate) in A431 cells. The IC₃₀ values of individual test compounds were determined to obtain the optimal level of anticancer efficacy, i.e., at least 50 % reduction (IC₃₀). The A431 cells were treated with different ratios of 5-FU:GA–SA conjugate (ie, 5:1, 4:1, 3:1, 2:1, 1:2, 1:3, 1:4 and 1:5; v/v). Table 3 and Figure 6 show the cytotoxicity values of the combination ratios of the test compounds (5-FU and GA–SA conjugate) in A431 cell line.

Table 1: Cytotoxicity of 5-fluorouracil in A431 cell line

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Absorbance (nm)</th>
<th>Cytotoxicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.626 ± 0.07</td>
<td>0.00</td>
</tr>
<tr>
<td>0.1</td>
<td>0.601 ± 0.04</td>
<td>4.12</td>
</tr>
<tr>
<td>1</td>
<td>0.446 ± 0.05</td>
<td>28.79</td>
</tr>
<tr>
<td>5</td>
<td>0.418 ± 0.08</td>
<td>33.32</td>
</tr>
<tr>
<td>10</td>
<td>0.373 ± 0.07</td>
<td>40.39</td>
</tr>
<tr>
<td>50</td>
<td>0.236 ± 0.02</td>
<td>62.37</td>
</tr>
<tr>
<td>100</td>
<td>0.170 ± 0.01</td>
<td>72.91</td>
</tr>
</tbody>
</table>

Compared with negative control, 5-FU treatment showed statistically significant cytotoxicity (p < 0.05). 

Table 2: Cytotoxicity of gallic acid–stearylamine conjugate in A431 cell line

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>Absorbance (nm)</th>
<th>Cytotoxicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.626 ± 0.07</td>
<td>0.00</td>
</tr>
<tr>
<td>0.1</td>
<td>0.543 ± 0.12</td>
<td>−5.53</td>
</tr>
<tr>
<td>1</td>
<td>0.633 ± 0.12</td>
<td>4.26</td>
</tr>
<tr>
<td>5</td>
<td>0.546 ± 0.04</td>
<td>10.06</td>
</tr>
<tr>
<td>10</td>
<td>0.503 ± 0.03</td>
<td>21.05</td>
</tr>
<tr>
<td>50</td>
<td>0.228 ± 0.06</td>
<td>72.38</td>
</tr>
<tr>
<td>100</td>
<td>0.185 ± 0.02</td>
<td>71.58</td>
</tr>
</tbody>
</table>

Compared with negative control, GA–SA conjugate treatment showed statistically significant cytotoxicity (p < 0.05). 

Results are presented as mean ± standard error of mean (n = 3); 5-fluorouracil
gallic acid– stearylamine conjugate
Figure 5: Cytotoxic effect of 5-FU and GA–SA conjugate on A431 cell line.

Toxicity of 5-FU and GA–SA conjugate

Table 4 shows the toxicity of 5-FU in HaCaT cell line.

Table 3: Cytotoxicity of 5 FU:GA–SA conjugate in A431 cell line

<table>
<thead>
<tr>
<th>Combination ratio (µg/mL)</th>
<th>Absorbance(nm)</th>
<th>Cytotoxicity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.903 ± 0.15</td>
<td>0.00</td>
</tr>
<tr>
<td>5:1</td>
<td>1.264 ± 0.18</td>
<td>40.94</td>
</tr>
<tr>
<td>4:1</td>
<td>0.896 ± 0.10</td>
<td>53.95</td>
</tr>
<tr>
<td>3:1</td>
<td>0.766 ± 0.07</td>
<td>57.05</td>
</tr>
<tr>
<td>2:1</td>
<td>0.954 ± 0.05</td>
<td>51.08</td>
</tr>
<tr>
<td>1:1</td>
<td>0.753 ± 0.08</td>
<td>60.29</td>
</tr>
<tr>
<td>1:2</td>
<td>0.633 ± 0.07</td>
<td>63.32</td>
</tr>
<tr>
<td>1:3</td>
<td>0.583 ± 0.02</td>
<td>68.94</td>
</tr>
<tr>
<td>1:4</td>
<td>0.693 ± 0.03</td>
<td>62.22</td>
</tr>
<tr>
<td>1:5</td>
<td>0.419 ± 0.02</td>
<td>77.93</td>
</tr>
</tbody>
</table>

Compared with negative control, 5 FU:GA–SA conjugate treatment showed statistically significant cytotoxicity (p < 0.05). Results are presented as mean ± standard error of mean (n = 3); 5-fluorouracil:gallic acid–stearylamine conjugate

DISCUSSION

5-Fluorouracil (5-FU) is a potent chemotherapeutic agent frequently chosen in combination therapy for the treatment of numerous cancers. However, the drug is disadvantaged by its short half-life and poor permeability in affected cells [10-12]. Consequently, significant research efforts have been directed towards enhancing the permeability, stability and half-life of the drug. With regard to permeability improvement, it was hypothesized in this study, that an adjuvant with long hydrocarbon chain would give the desired hydrophobicity for an optimal absorption of the drug molecule. It was also hoped that the chemical combination of such adjuvants with moieties possessing antioxidant and anticancer...
activities might help in obtaining desired synergistic cytotoxic activity against cancer cells. Therefore, this study set out to design a conjugate consisting of stearyl amine (possessing 18 methylene units) with gallic acid (known to possess antioxidant and anticancer activities against most cancer cell lines) as an optimal adjuvant formulation for 5FU. The choice of GA was based on the fact that it is known to be safe against normal skin cell line like HaCaT cells [13-15].

Besides, the proposed adjuvant should be cheap and easy to synthesize, such that the end formulation is economically feasible. In this study, GA-SA conjugate was synthesized using B(OCH$_3$CF$_3$)$_3$-facilitated amidation reactions. Amide conjugate was considered in this case owing to its abundance in nature and its biocompatibility with many of the cancer chemotherapeutic agents [6,16]. The GA-SA synthesis was carried out in open air with equimolar concentration (1mmol) of carboxylic acid and amine, which are easily soluble in acetonitrile (MeCN). Moreover, since the present approach did not require separation with column chromatography, it was believed that GA-SA could be an eligible cost-effective substitute for 5-FU topical formulations. The effectiveness of such a simple synthetic procedure was well reflected in the FTIR, NMR and mass spectra of the resultant GA-SA conjugate [16,17].

To test the efficacy of the synthesized GA-SA conjugate for the intended application i.e. topical formulations, two different cell lines viz. A431 and HaCat were chosen. The former is a widely used non-melanoma skin cancer cell line, while the latter is a well-known normal skin cell-line [18]. The cells were treated with GA-SA at different concentrations and the corresponding cytotoxicity results indicated that it was effective in cancer cell line and reasonably safe against normal cell lines. To test the efficacy of the produced GA-SA as a formulation adjuvant, cytotoxicity studies were conducted at different ratios of 5FU: GA-SA conjugate ranging from 5:1 – 1:5 (v/v), and significant synergistic cytotoxicity was observed against A431 cells at 1:1 ratio. It has been previously reported by several workers that synergism is dependent on the ratio of components [19, 20]. Importantly, it was observed that the cytotoxicity of the conjugate was as high as 60 % at 0.1µg/ml of 5-FU, in contrast to 5-FU when used alone. This indicates a 2-fold increase in the anticancer activity of 5-FU.

CONCLUSION
The results of this study show that B(OCH$_3$CF$_3$)$_3$ mediates the conjugation of GA with stearylamine via an amide conjugate. Furthermore, the combination of 5-FU and GA-SA conjugate in a ratio of 1:1 (v/v) is effectively cytotoxic against A431 cancer cell line, but it is non-toxic against HaCaT normal cell line. Thus, the combination of the GA–SA conjugate and 5-FU exerts synergistic anticancer effects in A431 cell line, and enhances the cytotoxicity of 5-FU, thereby achieving the desired therapeutic effects. The synergistic effect of 5-FU and GA-SA conjugate can thus minimise the clinical dosage of 5-FU, thereby reducing the toxicity associated with higher doses. Therefore, it may be suitable as an adjuvant in a topical formulation of 5-FU to improve permeation, localization of action and stability. However, further preclinical and clinical investigations are required to buttress these findings.

DECLARATIONS

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Conflict of Interest
No conflict of interest associated with this work.

Contribution of Authors
The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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