Original Research Article

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

R Rajagopalan1*, Sanjay K Jain2, Piyush Trivedi1
1Department of Pharmaceutics, School of Pharmaceutical Sciences, Rajiv Gandhi Technological University, Bhopal (M.P.),
2Department of Pharmaceutics, Dr. Harisingh Gour University, Sagar (M.P.), India

*For correspondence: Email: rajagopalan10@gmail.com; Tel: +91-9179626663

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Abstract

Purpose: To evaluate the individual and synergistic anti-cancer effects of 5-fluorouracil (5-FU) and synthesized gallic acid-stearylamine (GA-SA) conjugate in A431 human squamous cancer cell line.

Methods: Characterisation of the synthesised conjugate was performed using Fourier transform infrared spectroscopy (FT-IR), nuclear magnetic resonance (NMR), and mass spectrometry (MS). The synergistic effect of the combination therapy (5-FU/GA-SA) was assessed by determining their inhibitory concentration (IC50) whereby A431 cells were treated with 5-FU/GA-SA conjugate at various ratios ranging from 5:1 to 1:5.

Results: The cytotoxicity of 5-FU was 29 %, while that of the combination of 5-FU with GA–SA conjugate was as high as 60 %. Thus, this combination showed significant synergistic enhancement in cytotoxicity (p < 0.05). The results obtained also revealed that the IC50 values of 5-FU and the GA–SA conjugate were 1 and 10 µg/mL, respectively. The IC50 values of the combination ratios indicated that the dosages used in the study were safe in HaCaT normal cell line.

Conclusion: These results indicate that 5-FU/GA–SA conjugate at a ratio of 1:1 is effective against A431 cell line (cancer cells)) but safe in HaCaT cell lines (normal cells).

Keywords: 5-Fluorouracil, Gallic acid, Stearylamine, Skin cancer, Cytotoxicity, Synergism

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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 dihydroxyridine 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 localising the drug 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].
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 ($^1$H 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 $8 \times 10^3$ 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 % CO$_2$ incubator for 48 h [9].

**Determination of anticancer efficacy of combination of 5-FU and GA–SA conjugate**

The anticancer efficacy of the combination of the test compounds (5-FU and the GA–SA conjugate) was evaluated using A431 cell line and was determined based on the percentage cytotoxicity of the test compounds. The cells were trypsinized using Trypan blue method in Neubauer chamber, and plated in a 96-well plate at a density of $8 \times 10^3$ 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 % CO$_2$ 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 with 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 $^1$H NMR spectra of the GA–SA conjugate. The $^1$H NMR (d$_5$-DMSO) results were as follows:

$^1$H NMR 400 MHz (d$_5$-DMSO, δ ppm): 12.16 (OH$_s$, 2H, br), 9.14 (NH of conjugate, 1H, S), 8.74–8.9 (OH$_h$, 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).

Figure 2: FTIR spectra of gallic acid–stearylamine conjugate

**Mass spectrometry**

Figure 4 shows the mass spectra of the synthesized GA–SA conjugate. Based on the ionisation method, the results were as follows:

\[ C_{25}H_{43}NO_4 M^+ = 421 \text{ (calculated)}, [M-H^-] = 420.5 \text{ (actual)}. \]

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 IC\(_{30}\) 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 IC\(_{30}\) values obtained from the cytotoxic data of individual test compounds (5-FU and GA–SA conjugate) in A431 cells. The IC\(_{30}\) values of individual test compounds were determined to obtain the optimal level of anticancer efficacy, i.e., at least 50 % reduction (IC\(_{30}\)). The A431 cells were treated with different ratios of 5-FU:GA–SA conjugate (i.e., 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

| Concentration (µg/mL) | Absorbance (nm)\(^a\) | Cytotoxicity (%) |
|-----------------------|------------------------|-----------------|
| 0                     | 0.626 ± 0.07           | 0.00            |
| 0.1                   | 0.601 ± 0.04           | 4.12            |
| 1                     | 0.446 ± 0.05           | 28.79           |
| 5                     | 0.418 ± 0.08           | 33.32           |
| 10                    | 0.373 ± 0.07           | 40.39           |
| 50                    | 0.236 ± 0.02           | 62.37           |
| 100                   | 0.170 ± 0.01           | 72.91           |

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

\( ^a \)Results are presented as mean ± standard error of mean (n = 3); \(^b\)5-fluorouracil

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

| Concentration (µg/mL) | Absorbance (nm)\(^a\) | Cytotoxicity (%) |
|-----------------------|------------------------|-----------------|
| 0                     | 0.626 ± 0.07           | 0.00            |
| 0.1                   | 0.543 ± 0.12           | −5.53           |
| 1                     | 0.633 ± 0.12           | 2.66            |
| 5                     | 0.546 ± 0.04           | 10.06           |
| 10                    | 0.503 ± 0.03           | 21.05           |
| 50                    | 0.228 ± 0.06           | 72.38           |
| 100                   | 0.185 ± 0.02           | 71.58           |

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

\( ^a \)Results are presented as mean ± standard error of mean (n = 3); \(^b\)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

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

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
The results of this study show that B(OCH$_2$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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