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Article

Combating drug resistance in cancer cells: Cooperative effect of green tea and turmeric with chemotherapeutic drug

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SUMMARY

The major drawback of chemotherapy regimens for treating cancer is that the cancerous cells acquire drug resistance and become impervious to further dose escalation. Keeping in mind the studied success of herbal formulations with regard to alternative treatments for cancer, we hypothesized that the use of a chemotherapeutic drug and proprietary herbal formulation, HF1 (under patent by Sri Raghavendra Biotechnologies Pvt Ltd, Bangalore, India) would combat this phenomenon. 5-Fluorouracil (5FU) is a commonly administered chemotherapeutic drug that is used to treat many cancers but also has the problem of inducing drug resistance during its regimen. We undertook the present in vitro study to test the efficacy of HF1 (which is mainly composed of green tea and turmeric) in reversing drug resistance in a 5FU-resistant cell line. To do so, we conducted a cell viability assay known as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) assay. The MTT assay uses a reagent that produces a color proportionally to the percentage of living cells present in the culture. Firstly, a resistant cell line of HeLa cells (HeLa-R) was cultured by slowly exposing it to gradually increasing doses of 5FU. This resistant cell line was then subjected to three treatments: i) only 5FU, ii) only HF1, and iii) both 5FU and HF1. These results were then compared to determine the relative effects of each treatment on cell survival. Results demonstrated a cooperative effect between HF1 and 5FU on the drug resistant cell line, implying that administration of HF1 with 5FU results in cell death as measured by MTT assay.

INTRODUCTION

Cancer is one of the most common non-communicable diseases and one of the leading causes of death worldwide, with approximately 9.6 million deaths in 2018 (1). Despite the fact that chemotherapy is central to clinical management of cancer, failure in chemotherapy is not uncommon, mainly due to the occurrence of drug resistance. An attempt to find a way of overcoming this barrier is essential in improving the treatment and care given to patients, especially those who have relapsed.

Drug resistance is an increasingly common phenomenon that occurs when diseases are left unaffected and become tolerant to pharmaceutical treatments (2). Drug efflux is a highly studied method of drug resistance where drugs are pushed through protein pumps on the cell membrane to reduce drug accumulation (3). The ATP binding cassette (ABC) protein family is largely responsible for this phenomenon. The permeability glycoprotein (P-gp), from subfamily B, transports lipophilic substances. It is characterized by its wide substrate specificity, known to exceed over 300 compounds (4), and its ability to mediate ATP-dependent translocation of drugs across the plasma membrane against considerable concentration gradients.

In human cancer cells, multi-drug resistance refers to the intrinsic or acquired resistance to a variety of chemotherapeutic drugs that is triggered after having been exposed to just one cytotoxic compound, making the tumor cells increasingly hard to target without administering highly toxic concentrations of the drug (5). The chemotherapeutic drug of interest in this study is 5-fluorouracil (5FU), which works by inhibiting the nucleic acid synthesis required for cell division and is used to treat a wide range of solid tumors. In vitro, these drug resistant cancer cells can be cultured by exposing the cells to doses of one particular drug, increasing the concentrations as the passage numbers increase (6).

Interest is increasing in P-gp inhibitors that are formulated from natural resources and could be used in place of toxic synthetically produced inhibitors. By successfully inhibiting the action of these protein pumps, drug efflux can be suppressed and drug resistance decreased. This is essential to cancer treatment as it will then allow a regular dosage of the chemotherapeutic drug to be administered with intended effect. Among examples of natural inhibitors, there are curcumin and epigallocatechin gallate (EGCG), which are derived from turmeric (Curcuma longa) and green tea, respectively (7).

Green tea and turmeric are known to exhibit anti-oxidative, anticancer, chemo-preventive, chemo-protective, anti-proliferative, and anti-inflammatory properties (7, 8). Both spices have a long history in traditional food and medicine, and have been studied in great detail in the context of cancer therapy and/or prevention. Hence, the herbal formulation

called HF1 (FSSAI approved, patented by Sri Raghavendra Biotechnologies Pvt Ltd, Bangalore, India), which consists of a crude preparation made with green tea and turmeric, was hypothesized to demonstrate an increase in susceptibility to the drug.

Results suggest that the addition of HF1 and 5FU together have a greater cytotoxic effect against HeLa-R, making the combination effective even against drug resistant cells in vitro. Hence, the herbal formulation can be further studied as a potential direction for novel means of combating chemo-drug resistance in relapsed cancer.

RESULTS

We performed MTT assays to measure the percentage viability of the cell culture after subjecting it to a specific treatment for 24 hours. The percentage of living cells was a direct indicator of the cytotoxicity of the treatment provided: the greater the viability, the lesser the cytotoxicity. From these values, we calculated the inhibitory concentration at 50% (IC50) to compare efficacy between the treatments. This value indicates the concentration required to bring about 50% death—the larger the IC50 value, the less effective the treatment was. Three treatments were performed: HF1 alone on HeLa-R, 5FU alone on HeLa-R, and HF1+5FU together on HeLa-R.

Cytotoxic effects of HF1 and 5FU on HeLa-R cells

In the first treatment, we administered HF1 to resistant cells to determine its effect when used independently of 5FU and to calculate its IC50 value. The IC50 of HF1 on HeLa and HeLa-R cells was 0.6 mg/ml and 1.6 mg/ml, respectively (**Figure 1A**).

Similarly, in the second treatment, we tested the effect the chemotherapy drug 5FU on resistant HeLa cultures. Doses of 5FU that even greatly exceeded its IC50 value on HeLa cells (5000 µg/ml) did not lead to significant death of HeLa-R cells (**Figure 1B**), suggesting complete resistance of HeLa-R to 5FU.

Cooperative effect of combined HF1/5FU on HeLa-R cells

The third treatment was a combination of lower concentrations of both HF1 and 5FU. We sought to determine if the combination of HF1 and 5FU, in which each drug is administered at a concentration below that at which it is effective when used singly, would still provide effective treatment. Essentially, the first two treatments were necessary to determine the range of concentrations that had the potential for cytotoxicity in HeLa-R for both HF1 and 5FU. Despite lack of efficacy in the previous treatments, the cells' drug resistance was reversed by the combined effect of HF1 and 5FU on HeLa-R cells (**Figure 2**).

When 0.6 mg/mL HF1 (the IC50 of HF1 on HeLa cells) was added to various concentrations of 5FU, the IC50 of combined HF1/5FU on HeLa-R was significantly low ($p < 0.001$) as compared to 5FU alone on HeLa-R. The IC50 value of HF1 on regular HeLa cells was used as the purpose was to demonstrate that concentrations that should work on regular cultures, but had little effect on resistant cultures, are effective in tandem with the drug when used to treat resistant cultures. This was purposefully used to show that the death brought upon the HeLa-R culture is not by the sole toxicity of HF1 but is a result of the combinatory effect of the formulation and drug.

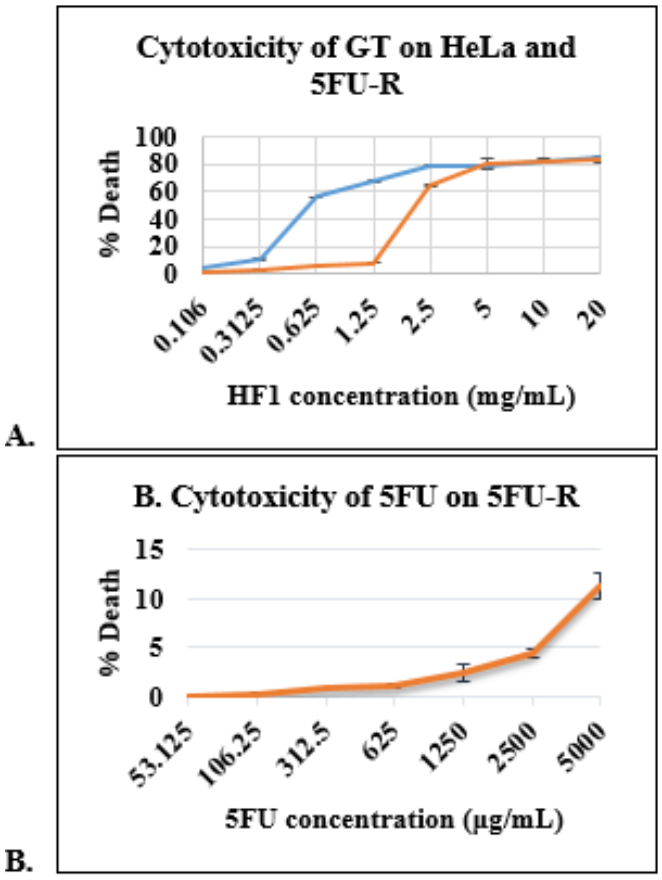

Figure 1. Cytotoxic effect of each treatment separately (HF1 alone and 5FU alone) on HeLa-R. Mean ± standard deviation is shown (n=3). A) Cytotoxicity of HF1 on HeLa (blue) and HeLa-R cells (orange). Both HeLa (blue line) and HeLa-R cells (orange line) were treated with various concentrations of HF1 only. The media control contained no HF1. Cell viability was assessed using an MTT assay. B) Inhibition curve of 5FU on HeLa-R cells. HeLa-R was treated with various concentrations of 5FU only. Control was media only. Cell viability was assessed using an MTT assay.

DISCUSSION

Chemotherapy is one of the mainstay treatments for cancer. However, its clinical efficacy is getting compromised due to the multi-drug resistant (MDR) property of some tumor cells. The mechanisms underlying MDR involve many factors and are complex. A major mechanism is transporter-mediated efflux, where drug entry into the cancer cells is limited by many proteins including P-glycoprotein (P-gp), and multidrug

resistance proteins (MRPs). Many anticancer drugs, including paclitaxel, doxorubicin, and 5FU are substrates of P-gp (6). HF1 treatment shows promise in overcoming drug resistance, as it was shown to effectively reverse the effect of P-gp (12-16).

This study shows a synergistic effect between HF1and 5FU, as IC80 is achieved at concentrations equal to the IC50 of 5FU and the IC50 of HF1. Clearly, an increase in effectiveness is seen when both are used simultaneously.

All these experiments were conducted on Hela-R, the resistant HeLa cultures, in order to create an in vitro representation of the resistant cancer that often relapses in patients. Hence, HF1 would be tested not as a supporting agent for chemotherapy to cure cancer, but rather as a compound to reverse drug resistance and re-sensitize the cancer cells to 5FU once again.

Furthermore, similar studies need to be done using other cell lines representing varying cancer types in order to validate HF1 for clinical management of cancer drug resistance. In this study, cells from the HeLa cell line, a human cervical cancer cell line, were used because they carry advantageous characteristics. They multiply rapidly, doubling every 24 hours, which makes them easier to work within a project containing a lot of cell culture work. Also, HeLa cells are robust enough to survive and grow successfully even after multiple passages, a requirement when cultivating cells to become resistant.

Investigating potential cooperative effects between HF1 and multiple other common chemotherapeutic drugs, such as paclitaxel, will be an important step in showing that this alternative is a viable approach to combat drug resistance.

The findings from this experiment imply that HF1 can be a potential cancer treatment when used in tandem with common chemotherapeutic treatments, as it may be able to re-sensitize resistant cancer cells to the original drug that was being used. This opens up a pathway for clinicians to be able to give patients a second chance at life, without having to resort to stronger treatments.

MATERIALS AND METHODS

Cell culture

Cells were cultured in DMEM-F12 medium, purchased from Thermo Fisher Scientific (Waltham, MA, USA) with 10% FBS (Thermo Fisher, Waltham, MA, USA) in a humidified incubator at 37˚C with 5% CO2 (9). Human cervical cancer cells (HeLa cells), obtained from NCBS, Pune, were grown to be HeLa-R (resistant) to 5-Flourouracil (5FU) in the laboratory as previously described, in 2 µg/ml of 5FU (10). Cells were harvested at 80% confluency.

MTT Assay

MTT assays are viability assays that indicate the percentage of living cells present in the culture. A colorimetric assay with MTT dye was used to measure the percentage of living cells present as MTT is processed into purple crystals within living, but not dead, cells. The intensity of color produced is proportionate to the percentage of living cells. The MTT dye was obtained in powder form from Sigma (St. Louis, MO, USA). Single cell suspensions were cultured in a 96-well plate, seeded at a density of 100 µL/well (2×104 cell/mL) for 24 hours. These cells were then exposed to the treatments for 24 h. After this, 20 µl of MTT dye (5 µg/ml) was added and incubated for 4h at 37oC. The complete solution was replaced with DMSO, an organic solvent, and incubated for 30 minutes at room temperature in order to dissolve the crystals and create a purple solution which can be quantified by measuring absorbance at 545nm (11). Three different treatments were tested using this assay.

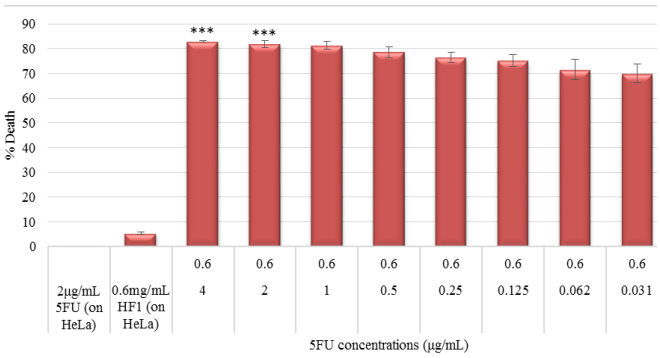

Figure 2. Synergistic effect of HF1 with 5FU on HeLa-R cells. HeLa-R cells were treated with 0.6 mg/mL HF1 (IC50 of HF1 on HeLa cells) and various concentrations of 5FU. Controls were treated with 2 µg/mL 5FU only (IC50 of 5FU on HeLa cells), or 0.6 mg/mL HF1 only. Cell viability was assessed using an MTT assay. p< 0.001 (***) when compared HeLa-R cells treated with 5FU to HF1 IC50 and 5FU IC50 on HeLa. Mean ± standard deviation is shown (n=3).

Cytotoxic effects of HF1 on HeLa and HeLa-R cells

HF1 (100 µL at different concentrations: 0, 20, 10, 5. 2.5, 0.625, 0.31, 0.15 mg/ml) was added to cultured cells for 24h. The MTT assay procedure as described above was followed. The percentage viability of the cells was plotted on a graph against the concentrations, and the IC50 value was interpolated and calculated using the online tool available from AAT Bioquest (Sunnyvale, CA, USA).

Cytotoxic effects of 5FU on HeLa and HeLa-R cells

5FU (100 µL of different concentrations: 0, 4, 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03, 0.015 µg/ml) was added to cultured cells for 24h. The MTT assay procedure as described above was followed. The percentage viability of the assay was plotted on a graph against the concentrations, and the IC50 value was extrapolated and calculated using the online tool available from AAT Bioquest (Sunnyvale, CA, USA).

Cooperative effect of combined 5FU/HF1 on HeLa-R cells

Cells were treated with different concentrations of 5FU and a concentration of HF1 equal to its IC50 value determined for regular HeLa cells. 100 µL of each mixture was used. The

MTT assay procedure as described above was followed.

Statistical analysis

GraphPad Prism 5.0 (San Diego, CA, USA) was used to analyze and represent graphical data. One-way ANOVA was performed, followed by Tukey's test post ANOVA. The experimental data is expressed as mean and standard deviation. Statistical significance was achieved when $p < 0.001$. Three independent experiments were performed in triplicate.

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