Research Article

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Synergistic role of thymoquinone and 5-fluorouracil in U-251MG glioblastoma cell line

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Abstract

Objectives: Glioblastoma is a fast-growing and aggressive brain tumor. Despite the current treatment methods, such as chemical and surgical operations, the prognosis is still poor. Therefore, combined therapeutic strategies are proposed to maximize therapeutic efficacy and reduce toxicity. Thymoquinone has been shown to have neuroprotective effects in addition to its anti-cancer effects on different types of cancer. 5-Fluorouracil, on the other hand, is a cytotoxic chemotherapy agent used to treat cancer. As a synergistic combinational approach, this study aimed to examine the antiproliferative effects and production of reactive oxygen species in a glioblastoma cell line.

Methods: We have tested thymoquinone and 5-fluorouracil alone and in their combination to observe cellular growth with MTT assay. The combinational effects of the agents were determined by the CompuSYN software program. Cell proliferation was assayed with crystal violet assay. Reactive oxygen species production was analyzed by 2′,7′-dichlorodihydrofluorescein diacetate in glioblastoma cells.

Results: Thymoquinone and 5-fluorouracil inhibited cell growth of glioblastoma cells with half maximal inhibitory concentrations (IC_{50}) of 45.93 and 14.02 µM for 48 h, respectively. At synergistic combinational concentrations, the crystal violet assay demonstrated that there is a positive correlation between combination index values and cell proliferation. Also, an increment in the production of reactive oxygen species was observed upon combinational treatments.

Conclusions: Our results indicate that the combinational strategy of these two agents reduced cell viability and proliferation in glioblastoma cells and showed strong synergistic anticancer efficiency.

Keywords: anticancer effect; thymoquinone; 5-fluorouracil; synergistic combination; ROS production

Introduction

Glioblastoma (GBM) is the most aggressive cancer type in adults [1]. It has a very fast growth rate with a high invasion capacity. Current treatments for GBM include surgical resection, radiotherapy, and the most common form of treatment is chemotherapy [2]. However, the average lifetime after the diagnosis is very short, which highlights the importance of developing novel treatment options for GBM with enhanced therapeutic effects. It has been reported that combinational therapeutic strategies can be a promising ways of treating cancer. In order to achieve this goal, combinational therapy strategies, which have been shown to perform better than single ones, are suggested to increase the therapeutic efficacy against GBM [3, 4]. The combinational approaches were designed to improve the therapeutic activity of the drug, in this case, 5-fluorouracil (5-FU), by reducing the dose and chemotherapy-related toxicity [5].

The abundance of bioactive substances found in fruits, vegetables, herbs, and spices has resulted in their use as a practical strategy to cure human cancers [6]. Among these,

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thymoquinone (TQ), which is a bioactive phytochemical found in black cumin [7], has been shown to exhibit anticancer, anti-inflammatory, and immunomodulatory features along with other important biological activities [7–11]. Studies on the anticancer activities of TQ have revealed that TQ can inhibit cancer cell proliferation and also stimulate apoptosis [7]. Various studies report the anticancer and anti-inflammatory effects of TQ for various cancer types, including breast [12], blood [13], lung [14], pancreatic [15], prostate [16], bone [17], and colorectal cancer [18]. Despite this, a small number of studies have revealed its effects on brain cancer and GBM. TQ has been reported to stimulate caspase-independent apoptotic cell death in GBM cells [19]. Additionally, TQ has been shown to decrease chemotherapeutic toxicity [19].

5-FU is known to have anticancer effects due to its ability to inhibit thymidylate synthase (TS) and also because its metabolites can be incorporated into nucleic acids, which inhibit cell growth [20]. However, due to it being cytotoxic, and reducing its dosage in combination therapies can be considered an advantage. Thus, this research aimed to elucidate the antiproliferative efficacy and percentage of ROS production upon treatment with TQ alone, 5-FU alone, and their combined administrations in the GBM cell line.

Materials and methods

Compounds and cell culture

Thymoquinone and 5-fluorouracil were purchased from Sigma. Human U251 GBM cells were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI-1640) at 37 °C in humidified air with 5 % CO_2.

Cell viability and crystal violet assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma-Aldrich, St Louis, MO, USA) was used to evaluate the cytotoxic effects of the compounds to indicate cell viability. The GBM cells with a number of 7×10^4 were transferred into each well on 96-well culture plates, and treated with TQ and 5-FU at different concentrations and time intervals. After incubation periods of 24 and 48 h, 0.5 mg/mL MTT solution was transferred to the wells and incubated for 4 h at 37 °C. Following this, DMSO was added to each sample and measurements were taken spectrophotometrically at 595 nm. Below equation (1) was used to determine the percent cell viability;

$$\text{Cell viability}\,\% = \frac{A_s}{A_c}\times 100 \quad (1)$$

where, A_s and A_c stand for absorbance of sample and control, respectively.

After treatments as aforementioned, ethanolic solution of crystal violet was used for the crystal violet assay. After 15 min of incubation at room temperature, wells were washed thoroughly and images were captured after air drying using an Etaluma microscope LS460.

Combination analysis

CompuSyn Software analysis was employed for the drug combination analysis to investigate the effect of the drugs on the tested cell lines. The combination index (CI) value which determines the synergistic, additive and antagonistic effects of the drugs was calculated with Chou & Talatay method using the CompuSyn Software analysis program. CompuSyn software can automatically simulate the combination index equation (CIE), which determines synergism (CI<1), additive effect (CI=1), and antagonism (CI>1). Even in animals or clinical trials, are required to assess quantitative synergy in two-drug combinations with adequate experimental precision [21].

Intracellular ROS measurement

Reactive oxygen species (ROS) can be found in cancer cells and cause oxidative damage, eventually leading to apoptotic signals. Here, ROS generation was evaluated using 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) fluorogenic dye. In each well, 10×10^4 cells were seeded on a 96-well plate. Cells were pretreated with 5 mM N-acetyl-L8 cysteine (NAC) for 1 h following a 24 h incubation period. Cells were then treated with 20 µM H_2DCFDA for 45 min at 37 °C in the dark. The relative fluorescence intensity of the samples was measured at 488 nm and at 525 nm for excitation and emission, respectively.

Statistical analysis

Biosoft CompuSyn 2.1 software was used to determine the combine effect and calculate the combination index (CI). A one way analysis of variance (ANOVA) and Dunnett's multiple comparison test was used to determine significant differences between the treatments and the corresponding controls in the MTT and ROS assays.

Results

GBM cell viability

Firstly, we determined the effect of TQ and 5-FU on GBM cell growth. We treated cells with different concentrations of TQ and 5-FU performed viability test as dose and time-dependent through the MTT assay on GBM cells as shown schematically in Figure 1. As shown in Figure 1A, after treatment with 5 µM, 10 µM, 20 µM and 40 µM TQ alone, there was no significant reduction but 60 µM TQ ($p<0.01$) reduced cell viability for 24 h. GBM cell viability decreased with all dosages of TQ ($p<0.0001$) for 48 h. As expected in Figure 1B, the treatment with 10 µM ($p<0.01$), 20 µM ($p<0.001$), 40 µM ($p<0.001$), and 60 µM ($p<0.0001$) 5-FU reduced cell viability at 24 h on GBM cells. Cell viability was decreased 5-FU treatment cells especially with all concentrations ($p<0.0001$) at 48 h on GBM cells. After MTT test,

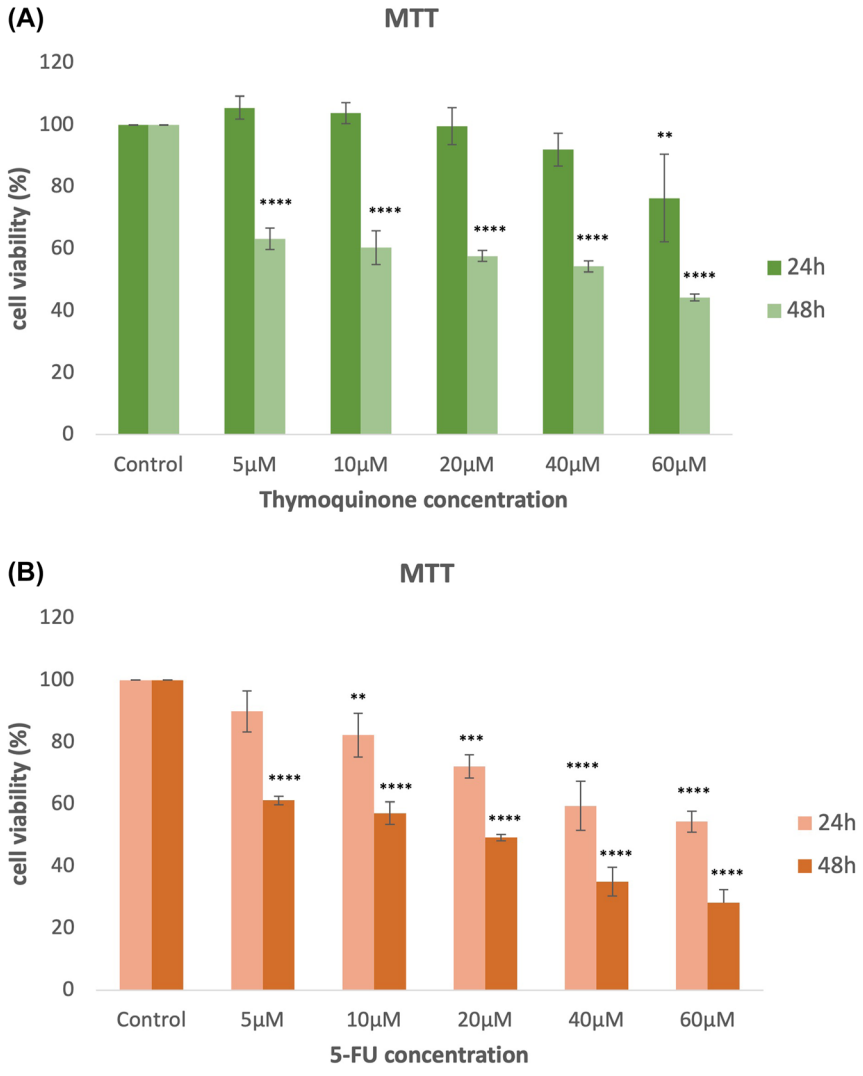

Figure 1: Percent cell viability of various concentrations of (A) thymoquinone and (B) 5-fluorouracil. Values are expressed as mean ± SD of three measurements. **p<0.01, ***p<0.001, ****p<0.0001 vs. the control group.

we calculated IC_{50} values using Compusyn Software program. The IC_{50} values of TQ and 5-FU were found to be 45.93 and 14.02 μM for 48 h treatment, respectively.

Effect of combination on cell proliferation

TQ and 5-FU combined treatment reduced GBM cell viability compared to TQ and 5-FU alone treatment, especially 40 μM TQ + 16.6 μM 5-FU ($p<0.5$), 60 μM TQ + 16.6 μM 5-FU ($p<0.01$), 33 μM TQ + 10 μM 5-FU ($p<0.0001$), 66 μM TQ + 20 μM 5-FU ($p<0.0001$), 38 μM TQ + 40 μM 5-FU ($p<0.0001$) as demonstrated in Figure 2. After that, The IC_{50} values for each combination were calculated to determine the synergistic effect of TQ and 5-FU. The combination index value (CI<1) was considered a drug selection criterion to observe the maximum synergistic effect. Low CI values (CI<0.5) show a high synergistic effect between the two drugs. CI values determined for various combinations of TQ and 5-FU are shown in Table 1 according to the Compusyn program. Two combinations were selected according to CI and affect value. Table 2 shows the CI values that were chosen to correspond to synergistic doses of the compounds. These combinations, which are designated as the combination-1 and combination-2 groups, respectively, are 60 M TQ + 16.6 M 5-FU (CI: 0.414, effect value: 0.676) and 33 M TQ + 10 M 5-FU (CI: 0.310, effect value: 0.651) (Table 2).

Cell proliferation was confirmed using a crystal violet staining assay on GBM cancer cells, as shown in Figure 3. The number of GBM cancer cells decreased significantly upon treatment with a combinations of TQ and 5-FU compared to TQ and 5-FU alone. The crystal violet assay demonstrated that there is a positive correlation between cell proliferation. During cell death, adherent cells separated from cell culture plates. This property can be used to assess changes in proliferation after stimulation with death-inducing agents and

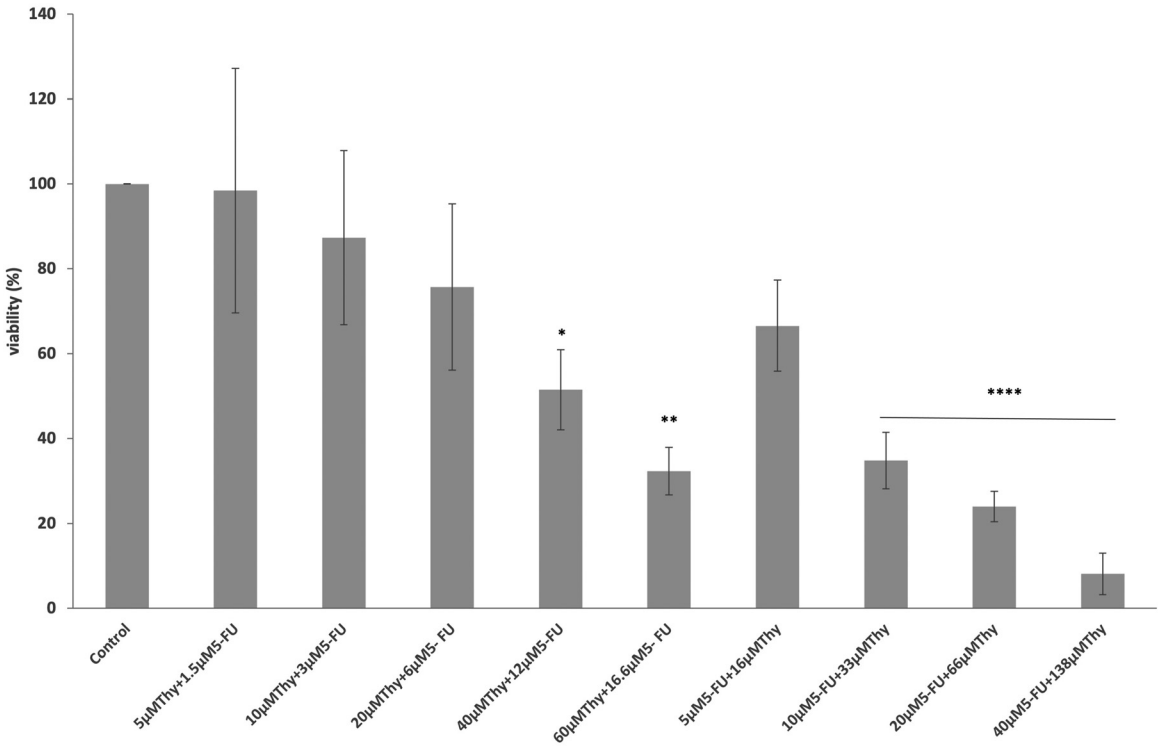

Figure 2: Cell viability results for different combinations of thymoquinone and 5-fluorouracil. Values are expressed as mean ± SD of three measurements. *p<0.5, **p<0.01, ****p<0.0001 vs. the control group.

Table 1: Combination index values.

Concentration of thymoquinone, µM	Concentration of 5-fluorouracil, µM	Effect	CI
5.0	1.5	0.015	472,423.0
10.0	3.0	0.126	273.680
20.0	6.0	0.242	31.713
40.0	12.0	0.485	2.035
60.0	16.6	0.676	0.414
16.0	5.0	0.334	5.590
33.0	10.0	0.651	0.310
66.0	20.0	0.759	0.212

Table 2: CI values of thymoquinone (TQ) and 5-fluorouracil (5-FU).

Combination	Concentration of thymoquinone (TQ), µM	Concentration of 5-fluorouracil (5-FU), µM	Effect	CI
1	60.0	16.6	0.676	0.414
2	33.0	10.0	0.651	0.310

to indirectly quantify cell death. Based on cell proliferation in combination groups, treatment with 5-FU and TQ

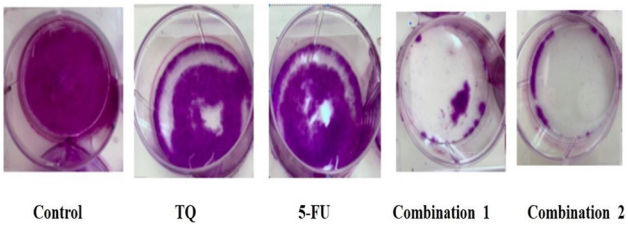

Figure 3: Crystal violet staining assay on GBM cell line.

significantly reduced the number of cells compared to treatment with TQ and 5-FU separately.

Determination of intracellular ROS levels

Intracellular ROS productions of TQ and 5-FU, combination-1 and combination-2 groups were evaluated using the H_2DCFDA assay on GBM cancer cells, as shown in Figure 4. Based on ROS production percentages, it was found that there is no significant increase in ROS production with TQ, 5-FU, and combination-1 compared to the control group. It was observed that combination-2 stimulates the production of ROS in GBM cells significantly ($p<0.001^{**}$) as opposed to nonsignificant (ns) ROS production with combination-1.

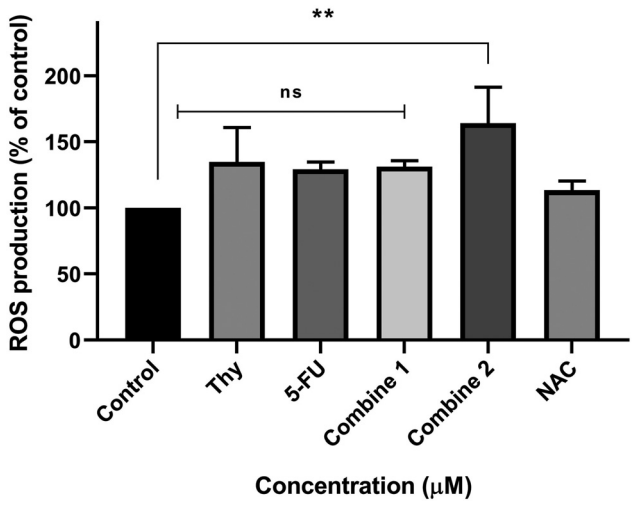

Figure 4: Intracellular reactive oxygen species (ROS) assay on GBM cancer cell line for TQ, 5-FU, combination 1, combination 2 and N-acetyl-L-cysteine (NAC).

ROS-stimulating drugs and their synergistic combinations could provide cancer-specific treatment. ROS-producing anticancer treatment methods indicate that a strategy combining these drugs in glioblastoma needs further research.

Discussion

5-FU is a chemotherapy drug and commonly used in the treatment of various cancers. Some of them can be listed as colorectal cancer [22], gastric cancer [23], and pancreatic cancer [24]. However, 5-FU has certain limitations and one limitation of using 5-FU is its potential for systemic toxicity, which may cause adverse effects in non-cancerous cells and tissues. To achieve the desired balance between efficacy and safety for 5-FU therapy, adjusting the dose, frequency, and duration of treatment may be needed [25]. 5-FU and other drug combinations' effectiveness has been assessed in clinical trials to achieve prolonged survival of glioma patients (NCT00281944, NCT01470794). However, no results were posted or published related to these studies [26, 27].

On the other hand, low bioavailability, high toxicity, and a short half-life can be considered drawbacks of 5-FU during chemotherapy treatment [28]. To overcome the poor ability of 5-FU to cross the blood-brain barrier, researchers have explored various strategies. These include stereotaxic implantation of 5-fluorouracil-releasing microspheres in glioma patients and the utilization of nanomedicine as liposomes [29, 30].

Additionally, scientists have developed a range of strategies, such as smart delivery systems and combinational drug therapies, to enhance the therapeutic efficiency of 5-FU and address these limitations. In the present study, the effects of 5-FU with TQ were analyzed in a combinational treatment model on GBM cells, and cell viability, proliferation and ROS production percentages were determined *in vitro*.

Thymoquinone has been shown to have anticancer activity. TQ is a multitargeted natural drug that has effects on multiple molecules in carcinogenic signaling pathways. In prostate cancer, TQ has been reported to have down-regulated regulatory activity for cell proliferation regulators (E2F-1) and androgen receptors [31]. Inhibition of cancer cell growth and stimulation of apoptosis have been detected due to the inhibition of signal transducer and activator of transcription 3 (STAT3) phosphorylation. Cyclin D1, Bcl-2, Bcl-xL, and vascular endothelial growth factor (VEGF) are STAT3-regulated gene products, and their expression can be inhibited upon TQ treatment [32]. In colon cancer cells (HCT-116), blockage of the STAT3 pathway and stimulation of apoptosis of cancer cells upon TQ treatment have been reported. Enhanced cleavage rate of poly-(ADP-ribose) polymerase (PARP), stimulation of caspases, and upregulation of Bcl-2 and Bax genes, which eventually cause inhibition of cancer cell growth [20]. Due to its simple chemical structure and lipophilic properties, TQ significantly crosses the blood-brain barrier [33].

5-FU mimics the chemical structure of uracil with a fluorine atom at the C-5 position instead of a hydrogen atom. 5-FU can disrupt the metabolism of nucleosides, and it can be incorporated into nucleic acids instead of uracil. This eventually triggers cell death due to the cytotoxicity of 5-FU [34].

Drug resistance limits the use of anticancer drugs or reduces their efficiency. The rate of response to 5-FU alone is around 10–15 % in colorectal cancer. Drug combination strategies with 5-FU have been shown to improve the response rate of colorectal cancer cells by overcoming drug resistance [34]. The cytotoxicity of 5-FU can be lowered by using a combinational strategy with natural agents to reduce the amount of 5-FU required for cancer treatments. For instance, the combination of 5-FU with Manuka honey induced apoptosis and chemosensitized HCT-116 colon adenocarcinoma spheroids [35]. Strawberry tree honey co-administration with 5-FU increased oxidative stress biomarkers, and caused cell cycle arrest and apoptosis in different colon cancer cells [36]. Also, resveratrol has been reported to help to modulate the chemosensitisation of HCT-116 colon adenocarcinoma cells [37]. Additionally, co-crystallization of 5-FU with bioactive compound, gallic acid, resulted in superior cytotoxicity in breast cancer cells [38].

In the present study, TQ was combined with 5-FU and the synergistic activity was observed against the GBM cancer

cell line via a significant reduction in the requirement for 5-FU. As previously reported, TQ was found to be non-cytotoxic to normal human astrocytes, can selectively inhibit cancer cells, and readily passes through the blood-brain barrier. The microtubule-targeting ability of TQ was also demonstrated, and it was found to have a dose-dependent effect on the α and β-tubulin degradation of glioblastoma cells [39].

Cell death signaling pathways, namely apoptosis and necroptosis, are triggered by the activation of signaling pathways when exposed to elevated levels of ROS. It has been reported that TQ stimulates the accumulation of ROS, which eventually enhances cytotoxicity, and genotoxicity and results in apoptosis induction in glioma cells [2]. In our study, it was demonstrated that combination 2 with 33.0 μM TQ and 10.0 μM 5-FU has the lowest CI value (0.310) and the highest ROS production percentage. This concludes that the generation of intracellular ROS was enhanced upon combinational treatment with TQ and 5-FU, which affected the viability of the GBM cells and reduced cell proliferation which indicates good anticancer activity *in vitro*. However, additional analysis is warranted to comprehensively elucidate the intricate mechanisms underlying the cell death pathways subsequent to treatment with TQ and 5-FU in GBM.

As shown in Table 3, the combination index values of various drug combinations, either with TQ or with 5-FU, from recent literature studies were represented in increasing order on different types of cell lines. CI values of these two combinations were previously determined as 0.414 and 0.310 as given in Table 2. Even with these two combinations, CI values were quite low compared to recent studies. This proves that the combination of TQ and 5-FU has almost equal or sometimes even better synergistic anticancer activity than previous drug combination strategies.

Table 3: Combination of agents with different concentrations of TQ and 5-FU toward different cell lines.

Combination of agents with concentration	CI	Cell line	References
33 μM TQ – 10 μM 5-FU	0.31	Glioblastoma multiforme cells (U87MG)	This study
50 μM quercetin – 25 μM 5-FU	0.33	Breast cancer (MDA-MB-231)	[31]
3.4 μM 2-pyridin-4-yl methylene β-boswellis acid (PMBA) – 29.4 μM 5-FU	0.35	Colorectal carcinoma cells (HCT-116)^{G13D}	[32]
50 μM temozolomide – 21 μM TQ	0.37	Glioblastoma multiforme cells (U87MG).	[20]
60 μM TQ – 16.6 μM 5-FU	0.41	Glioblastoma multiforme cells (U87MG)	This study
4.4 μM 2-pyridin-4-yl methylene β-boswellis acid (PMBA) – 156 μM 5-FU	0.43	Colorectal carcinoma cells (SW-620)^{G12V}	[32]
72.98 μM kaempferol – 706 μM 5-FU	0.6	Colon cancer cells (LS175-R)	[33]
425 μM piperine – 80 μM TQ	0.788	Mouse breast cancer cells (EMT6/P cells)	[34]

In the present study, preclinical trails have been evaluated to select specific synergistic concentrations of TQ and 5-FU. However, upon direct administration of the synergistic dosages to multicellular organisms, the levels of TQ/5-FU decreases as they will be used and dosages of TQ/5-FU may reach to antagonistic levels. Therefore, further studies are required to design a controlled release mechanism such as polymeric systems. Smart polymeric systems may be used as controlled drug delivery system and this will keep the levels of drugs at required synergistic dosages prior to clinical studies.

Conclusions

In summary, the present combination of TQ and 5-FU can be used as a novel strategy to improve the anticancer activity of the agents by decreasing the concentration of 5-FU requirements, which in turn reduces the systemic cytotoxic effect of 5-FU in noncancerous cells and tissues. Combination index values were determined as 0.41 and 0.31 for TQ and 5-FU against GBM cells, respectively, which were classified as strong synergistic. In addition, the combinational strategy enhanced ROS production, which resulted in higher cytotoxicity and lower cell proliferation capacity at lower concentrations in the GBM cells. This study showed that the combination of TQ and 5-FU can be used as a potential strategy to improve the anticancer activity of 5-FU without increasing the dosage during drug development for GBM treatment. However, additional comprehensive studies which are focusing on molecular pathways and using models such as co-cultured different cells and *in vivo* models are essential to enhance the clinical applicability and translational relevance of the findings obtained in our study.

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