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ANTIPROLIFERATIVE EFFECT OF QUERCETIN AND RITA (P53 ACTIVATING SMALL MOLECULE) IN MULTIPLE CANCER CELL LINES

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Abstract

Naturally occurring compounds are considered the most interesting agents to test for cancer prevention and therapy, due to their anticipated multimodal actions and limited toxicity, these compounds could be developed further as a potential anti-cancer agent, both in conventional and combination therapy.

To overcome challenges like slow response with small molecules, combination therapy with natural compounds like plant flavonoid quercetin and synthetic anti-cancer drug RITA could be considered effective for cancer therapy.

The current study tested synergistic antiproliferative activity/anticancer activity of natural compound quercetin and p53 activator by cell viability assays in COLO-320 (colon) NCI-H23 (lung) and HeLa (cervical). The results revealed that the combination has enhanced antiproliferative activity than the individual drugs in multiple cancer cell lines. Among the tested cell lines, NCI H23 cell lines showed antiproliferative effect with the drug combination.

KEYWORDS: *Quercetin, p53 activator, combination therapy*, *RITA- Reactivating p53 and inducing tumor apoptosis*

Introduction

After cardiovascular diseases, cancer is among the absolute leading causes of mortality and morbidity globally; therefore, in this aggressive disease, uncontrolled growth & dispersal of transformed cells are key characteristics. More than 70% of anticancer agents are either natural products or their derivatives. One of the most outstanding needs in cancer research is to develop or identify compounds that have high toxic effects on transformed (cancer) cells, but little toxicity for the normal cells (1,3). Over the past few years plant flavonoids have been recognized to be excellent sources of anticancer compounds, with enormous therapeutic potential for cancer treatment (1,2).

The role of quercetin as an anticancer agent has been studied and found effective over different cancer cells such as lung (4)(9), cervical (5)(8) colorectal carcinoma (7)(10), and prostate cell lines.

The small molecule reactivating p53 and inducing tumor apoptosis (RITA) is described as an activator of wild-type and reactivator of mutant p53 function, resulting in elevated levels of p53 protein, cell growth arrest, and cell death. Additionally, it has been shown that RITA can induce DNA damage signaling. It also showed enhanced anti proliferative activity when tested in combination with of 5FU and oxaliplatin by enhancing DNA damage signaling pathways (12).

Combination treatment with plant-derived compounds can also help against resistance to chemotherapy drugs, so new putative targets or their combination with existing anticancer drugs must be developed for enhanced treatment and to overcome side effects and drug resistance.

Materials and methods

Cell lines were procured from ATCC Dulbecco's modified Eagle's (DME) Medium, RPMI-1640, antibiotic solutions-Penicillin, Streptomycin, Amphotericin B, Trypsin-EDTA, Phosphate buffer saline (PBS, pH 7.4) from Sigma-Aldrich, India, Fetal bovine serum purchased from Gibco, USA. Human cell lines HeLa were cultured in DME medium. COLO-320 and NCI-H23 were cultured in RPMI-1640 culture media.
Que (117-39-5) was purchased from Sigma, and RITA (213261-59-7) was purchased from Merck Millipore. Que and RITA stock solutions (1 millimolar) were prepared in the solvent DMSO and stored at -20°C until use. All the other chemicals were procured from Sigma, USA.

MTT reagent preparation

MTT dye (3-(4,5-Dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide-Sigma) powder was weighed and dissolved in DPBS (5mg/ml-stock) sterilized by filtration with 0.22µm syringe filter (Millipore) and stored at -20 ºC freezer. Working stock (1mg/ml) was prepared with suitable medium stored in the dark at 4ºC until use. Working stock was prepared freshly and used on the same day.

Cell viability (MTT) assay

The cell viability or cytotoxic activity of quercetin and/or RITA in cultured Human cell lines COLO-320, HeLa and NCI-H23 cells was determined using MTT cell viability assay.

MTT has the advantage of being easily processed in a 96-well plate without a washing step. Briefly, the yellow dye MTT is converted to insoluble formazan crystals by mitochondrial dehydrogenases in the mitochondria of living cells. Mitochondrial dehydrogenases cleave the tetrazolium ring of MTT dye, producing insoluble dark purple formazan crystals. These free crystals are dissolved in an organic solvent and the purple-colored formazan solution released, is measured using a spectrophotometer at a wavelength of 570 nm. Since MTT reduction occurs naturally in living cells, it is considered an activity that measures cell viability.

Cells were seeded in 96-well plates (Thermo Fisher, USA) at a density of ten thousand cells per well in 100 µl of culture medium and incubated at 37°C with 5% CO_2 in an incubator. The next day, the cells were treated with Que or RITA at different concentrations (1-250 µM) and incubated for an additional 48 hrs. at 37ºC with 5% CO_2. After treatment, cell viability was determined by an MTT cell viability assay. Briefly, cells were incubated with 100 µg of MTT solution (Sigma, USA) for 4 hrs. the formazon crystals formed were dissolved using DMSO, and the absorbance at 570 nm was measured with a microplate reader (Synergy 4-BioTEK, USA)

The experiment was repeated at least three times. Percent cell viability was calculated by the formula: OD of Test/OD of Controlx100. 0.1% DMSO was used as solvent control and Considered cell viability as 100%.

Using GraphPad Prism statistical software, the IC50 values (half-maximal inhibitory concentration) were nonlinearly regressed and fit to a sigmoidal dose-response curve (log of compound concentration versus normalized response).

Combination effect of Quercetin and RITA on cell viability

The combination effect of quercetin and RITA was evaluated in COLO-320, HeLa, and NCI-H23 human cancer cell lines.

Cells were seeded in 96-well plates (Thermo Fisher, USA) at a density of ten thousand cells per well in 100 µl of culture medium and incubated at 37°C with 5% CO_2 in an incubator. The next day, the cells were treated various possible combinations of quercetin and RITA based on the IC50 values and incubated at 37ºC with 5% CO_2 for 48h. Each concentration or combination of drugs was performed in triplicate. Inhibition of cell proliferation or cell viability was calculated as described previously using MTT assay.

Analysis of drug combination effects (synergistic/additive) on cell using CompuSyn software

The effects of the combination of quercetin and RITA on cell viability in cultured cancer cells were further analyzed to conclude drug interactions for synergistic or additive effects based on the value of the combination index (CI), calculated using CompuSyn software. In theory, the combination of drugs exhibited more antiproliferative effect than the sum of the individual effects of the drugs is called "synergistic/additive effect".

Results

MTT cell viability assay

Anti-proliferative or cytotoxic activity of quercetin and RITA in cultured COLO-320, NCI H23 and HeLa cells was determined by MTT cell viability assay. The maximum cell death was observed at 48 h for all the doses studied. The observed anti-proliferative activities were 63% at 75µM of quercetin and 41% at 50µM of RITA in COLO-320 (Fig. 1) 78% at 100µM of quercetin and 64% at 25µM of RITA in NCI-H23 (Fig. 2) and 65% at 100µM of quercetin and 40% at 75µM of RITA in HeLa (Fig. 3) at 48 h. Complete cell death was observed at greater test concentrations.

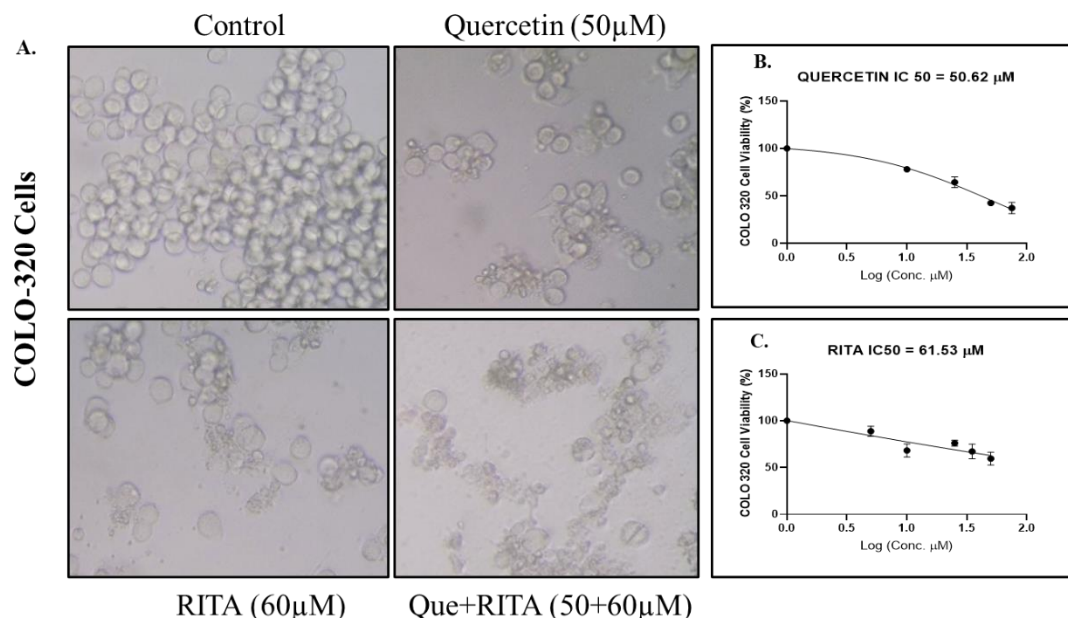


Fig 1: Cell viability assay results. **A.** Cells were treated with 50 µM Que, 60 µM RITA and their combination for 48 hrs. and photographed under an Olympus microscope. Scale bar, 100 µm. **B.** Dose-dependent curve for the IC50 calculation of Que. **C.** Dose-dependent curve for the IC50

calculation of RITA. The data are expressed as the mean ± SD (n=3). Post-treatment cells were incubated for 48 hrs. and the cell viability was determined by MTT cell viability assay. Que and RITA exhibited anti-proliferative activity directly proportional to dose in the tested COLO-320 cells compared to that of the control. DMSO (0.1%) was used as a control.

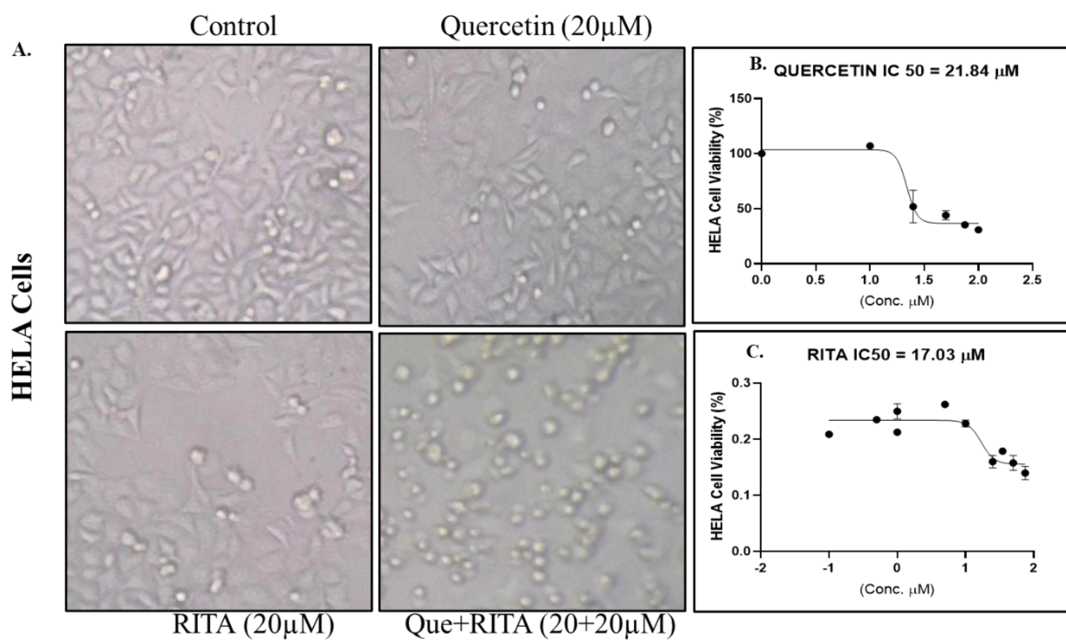


Fig 2: Cell viability assay results. **A.** Cells were treated with 20 μM Que, 20 μM RITA and their combination for 48 hrs. and photographed under an Olympus microscope. Scale bar, 100 μm. **B.** Dose-dependent curve for the IC50 calculation of Que. **C.** Dose-dependent curve for the IC50 calculation of RITA. The data are expressed as the mean ± SD (n=3). Post-treatment cells were incubated for 48 hrs. and the cell viability was determined by MTT cell viability assay. Que and RITA exhibited anti-proliferative activity directly proportional to dose in the tested HeLa cells compared to that of the control. DMSO (0.1%) was used as a control.

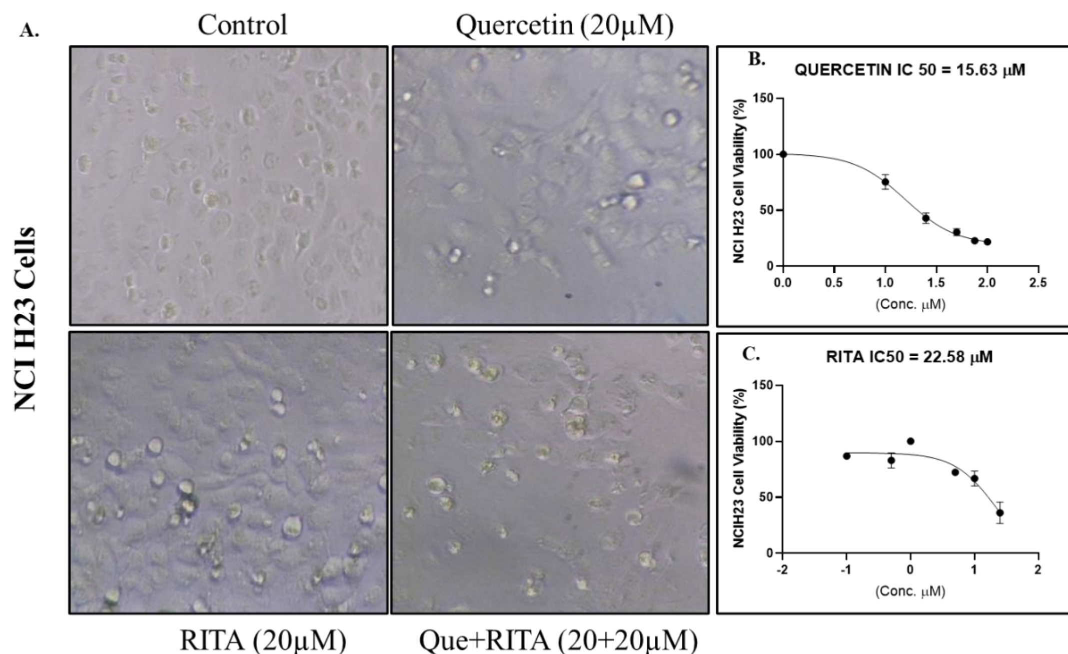


Fig 3: Cell viability assay results. **A.** Cells were treated with 20 µM Que, 20 µM RITA and their combination for 48 hrs. and photographed under an Olympus microscope. Scale bar, 100 µm. **B.** Dose-dependent curve for the IC50 calculation of Que. **C.** Dose-dependent curve for the IC50 calculation of RITA. The data are expressed as the mean ± SD (n=3). Post-treatment cells were incubated for 48 hrs. and the cell viability was determined by MTT cell viability assay. Que and RITA exhibited anti-proliferative activity directly proportional to dose in the tested NCI-H23 cells compared to that of the control. DMSO (0.1%) was used as a control.

Under the experimental conditions, the calculated 50% anti-proliferative activity (IC50 value) of quercetin and RITA 50.62 and 61.53 µM in COLO-320, 21.84 and 17.03 µM in HeLa and 15.63 and 22.58 µM in NCI-H23 cells respectively (Table 1).

Table 1: IC50 values for anti-proliferative activity

Cell line	Quercetin (µM)	RITA (µM)
COLO-320 (colon)	50.62	61.53
HeLa (Cervical)	21.84	17.03
NCI-H23 (Lung)	15.63	22.58

Effect of quercetin and RITA combination on antiproliferative activity

The combination effects of quercetin and p53 activator (RITA) were tested at several combinations by treating the quercetin and p53 activator at the same time for 48 hrs. Inhibition of cell proliferation was calculated as described previously using the MTT assay. These results strongly indicate that combination treatment is more effective than either of the drugs alone (Fig. 4).

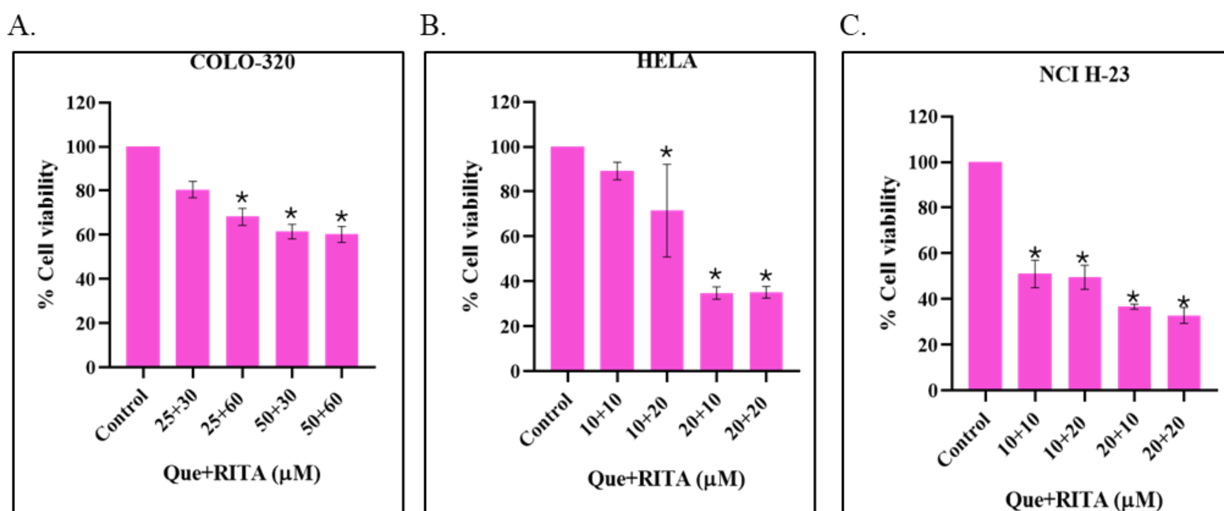


Fig 4: Combination effect of quercetin and RITA on cell viability. **A.** COLO-320 **B.** HELA **C.** NCI H-23 cells. The cell viability of untreated control was set at 100% to calculate the % cell viability of drug treated groups. Data were expressed as mean ± SD (n=3). *$p < 0.05$

Analysis of combination effect for Additive/Synergistic effect using CompuSyn software

Drug combination effects for quercetin and RITA were analyzed using CompuSyn software program using CI values. CI value <1 is Synergistic effect, ≤ 1.0 is Additive and >1 is Antagonistic effects. The observed CI Value for antiproliferative activity against different combinations of quercetin and RITA in HCT-116, COLO-320, HELA and NCI-H23 cells were presented in table 2, Fig. 5.

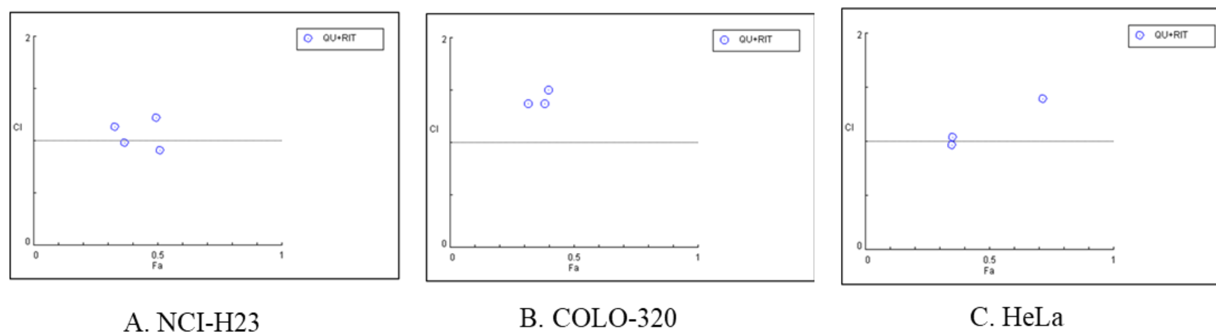
Table 2: Summary of combination assay results on antiproliferative activities in COLO-320, HELA and NCI-H23 cells and CI values

Cell line	Combination QUE+RITA (μM)	CI Value for antiproliferative activity

COLO-320	25+30	2.08
	25+60	1.37
	50+30	1.37
	50+60	1.50
NCI-H23	10+10	0.91
	10+20	1.22
	20+10	0.98
	20+20	1.13
HeLa	10+10	2.17
	10+20	1.39
	20+10	0.96
	20+20	1.04

Synergistic/Additive combination CI values were highlighted in the table.

Quercetin+RITA combination effect (CI values) using CompuSyn software - CI-Fa Plot:



A. NCI-H23 B. COLO-320 C. HeLa

Fig 5: A. NCI-H23; Quercetin and RITA combinations at 10+10 and 20+10 µM are closely synergistic. **B.** COLO-320; Synergistic drug interaction was not observed in all the combination of drugs. **C.** HeLa: Quercetin and RITA combinations at 20+10 µM were found to be closely synergistic.

Based on the above results Quercetin and RITA combinations 10+10 and 20+10 µM showed additive effect in NCI-H23 and 20+10 µM showed additive effect HeLa cells.

Discussion

Quercetin has the potential as a chemosensitizer and immune modulator to enhance the antitumor efficacy in various cancers in combination with other therapeutic compounds (3,4,12). Quercetin has shown satisfactory antitumor efficiency by inducing apoptosis, arresting the cell cycle, reducing angiogenesis and metastasis, reversing drug/radiotherapy resistance, and modulation tumor immunity. Quercetin can act as aurora B (highly expressed in several cancer cells) (9,10,11). When tested for cytotoxicity against A549/Taxol cells, quercetin significantly reversed the Paclitaxel resistance (by inhibiting Akt and ERK phosphorylation and membrane potential (MMP) depolarization (3,4). When combined with cisplatin, a commonly used chemotherapeutic drug, quercetin showed a synergistic effect in inhibiting cell proliferation. The proteins known to be involved in cancer cell growth, migration, invasion, and drug resistance are downregulated by quercetin (5,6). Quercetin and aconitine act synergistically to induce HeLa (human cervical carcinoma) cell apoptosis by inhibiting the proliferation of the MDR1 gene and the P-glycoprotein (P-gp) transporter.) (8).

The molecular mechanisms involved in the responses to small molecules remain enigmatic. Several groups reported the induction of a p53-dependent DNA damage response and a recent study showed synergistic induction of apoptosis by a p53 activator in combination with small molecules like Nutlin-3 (15,17,19).

In a study, small molecule (RITA)-induced DNA damage signaling, increased the antiproliferative response to 5FU and Oxaliplatin when tested with CRC cells (12). Another study has demonstrated that disruption of Mdm2-p53 binding activates p53, induces apoptosis, and sensitizes lung cancer cells to chemotherapy (13,14,16,18).

From the above data, it was clear that quercetin has additive/synergistic effect with other anticancer drugs. In the current study, we demonstrated that the antiproliferative effect of the combination of selected drugs is more effective than the individual drugs.

Conclusion

In conclusion, Combination therapy of either herbal medicine or chemotherapeutic agents enhances tumor suppression due to the multimodal action and could be developed further as a

potential anti-cancer agent, in cancer therapy, to sensitize the cells to chemotherapy and to overcome drug resistance.

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Disclosure statement

No potential conflicts of interest were reported by the authors.

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