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Research Article

Studies on Fixed Dose Combination of Ibrutinib and Quercetin Self-Nanoemulsifying Drug Delivery Systems in Human Cancer Cell Lines

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ABSTRACT

Ibrutinib (IB), irreversibly inhibits Bruton's tyrosine kinase which plays a crucial role in the tumor microenvironment and quercetin (QC) has shown apoptosis induction, angiogenesis inhibition, and anti-proliferative action against several human carcinoma cells. The self-nano-emulsifying drug delivery system (SNEDDS) is suitable for loading insolubilized oil-based compounds such as ibrutinib and quercetin. In the current study IB with QC was formulated into SNEDDS and cytotoxicity was determined by using human malignant melanoma (A-375) and human lung adenocarcinoma (A549) cell lines. The optimized loaded formula consisted of castor oil, Kolliphor® RH 40, and PEG-600. The optimized formulation was evaluated for physical parameters and the results were satisfactory. For cytotoxicity studies MTT assay was conducted for these combinations, IC_{50} values were calculated for the tested compound. In A-549 adenocarcinoma cell line, the calculated IC_{50} values (µM) for the test compounds T1 (pure IB ± QC) and T2 (IB ± QC SNEDDS) were 70.34 ± 0.8 and 85.46 ± 0.93 µM at 24 hours study, respectively. In A-375 cancer cell line, IC_{50} values for the compounds T1 and T2 were 59.52 ± 0.87 and 88.43 ± 1.03 µM for 24 hours study, respectively. It was observed that the IC_{50} of IB-QC loaded SNEDDS was higher than pure drug combination and these enter the cells by active transport and induce cytotoxicity to the cells. The overall results from the studies suggest that IB-QC-loaded SNEDD provided synergistic effects, which could play a significant role in the percentage of cell death.

Introduction

In recent years, not only has cancer been recognized as one of the major causes of death worldwide, but its incidence and mortality rate have grown rapidly.[1] The reasons behind that are complex and multifactorial. Still, they reflect the growth and aging of the worldwide population, as well as the increase in the prevalence and distribution of several cancer risk factors.[2] Although, currently, a plethora of studies researching new treatment methods are being conducted, we should also consider other possibilities for repurposing already established medications. As the most widely adopted approach in cancer therapy, chemotherapy is subject to many *in-vitro* and *in-vivo* barriers, such as tumor microenvironment and multidrug resistance (MDR). In particular, during the chemotherapy processes, chronic damage to cells elicits the secretion of damage response program molecules to promote the survival and growth of neighboring cells, thus causing acquired MDR to the chemotherapies.[3]

Combination chemotherapy for cancer therapy is considered an important protocol to enhance therapeutic effects and reduce systemic toxicity by simultaneously modulating multiple cell-signaling pathways. In recent years, the combination of chemotherapeutic drugs *via* nanocarriers has emerged as a promising strategy for treating cancer.[4] These co-delivery systems can address the issues of poor solubility and stability associated with such drugs, transport simultaneously both drugs to the target site, release the payloads in a controlled manner and

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accurate dose, synchronize the drug exposure, maximize the therapeutic efficacy, and reduce the toxicity. Several drug delivery platforms have been explored for the co-delivery of various combinations of drugs, and their efficacy has been tested both *in-vitro* and *in-vivo.*[5]

Co-administration of an antioxidant, having anti-proliferative and antioxidant properties could be of great interest for augmenting overall antitumor efficacy and reducing the toxicity of anticancer drugs. The drug combination we are interested in this study is ibrutinib (IB) with quercetin (QC).[6]

IB, also known as PCI-32765, is a first-of-its-kind agent, irreversibly inhibiting Bruton's tyrosine kinase (BTK).[7] BTK has been found to play a crucial role in the tumor microenvironment, complex and meticulous network of many types of cells and their precursors, such as pericytes, smooth muscle cells, fibroblasts of various phenotypes, myofibroblasts, neutrophils, eosinophils, basophils, mast cells, T-cells, B-cells, natural killer (NK) lymphocytes, as well as antigen-presenting cells such as macrophages and dendritic cells. All these cells take part in the pathophysiology of cancer.[8] These observations consequently make BTK a potential target in the treatment of solid tumors. Furthermore, IB is not entirely selective towards BTK, it has been discovered that over ten other kinases are inhibited by this drug, including those commonly associated with several solid tumors. Taking advantage of the aforementioned aspects of BTK biology and IB's non-selectiveness, several studies have been conducted focusing on indication characteristics other than hematological malignancies.[9]

QC is a polyphenolic flavonoid compound that has shown different promising biological activities, including apoptosis induction, angiogenesis inhibition and anti-proliferative action against several human carcinoma cells. Besides, QC can competitively inhibit the members of MDR family, such as P-glycoprotein (P-gp), MRP1, and BCRP, which are responsible for the recognition and efflux of chemical drugs.[10] Since the efficacy of QC is limited by hydrophobicity, instability in physiological media, poor gastrointestinal absorption, and extensive xenobiotic metabolism in the intestines and liver, the formulation in a suitable delivery system may improve their oral bioavailability, ensuring its protection from degradation and prevent premature release.[11] Further, the co-delivery may be exploited to gain either additive or synergistic effects, with the final goal to maximize the therapeutic efficacy.[12]

Cell lines are *in-vitro* model systems that are widely used in different fields of medical research, especially basic cancer research and drug discovery. Their usefulness is primarily linked to their ability to provide an indefinite source of biological material for experimental purposes. Under the right conditions and with appropriate controls, authenticated cancer cell lines retain most of the genetic properties of the cancer of origin. Human cancer cell lines continue to play a critical role in modern cancer research. Indeed, they are widely used as preclinical model systems for gaining mechanistic and therapeutic insight.[13] Notably, with the advent of -omics technologies, recent studies have provided comprehensive databases dedicated to the characterization of most existing cell lines.[14] Furthermore, the online availability of the information that was derived from these studies created an important resource for the study of cancer cell lines and facilitated researchers in selecting the most appropriate *in-vitro* model system for their research projects.[15]

In-vitro, cell viability and cytotoxicity assays with cultured cells are widely used for cytotoxicity tests of chemicals and for drug screening. Application of these assays has been of increasing interest over recent years. Currently, these assays are also used in oncological research to evaluate both compound toxicity and tumor cell growth inhibition during drug development. It is important to know how many viable cells are remaining and/or how many cells are dead at the end of the experiment. A broad spectrum of cytotoxicity and cell viability assays is currently used in the fields of toxicology and pharmacology. This analysis would support the researchers in understanding the efficiency of the prepared formulations and provide a basis for further studies.

IB with QC was formulated into a self-nanoemulsifying drug delivery system (SNEDDS) and cytotoxicity was determined by using human malignant melanoma (A-375) and human lung adenocarcinoma (A549) cell lines.

Material and Methods

Materials

Human malignant melanoma (A-375) and Human lung adenocarcinoma (A549) were acquired from American Type Culture Collection and were sub-cultured in-house. IB with QC were procured by Hetero Drugs Pvt Ltd, Hyderabad, India. Kolliphor® RH 40 was procured from MSN Labs, Hyderabad, India. Castor oil and PEG were obtained from SD Fine Chemical Ltd., Mumbai. Dulbecco's Modified Eagles medium, trypisn EDTA, and MTT cell viability assay kit were from Sigma Aldrich, New Delhi, India.

Preparation of IB and QC SNEDDS

The initial screening was done and castor oil (oil phase), Kolliphor® RH 40 (surfactant) and PEG-600 (co-surfactant) were selected from the solubility study. The formulations were further optimized using a pseudo-ternary phase diagram in which an area of emulsification was identified.[16] SNEDDS formulation was prepared by mixing the components in optimized concentrations by stirring, vortex mixing and heating at 37°C on a magnetic stirrer. Drug-loaded SNEDDS formulation was prepared by dissolving specified quantities of both drugs in the mixture of Castor oil, Kolliphor® RH 40 and PEG-600.

The components were mixed by stirring, vortex mixing and heating at 37°C on a magnetic stirrer until both the drugs were dissolved completely.[17] The SNEDDS formulations were characterized and evaluated for their physico-chemical properties like drop size, zeta potential, thermodynamic stability, drugexcipient compatibility, surface morphology, *invitro* drug release, and stability. [18] The results were published in International Journal of Applied Pharmaceutics.

In-vitro Cell Line Studies

Morphological assay

- *Cell culture*

The cancer cell line used for the study were human malignant melanoma (A-375) and human lung adenocarcinoma (A549), which were sub-cultured in-house, in CSIR-Centre for Cellular and Molecular Biology (CCMB), Hyderabad. The original source of the cell line is American Type Culture Collection (ATCC). Cells were grown in 75 cm^2 bottle canted necked vented flasks (Corning) with Dulbecco's Modified Eagle Medium (DMEM) and the cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Cells (passages 30–50) were grown in DMEM (Gibco Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% penicillin (1000 U/mL), 1% streptomycin (1000 µg/mL) and 1% amphotericin (250 U/mL).[19] The cells were passaged enzymatically with 0.25% trypsin- 1 mM Ethylenediamine tetraacetic acid (EDTA) and sub-cultured on 75 cm^2 plastic flasks at 2.2x104 cells/cm2 density. The culture medium was replaced every 2 days. Cell confluence (80%) was confirmed by microscopic observance. Experiments were performed 24 hours post-seeding to prevent cell differentiation.[20] All the molecules used were 95 to 97% pure and were gauged by HPLC and verified by mass spectrometry.

- *Sub culturing protocol*

Volumes used in this protocol are for a 75 cm^2 flask from which the culture medium was discarded. The cell layer was briefly rinsed with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contained trypsin inhibitor. 2.0 to 3.0 mL of trypsin-EDTA solution was added to the flask and cells were observed under an inverted microscope until the cell layer was dispersed (usually within 5–15 minutes). A complete growth medium was added and cells were aspirated by gently pipetting. Appropriate aliquots of the cell suspension were added to new culture vessels and cultures were established between 2 x 10^3 and 1 x 10^4 viable cells/cm^2. The prepared cultures were incubated at 37°C.

- *Screening of test formulations against morphology of A-375 and A-549*

A-375 and A-549 cell were treated with 100 µM concentration of test formulations which were characterized using fourier transform infrared (FTIR), differential scanning calorimetry (DSC) and X-ray diffraction (XRD) spectral techniques prior to use for morphological study. Cells were observed for 24, 48, 72 hours, after treatment of test molecules. Images were taken by Axiovert 200 M phase contrast microscope at the magnification of 10X. Axiovision Rel.4.2 software was used to acquire the images. The test molecules in IB-QC–SNEDDS formulation have shown a better cytotoxic effect on both A-375 & A549 cells in time-dependent manner at a concentration of approximately 100 µM and whereas simple combinations at below 72 hours have not showed significant morphological changes against A375 and A549 cells.[21]

- *Determination of cytotoxic concentration of test formulations against A-375 and A-549 using MTT assay*

The MTT cell viability assay kit provides a convenient, sensitive, quantitative and reliable assay for determining the number of viable cells in a given culture. This homogeneous colorimetric assay is based on the conversion of a tetrazolium salt MTT, a pale-yellow substrate, to formazan, a purple dye. This cellular reduction reaction involves the pyridine nucleotide cofactors NADH/NADPH and is only catalyzed by living cells. The formazan product has a low aqueous solubility and is present as purple crystals. Dissolving the resulting formazan with a solubilization buffer permits the convenient quantification of product formation. The intensity of the product color, measured at 550 to 620 nm, is directly proportional to the number of living cells in the culture. Reagents in the kit have been carefully formulated and optimized for sensitivity, assay robustness and automation.[22]

Assay Principle

The study of cell proliferation and cell viability requires the accurate quantification of the number of viable cells in a cell culture. Therefore, assays for calculating cell viability are necessary to optimize cell culture conditions, evaluate cell growth factors and nutrients, discover novel antibiotics and anticancer drugs, evaluate toxic effects of environmental pollutants and cell-mediated toxicity, and study programmed cell death (apoptosis).[23]

MTT cell proliferation assay provides a colorimetric format for measuring and monitoring cell proliferation. The kit contains sufficient reagents for the evaluation of 960 assays in 96-well plates or 192 assays in 24-well plates. Cells can be plated and treated with compounds or agents affecting proliferation.[24] Cells are then detected with the proliferation reagent, which is converted in live cells from the yellow tetrazole MTT to the purple formazan form by a cellular reductase. An increased signal accompanies an increase in cell proliferation, while a decrease in cell proliferation (and signal) can indicate the toxic effects of compounds or suboptimal culture conditions. The

basic assay principles can be applied to most eukaryotic cell lines, including adherent and non-adherent cells and certain tissues. This cell proliferation reagent can detect proliferation in bacteria, yeast, fungi, protozoa, and cultured mammalian and piscine cells.[25]

Determination of cytotoxic Concentration of TI and T2 against A-375 and A-549 using MTT Assay

Assay protocol

A549 and A-375 cells (100 µL per well) were plated and cultured in clear bottom 96-well tissue culture plates. The number of cells can vary from 1,000 to 80,000 per well and the volume can vary from 50 to 150 µL, although 100 µL is used in this experiment. The test compounds and control were added and incubated for a 48 hours period of time.[24] To the above 15 µL (per 100 µL cell culture) of reagent per well was added and incubated for 4 hours at 37°C. The volume of the reagent has been adjusted depending on the volume of the cell culture. The solubilizer (100 µL) was added and mixed gently on an orbital shaker for one hour at room temperature. The volume of the solubilizer has been adjusted depending on the volume of the cell culture. The absorbance was measured at OD 570 nm for each well on an absorbance plate reader. Maximum absorbance of the formazan dye lies between 560 and 590 nm.[25]

Percent proliferation inhibition was calculated using the formula

$$\text{Viability cell inhibition (\%)} = 100 - \frac{At - Ab}{Ac - Ab} \times 100$$

At = Absorbance of the test compound,
Ab = Absorbance of the blank,
Ac = absorption of control.
IC_{50} values were calculated by analyzing the relationship between concentrations and percent inhibitions using the GraphPad Prism 7 version 7.00 for Windows, GraphPad Software.

Results and Discussion

Evaluation of IB and QC SNEDDS

The combined dosage form of IB-QC SNEDDS formulation was successfully developed with increased drug solubilization and enhanced dissolution rate. The formulation variables were optimized by response surface methodology. The optimized loaded formula consisted of 56.32% castor oil, 31.32% Kolliphor® RH 40, and 15.91% PEG-600, forming an aqueous thermodynamically stable nanoemulsion. The optimized SNEDDS formulations showed a particle size range of 71.12 to 76.38 nm, polydispersity index of 0.126 to 0.312, zeta potential of 24.6 to 28.4, and encapsulation efficiencies of 88.98 to 90.22% and 84.96 to 86.78% for IB and QC, respectively. According to *in-vitro* testing, the medication released from SNEDDS was released more quickly (> 90% 600 minutes). The formulation was further evaluated using FTIR, XRD, DSC, SEM, and stability investigations, which validated the complexation of IB and QC in the drug's amorphous state and stability for six months.[16,18]

Screening of Test Formulations against Morphology of A-375 and A-549

The following test formulations were analyzed at 24, 48 and 72 hours against the morphology of A-375 and A-549 and the results were presented in Figs. 1 to 2 (T1 [IB ± QC pure drug]; T2 [IB ± QC-optimized SNEDDs]),

Determination of Cytotoxic Concentration of Test Formulations against A-375 and A-549 using MTT Assay

Cytotoxic concentrations of T1 (QC ± IB) and T2 (QC ± IB-SNEDDs) against A-375 and A-549 using MTT Assay were determined and the results were graphically presented in Figs 3-6.

The inhibitory effect of the pure drug and drug-loaded SNEDD formulations on A-375 and A549 cells was examined by MTT assay. The cytotoxic effect in terms of IC_{50} (the

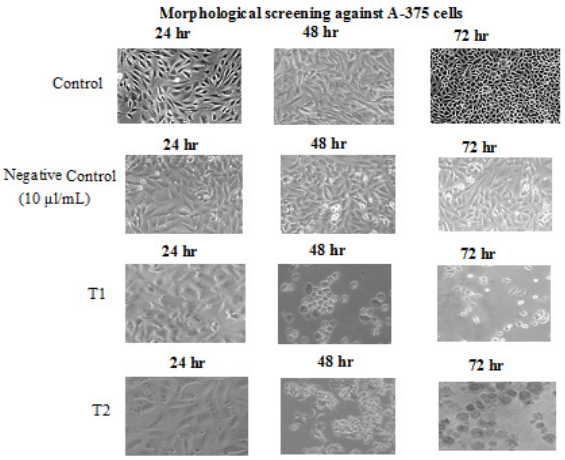

Fig. 1: Morphological screening against A-375 cells

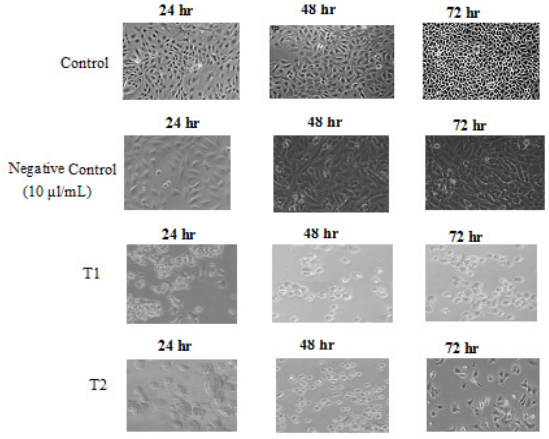

Fig. 2: Morphological screening against A-549 cells

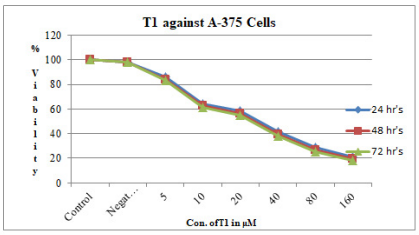

Fig. 3: Cytotoxic concentration of T1 against A-375 cells

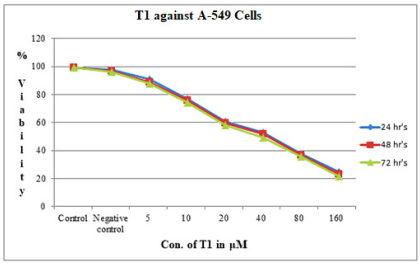

Fig. 4: Cytotoxic concentration of T1 against A-549 cells

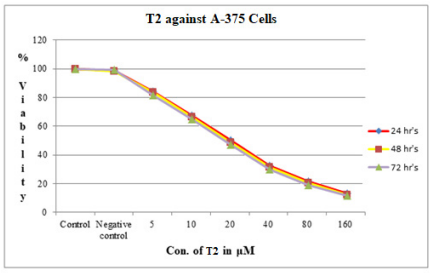

Fig. 5: Cytotoxic concentration of T2 against A-375 cells

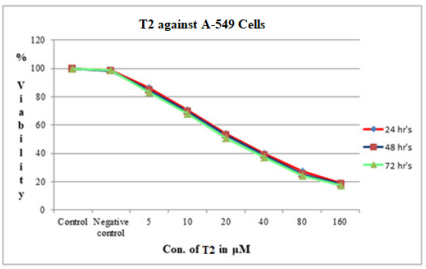

Fig. 6: Cytotoxic concentration of T2 against A-549 cells

concentration that kills 50% of cells) was calculated using the GraphPad Prism 7 version 7.00 for Windows, GraphPad Software. The result of the screening of T1 (QC ± IB) and T2 (QC ± IB-SNEDDs) against morphology of A-375 and A-549 shows that QC and IB combinations of pure drug and also the SNEDDs have shown significant cytotoxic effects on both A-375 and A-549 cells in a time-dependent manner.[20] The compound's pure drug combinations moderately inhibit the proliferation of A-549 cells and therefore exhibited moderate activity against A-549 cells. The SNEDDs combinations were also found effective in A-375 cells as the morphological figures show that there is a prominent inhibition of the A-375 cancer cell proliferation.[21]

Morphological studies clearly show the moderate inhibition of A-549 cell proliferation due to the effect of T1 and T2 compounds.[23] After morphological study, the results of the cytotoxic activity of the tested compounds using MTT assay confirmed that the compounds T1 and T2 exhibited satisfactory cytotoxic activity in A-549 lung adenocarcinoma cancer cells. These results are due to the advanced drug delivery system.[24]

MTT assay was conducted for these combinations, IC_{50} values were calculated for the tested compound.[25] In A-549 adenocarcinoma cell line, the calculated IC_{50} values (µM) for the test compounds T1 and T2 were 70.34 ± 0.8 µM and 85.46 ± 0.93 µM at 24 hours study, respectively. This study was further extended to 48 and 72 hours. In A-375 cancer cell line, IC_{50} values for the compounds T1 and T2 were 59.52 ± 0.87 µM and 88.43 ± 1.03 µM for 24 hours study, respectively.

It was observed that the IC_{50} of IB-QC loaded SNEDDS was higher than pure drug combination. Although IB-QC have a lower IC_{50} value to kill A-375 and A-549 cells compared to IB-QC loaded SNEDDS, IB-QC combination may still not be suitable for further treatment *in-vivo* due to the solubility and bioavailability limitation. It is believed that free IB-QC combination is easily available and enters the cells immediately via passive diffusion causing it to be more toxic to the cancer cells, IB-QC that was encapsulated in the SNEDDS nanocarrier system, to increase the solubility and bioavailability must first be internalized by active transport or *via* endocytosis in order for it to enter the cells and induce cytotoxicity to the cells.[26] Previous studies justified that the incorporation of drug active compound into nanoparticle or a nanocarrier system did not affect or increase the efficiency of the drug on inhibiting cancer cells to proliferate.[27] Further, the effect and function of drug compound in a nanocarrier is more effective and can be observed better *in-vivo* as the purpose of encapsulation to make the drug compound soluble and enhance the oral bioavailability might not be seen *in-vitro*.[28] The research that broadcasted similar results includes; SNEDDS formulation of α-tocopherol (α-TOH), resveratrol (RES), and coenzyme Q10 (CoQ10) which showed potential in improving the prophylactic activity of antioxidants.[29] QC-loaded SNEDDS exhibited significantly higher therapeutic anticancer efficacy.[30] QC-SEDDS showed a significantly higher *in-vivo* antioxidant potential compared to free QT when evaluated as a function of ability to combat doxorubicin- and cyclosporin A-induced cardiotoxicity and nephrotoxicity, respectively.[31] The overall results from the studies suggest that IB-QC loaded SNEDD provided synergistic effects, which could play a significant role in the percentage cell death.

Conclusion

The combined dosage form of IB-QC SNEDDS formulation was successfully developed and was optimized. The optimized loaded formula consisted. *In-vitro* release studies of optimized formulation showed that optimized

formula had faster release than that of pure drugs, confirming the efficiency of SNEDDS for improving the solubility and dissolution rate of poorly water-soluble drugs (IB/QC) combination. Further the cytotoxicity was confirmed by cell line studies using A-375 and A-549 cancer cell lines. The inhibitory effect of the pure drug and drug-loaded SNEDD formulations on A-375 and A-549 cells was examined by MTT assay. It was observed that the IC_{50} of IB-QC loaded SNEDDS was higher than pure drug combination. IB-QC was encapsulated in the SNEDDS nanocarrier system, to increase the solubility and bioavailability must first be internalized by active transport for it to enter the cells and induce cytotoxicity to the cells. In conclusion, our results show that combination therapy of ibrutinib and quercetin loaded SNEDDS significantly reduced the cell viability, and altered the cellular morphology of A-375 and A-549 cells.

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