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Original Article

PREPARATION, CHARACTERIZATION, AND *IN VITRO* ANTI CANCER ACTIVITY EVALUATION OF IRINOTECAN AND SUNITINIB-LOADED NANOBUBBLES

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ABSTRACT

Objective: The objective of the present research is to develop, analyze, and evaluate the cytotoxic effects of nanobubbles loaded with irinotecan and sunitinib for colorectal cancer therapy.

Methods: Drug-loaded dextran sulfate nanobubbles were formulated using the emulsification technique and the prepared nanobubbles were evaluated for qualitative and quantitative parameters. Cell viability MTT assay was performed to evaluate the irinotecan and sunitinib-loaded nanobubbles for cytotoxicity or ability to inhibit cell proliferation spectrophotometrically as a function of mitochondrial activity in living CR4 and A-549(irinotecan), CR4 and A-498 (sunitinib) cell lines.

Results: The irinotecan and sunitinib-loaded nanobubbles were successfully prepared, with all qualitative and quantitative parameters within the acceptable range. The optimized nanobubbles demonstrated excellent physicochemical properties, with particle sizes of 177.8±5.2 nm for sunitinib and 89.8±9.5 nm for irinotecan. *In vitro* drug release studies showed significantly enhanced release profiles, with ultrasound-triggered drug release reaching 99% for both drugs, compared to only 39% for plain sunitinib and 35% for plain irinotecan. This highlights the ability of nanobubbles to enable controlled and targeted drug delivery, potentially improving therapeutic precision. The *in vitro* anticancer activity results revealed IC50 values of 70.41 and 73.26 µg/ml for irinotecan against CR4 and A-549 cell lines, respectively, and 84.34 and 60.08 µg/ml for sunitinib against CR4 and A-498 cell lines, respectively, demonstrating strong cytotoxic effects.

Conclusion: The nanobubble-based delivery system enhances drug bioavailability, cellular uptake, and tumor penetration, thereby improving cytotoxic efficacy compared to conventional drug formulations. These findings underscore the potential of ultrasound-responsive nanobubbles as a promising strategy for targeted colorectal cancer therapy, potentially leading to improved treatment outcomes with reduced systemic toxicity.

Keywords: Nanobubbles, Irinotecan, Sunitinib, Toxicity, Cell lines, Cancer

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INTRODUCTION

Colorectal Cancer (CRC) is the third most frequent disease worldwide and the major cause of cancer deaths, with 400,000 new cases annually [1]. Colorectal Liver Metastases (CRLM), one of the most common distant metastases, affect 50-60% of CRC patients [2]. CRC is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths worldwide. According to the World Health Organization (WHO), CRC accounted for approximately 1.9 million new cases and 935,000 deaths in 2022, with incidence rates steadily increasing due to aging populations, lifestyle factors, and genetic predispositions [1]. Current treatment strategies for CRC, including surgery, chemotherapy, targeted therapy, and immunotherapy, have improved patient survival. However, these approaches are often limited by systemic toxicity, poor drug bioavailability, and the development of drug resistance, necessitating the development of more efficient and targeted drug delivery systems [2]. Tumor cells interact with immune cells through cytokines, growth factors, and proteases to remodel the tumor microenvironment, aiding in escape, circulation survival, and metastasis. Only 10–15% of patients qualify for surgical removal of metastatic lesions, the most effective treatment. However, 50% of liver metastasis resection cases experience recurrence. Most unresectable CRLM cases pose a clinical challenge, highlighting the need for safer, more effective treatments [3].

Irinotecan (IRO), commonly known as CPT-11, is FDA-approved for colon cancer therapy. Anti-tumour effects come from topoisomerase-1 inhibition [4]. While it is more prevalent in cancer tissue than healthy tissue, its non-specific action kills healthy cells also. It is usually infused intravenously and metabolized by the liver and bile ducts.

Sunitinib, a novel inhibitor targeting the vascular endothelial growth factor receptor, has shown remarkable effectiveness in the treatment of renal cell carcinoma (RCC) and is currently extensively utilized for patients with advanced-stage cancer. It has been recently authorized for the management of advanced renal cell carcinoma and gastrointestinal stromal tumors following disease progression or intolerance to imatinib mesylate treatment [5].

Some of the following methods struggle to penetrate deep tissue and distribute drugs effectively despite advances. To fully explore dog's therapeutic potential in treating numerous illnesses, research and innovation must address these constraints.

Nanotechnology-based drug delivery enhances anticancer therapy by improving drug solubility, stability, and targeting. While nanoparticles like liposomes and polymeric carriers offer benefits, issues such as premature release, poor tumor penetration, and off-target effects limit their efficacy [6]. Nanobubbles present a superior alternative with a gaseous core and lipid or polymer shell, enabling ultrasound-triggered release and enhanced tumor penetration. Their external activation via ultrasound improves drug localization, reduces toxicity, and enables real-time imaging-guided therapy, making them a promising platform for targeted cancer treatment [7].

Beyond improving solubility, drugs must target diseased cells while sparing healthy tissues. This approach enhances blood concentration pharmacokinetics and reduces side effects. Nanobubbles are investigated in this study as an innovative cancer drug delivery method [6].

Nanobubbles are small cavities filled with gas in an aqueous solution, with a size range less than 1 µm. Bubbles are spherical particles with a gas-filled core and a shell that make them easily recognizable due to their dynamic features [7]. The core base may be charged with various gases such as air, carbon dioxide, sulfur hexafluoride, fluorocarbon, and sulfur dioxide [8]. The stability of nanobubbles is influenced by certain properties, such as the

thickness and flexibility of their shell [9]. The purpose of developing these nanobubbles, which are smaller than a micron, is to enhance the drug's biodistribution specifically at the targeted sick site, while also improving its stability and bioavailability [10].

Nanoparticles migrate to tumors, coalescing into microbubbles [11, 12]. Intense ultrasound deforms these microbubbles, triggering targeted drug release with higher efficacy and lower toxicity [13]. Further studies are needed to assess their effectiveness across cancer types [14]. Nanobubbles show promise for delivering irinotecan and sunitinib *in vitro* and *in vivo*. Cells exposed to nanobubbles undergo shape changes, altered proliferation, and increased cell death [15], necessitating viability monitoring in pharmacology. Mitochondrial activity changes can be detected using MTT and resazurin assays [16]. This study evaluates the *in vitro* cytotoxicity of irinotecan and sunitinib-loaded nanobubbles. This study hypothesizes that irinotecan and sunitinib-loaded nanobubbles will exhibit enhanced drug release, improved cellular uptake, and superior anticancer activity compared to conventional drug formulations. Specifically, the research aims to evaluate the physicochemical properties, drug release kinetics, and *in vitro* anticancer efficacy of these nanobubble-based formulations against colorectal cancer cell lines. By demonstrating their potential advantages, this study seeks to establish nanobubbles as a viable strategy for improving therapeutic outcomes in CRC treatment.

MATERIALS AND METHODS

Materials

Irinotecan is a pure drug given by Hetero Laboratories, a private limited company in Hyderabad. Sunitinib is a pure drug given by Dr. Reddy's Laboratories, a private limited company in Hyderabad. Degussa (Hamburg, Germany) generously provided us with soybean lecithin (Epikuron 200®). C3F8 (perfluoropropane) was procured from pharm affiliates Pvt ltd, Haryana, India. Sigma Aldrich, US, supplied Dextran sulfate (DEX) with an intrinsic viscosity of 0.22 dl/g and Mw 25,000 and palmitic acid. All other solvents were purchased from Qualigens, India.

Fetal Bovine Serum [#RM10432] and D-PBS [#TL1006], DMEM [#AL007A], EMEM [#AL047S] were from HiMedia. MTT Reagent [# M5655] and DMSO [#PHR1309] were from Sigma. 96-well plate for culturing cells was from Corning, USA. The cell lines were obtained from the National Center for Cell Sciences, Pune, India, and grown on Eagle's minimum essential medium.

Methods

Preparation of drug-loaded nanobubbles

Drug-loaded nanobubbles were developed using Perfluoropentane (PFP) as the inner core and dextran sulfate as the outer shell through a multi-step synthesis process. To prepare empty nanobubbles, a pre-emulsion was formed by mixing ethanol containing Epikuron® (1% w/v) and palmitic acid (0.5% w/v) with PFP, followed by homogenization (5600 rpm) with ultrapure water using an Ultra-Turrax homogenizer. Dextran sulfate (2.0% w/v) was added gradually under continuous stirring for 3 h to stabilize the formulation. For drug-loaded nanobubbles, a similar pre-emulsion was prepared using an ethanolic lipid solution containing 9.8% w/v of the drug, homogenized (100 rpm), heated to 37 °C, and stabilized by the dropwise addition of dextran sulfate. These drug-loaded nanobubbles were purified by dialysis to remove unbound molecules and subsequently freeze-dried for 36 h before storage. Three independent variables—dextran concentration (w/v), drug concentration (w/v), and homogenization speed (rpm)—were tested at low (-1), middle (0), and high (1) levels to study their effects. The response variables included particle size (Y1), polydispersity index (Y2), zeta potential (Y3), and encapsulation efficiency (Y4), which were analyzed to evaluate the formulations [8].

Characterization and evaluation of drug-loaded nanobubbles

The characterization of the synthesized nanobubbles involved several analytical techniques to ensure their quality and functionality. Particle size, polydispersity index (PDI), and zeta potential (ZP) were measured using dynamic light scattering (DLS), a method that provides insights into the size distribution, stability, and surface charge of the nanobubbles. Encapsulation efficiency (EE%) was determined using UV-visible spectrophotometry, which quantified the amount of drug successfully encapsulated within the nanobubbles.

The morphology of the plain nanobubbles, as well as irinotecan and sunitinib-loaded nanobubbles, was visualized using scanning electron microscopy (SEM). This technique revealed detailed surface characteristics and structural integrity of the nanobubbles at the nanoscale level. Drug-excipient compatibility was evaluated using Fourier-transform infrared (FTIR) spectroscopy and differential scanning calorimetry (DSC). FTIR identified potential chemical interactions by analyzing changes in characteristic functional groups, while DSC provided thermal profiles to detect any modifications in the physical state or stability of the formulations.

In vitro drug release studies were performed using the dialysis bag technique, which mimics controlled drug release under physiological conditions. The nanobubbles were placed inside dialysis bags and submerged in a release medium, with drug release quantified over time. These comprehensive analyses provided valuable information about the structural, physicochemical, and functional properties of the nanobubbles, ensuring their suitability for further applications.

In vitro cytotoxic study

Cell viability MTT assay was performed to evaluate the irinotecan and sunitinib loaded nanobubbles for its cytotoxicity or ability to inhibit cell proliferation spectrophotometrically as a function of mitochondrial activity in living CR4 and A-549 (irinotecan), CR4 and A-498 (sunitinib) cell lines The selection of the CR4, A-549, and A-498 cell lines for cytotoxic studies was based on their relevance to colorectal and other cancer types. CR4 cells are commonly used as a model for colorectal cancer, while A-549 and A-498 cells represent non-small cell lung cancer and renal cancer, respectively. These cell lines were chosen to assess the broader anticancer potential of the irinotecan and sunitinib loaded nanobubbles across different tumor types [15].

Preparing cell line

A vial of each cell lines was taken out from liquid nitrogen storage and thawed rapidly to room temperature [16]. The contents in the vials were added to 9 ml of complete medium and centrifuged at 125 g for 5 min. After centrifugation, the supernatant was discarded and pellet was mixed with 10 ml of complete medium and suspended in a T-25 flask and incubated at 37 °C with 5% CO_2. When the cell confluence reached ~80%, the cells were centrifuged at 125 g for 5 min; pellet was mixed with 15 ml of complete medium and transferred to two T-75 flasks. When the cell confluence reached around 80-90%, cells in the flask were used for the assay [17, 18].

MTT assay

200 µl cell suspension (in complete culture medium with 10% FBS) was seeded in a 96-well plate (20,000 cells per well), without the test agent and allowed to grow for 24 h. After 24 h of incubation, spent media in the wells of 96-well plate were replaced with appropriate concentrations of the irinotecan and sunitinib loaded nanobubbles and incubated for 48 h at 37 °C in a 5% CO_2 atmosphere [19]. After the incubation period, the plates were removed from incubator; spent media was removed followed by addition of MTT reagent to a final concentration of 0.5 mg/ml (0.2 µm filter sterilized). The plates were wrapped with aluminium foil to avoid exposure to light, and placed in the incubator for 3 h. After incubation, MTT reagent was removed and 100 µl of dimethylsulfoxide was added. Absorbance was measured on spectrophotometer (Tecan™ Infinite 200Pro) at 570 nm [20].

Data analysis

The percent viability of cells in the untreated (negative control) group was set to 100% and the % viability of cells in the treated groups was estimated relative to the negative control. The % viability was plotted against the concentration and evaluated for

dose response. Based on the dose-response relationships, an appropriate model was fit to estimate the I_{max} and IC_{50}.

Percentage viability was calculated using the following formula:

$$\% Viability = \frac{100\ X\ OD570e}{OD570b}$$

Where,

OD570e is the mean value of the measured Optical Density of the dilutions of test item;

OD570b is the mean value of the measured Optical Density of the negative control

For data analysis, statistical tests, such as one-way ANOVA, will be applied to compare the cytotoxic effects between treated and control groups. P-values<0.05 will be considered statistically significant to ensure the reliability of the results. Additionally, vehicle controls (cells treated with the solvent used for drug loading) and blank nanobubbles (without any drug) will be included in all cytotoxicity assays to account for any non-specific effects of the nanobubble carrier system. This will help to ensure that any observed cytotoxicity is due to the drug delivery mechanism rather than the nanobubbles themselves.

RESULTS AND DISCUSSION

Preparation of drug-loaded nanobubbles

In recent years, significant efforts have been directed toward developing novel drug delivery systems utilizing nanobubbles to address challenges associated with conventional drug formulations. These systems aim to minimize toxicity, enhance stability and specificity, improve bioavailability, and achieve sustained release of hydrophilic and hydrophobic drugs. Nanobubbles have emerged as promising carriers owing to their unique properties, including their ability to encapsulate therapeutic agents within their core or shell, protect drugs from degradation, and target delivery to specific sites, thereby reducing off-target effects.

The preparation of nanobubbles is highly dependent on the physicochemical properties of both the polymer used for the outer shell and the drug to be encapsulated. Factors such as solubility, molecular weight, hydrophilicity, and compatibility between the drug and polymer play a crucial role in determining the choice of the preparation technique. Among various methods available, the emulsification technique has been extensively used for its versatility and efficacy in forming stable nanobubbles.

For the formation of irinotecan and sunitinib loaded nanobubbles, the emulsification method was employed due to its suitability for drugs with distinct solubility profiles. This technique involves creating a pre-emulsion by combining lipids or surfactants with the drug and a volatile core-forming agent, such as PFP. High-shear homogenization is used to reduce droplet size, ensuring uniformity and stability. The gradual addition of a stabilizing polymer, such as dextran sulfate, under controlled conditions, helps form a robust shell around the core, encapsulating the drug within.

This method ensures precise control over nanobubble size, surface charge, and encapsulation efficiency, making it ideal for tailoring drug delivery systems for specific applications. The incorporation of irinotecan, a hydrophilic drug, and sunitinib, a hydrophobic drug, demonstrates the adaptability of the emulsification approach in handling diverse drug chemistries. By optimizing parameters such as homogenization speed, polymer concentration, and temperature, researchers have successfully developed nanobubbles capable of enhancing the therapeutic efficacy of these drugs while minimizing adverse effects [8, 11].

Characterization and evaluation of drug-loaded nanobubbles

The optimised sunitinib-loaded nanobubbles showed good physicochemical features and results in *in vitro* and *in vivo* experiments, indicating their promise as an enhanced drug delivery method. The PS of these nanobubbles was 177.8±5.2 nm, enabling efficient cellular absorption and circulatory circulation. The zeta potential of-21.1±0.43 mV indicates high colloidal stability, inhibiting particle aggregation by electrostatic repulsion. The PDI of 0.262±0.089 indicates a restricted size dispersion, indicating nanobubble homogeneity. EE% of 69.12±1.41% indicates successful sunitinib integration into nanobubbles. The drug loading of 26.29±4.01% ensures adequate therapeutic dosage while maintaining nanobubble structure. *In vitro* release tests showed that nanobubbles can release drugs under ultrasonic stimulation (99%) better than simple drugs (39%). This allows site-specific medication delivery and reduces systemic toxicity. FTIR and DSC were used to investigate drug-polymer interactions. Encapsulation protected the drug's chemical and thermal stability without polymer interaction, as shown by the absence of new FTIR peaks or shifts and constant DSC temperature profiles. SEM pictures showed consistently spherical and nanosized particles, ensuring biological system predictability. Particle size, zeta potential, and encapsulation efficiency did not change after 30 days of stability testing, showing excellent formulation stability under storage circumstances. Pharmacokinetic studies showed increased therapeutic potential, with Cmax rising to 4.52 and AUC0–t rising to 5.27. Sunitinib administered via nanobubbles appears to have increased solubility, absorption, and half-life. These characteristics show that optimised sunitinib-loaded nanobubbles can increase therapeutic efficacy, dose frequency, and clinical outcomes.

The optimized NBs exhibited a particle size of 89.8±9.5 nm, zeta potential of-18.6±1.2 mV, and PDI of 0.284±0.108. They achieved an entrapment efficiency of 72.86±1.54% and drug loading of 33.57±2.10%. *In vitro* studies showed a superior drug release of 99% with ultrasound compared to 35% for the plain drug. FTIR and DSC analyses confirmed no drug-polymer interaction.

In vitro cytotoxic study

The *in vitro* cytotoxicity studies give us a good idea about advantages and disadvantages of the model for screening and mechanistic analysis of potentially toxic compounds. A wide array of morphologic and biochemical markers is available for obtaining information at the cellular and molecular levels to detect chemical-induced disruption. Among the methods, *in vitro* cell line models are quite easy to perform in laboratory conditions. Cellular screening in cancer research mainly consists of human tumor cell lines. It is the most suitable system in terms of management and reproducibility [20]. Different concentrations of irinotecan and sunitinib and irinotecan and sunitinib loaded nanobubbles were tested for toxicity via cell viability on CR4 and A-549 (irinotecan; table 1), CR4 and A-498 (sunitinib; table 2) cell lines by MTT assay and calculated IC_{50} (table 3 and fig. 1 and fig. 2).

Table 1: Cytotoxicity of irinotecan and irinotecan-loaded nanobubbles on CR4 and A-549 cell lines

Concentrations (µg/ml)	% mean viability			
	CR4		A-549	
	Irinotecan pure drug	Irinotecan nanobubbles	Irinotecan pure drug	Irinotecan nanobubbles
Blank	-	-	-	-
Vehicle control	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
5.00	95.24±2.84	97.24±3.85	96.35±3.24	98.62±1.54
10.00	86.27±1.64	89.63±2.95	89.36±2.64	88.36±2.58
20.00	71.24±3.65	75.95±2.45	71.26±2.78	76.64±3.74
30.00	62.37±2.78	62.64±2.64	63.54±1.64	65.35±3.24
50.00	52.39±2.94	52.31±1.26	52.14±2.94	51.26±2.64
100.00	36.41±1.24	41.26±1.47	36.15±1.24	43.21±2.48

All the values were expressed in (n=3) Mean±SD

Table 2: Cytotoxicity of sunitinib and sunitinib-loaded nanobubbles on CR4 and A-498 cell lines

Concentrations (µg/ml)	% mean viability			
	CR4		A-498	
	Sunitinib pure drug	Sunitinib nanobubbles	Sunitinib pure drug	Sunitinib nanobubbles
Blank	-	-	-	-
Vehicle Control	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
5.00	96.35±2.52	98.56±2.58	95.42±2.78	97.25±3.65
10.00	85.65±1.95	92.35±1.28	85.15±2.95	85.24±2.84
20.00	72.36±1.28	85.63±2.52	76.14±3.54	74.24±3.64
30.00	61.26±1.51	76.35±1.98	52.94±1.54	54.26±1.85
50.00	54.26±1.24	62.41±1.95	41.24±2.64	41.26±2.67
100.00	21.51±1.26	45.26±1.26	21.85±2.48	36.24±1.39

All the values were expressed in (n=3) Mean±SD

Table 3: IC50 value of irinotecan and sunitinib and irinotecan and sunitinib loaded nanobubbles

Compound	IC50 value (µg/ml)		
	CR4 cell line	A 498-cell line	A-549 cell line
Irinotecan nanobubbles	70.41±5.84	-	73.26±7.33
Irinotecan pure drug	65.94±7.21	-	66.15±8.89
Sunitinib nanobubbles	84.34±3.94	60.08±5.02	-
Sunitinib pure drug	56.71±2.66	51.98±9.65	-

All the values were expressed in (n=3) Mean±SD

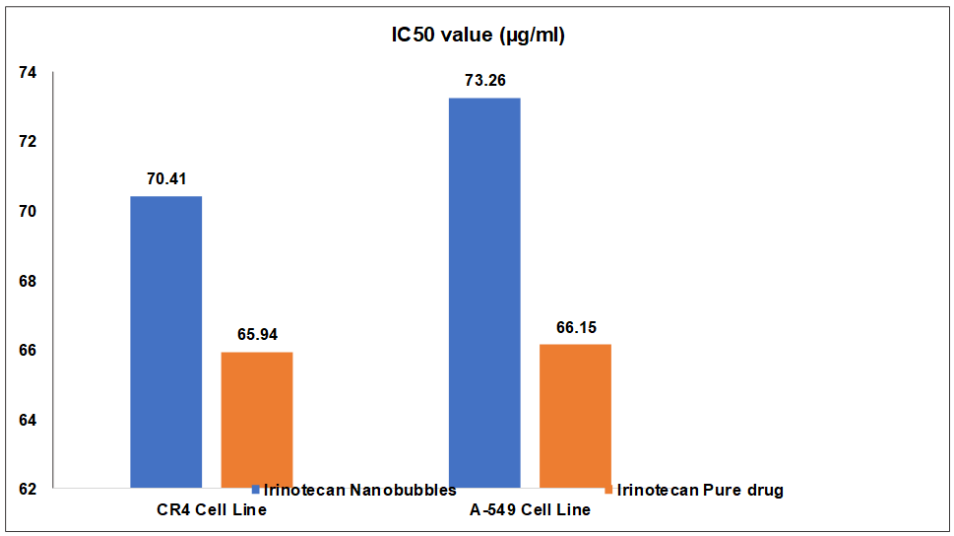

Fig. 1: IC50 value of irinotecan and irinotecan loaded nanobubbles

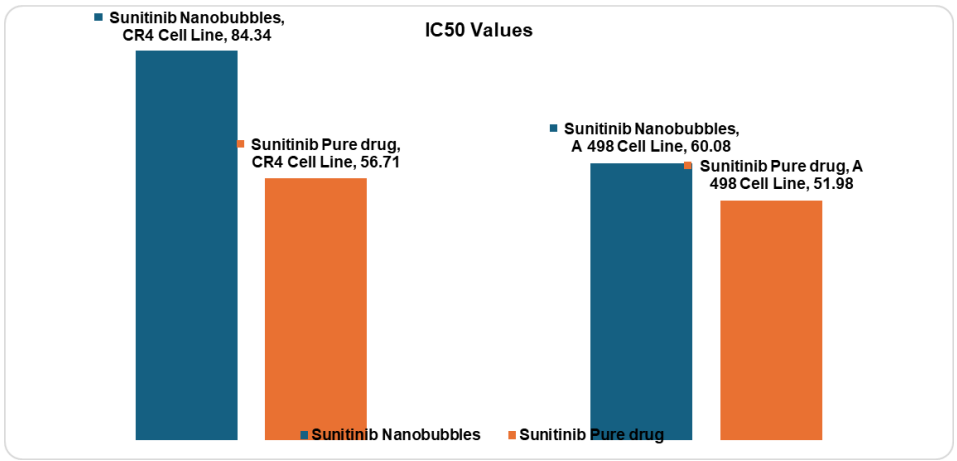

Fig. 2: IC50 value of sunitinib and sunitinib-loaded nanobubbles

After treatment with different concentrations of irinotecan and sunitinib and irinotecan and sunitinib loaded nanobubbles, the morphology of cell lines was visualized (fig. 3 and fig. 4). Marked deformations and alterations were observed on the cell surface, which could be attributed to the impact of tested nanobubbles [20]. While pure drugs showed less effect on cell morphology. The increment of irinotecan and sunitinib-loaded nanobubbles concentration was accompanied to the increment of cell morphology and apoptotic alteration. Mechanisms of irinotecan and sunitinib-loaded nanobubbles antitumor activity are associated with membrane-disrupting and apoptosis-inducing actions. When compared to conventional delivery systems, such as liposomes or polymeric nanoparticles, the nanobubble formulations demonstrated superior ultrasound-triggered release profiles, which may contribute to increased drug concentration at the tumor site. This targeted release could enhance the therapeutic efficacy while minimizing off-target effects, as seen in the improved cytotoxicity of nanobubble formulations in the CR4 and A-549 cell lines compared to pure drug treatments [21]. The observed cytotoxicity of irinotecan and sunitinib nanobubbles can be attributed to several mechanisms. First, the ultrasound-triggered release of the drugs from nanobubbles enhances drug uptake by tumor cells, leading to increased intracellular drug concentration [20]. This can promote apoptosis through mechanisms such as DNA damage, mitochondrial dysfunction, and activation of caspase pathways. The non-invasive nature of ultrasound further allows for precise spatial control, potentially leading to more effective tumor cell kill with minimal systemic toxicity [22]. The slightly higher IC50 values observed for nanobubbles compared to pure drugs could be attributed to differences in drug release kinetics and cellular uptake mechanisms. Unlike free drugs, which are readily available for immediate cellular uptake, nanobubbles encapsulate the drug and may exhibit a delayed or controlled release profile [11]. This could lead to a slower accumulation of the active drug at the target site, thereby increasing the IC50. Additionally, the uptake of nanobubbles by cells might be influenced by factors such as endocytosis pathways, vesicle degradation rates, and intracellular trafficking, which differ from the passive diffusion mechanism often seen with free drugs [9]. Studies have shown that nanocarriers, including nanobubbles, can exhibit a delayed intracellular release profile due to their structural properties, affecting the overall cytotoxic response [10].

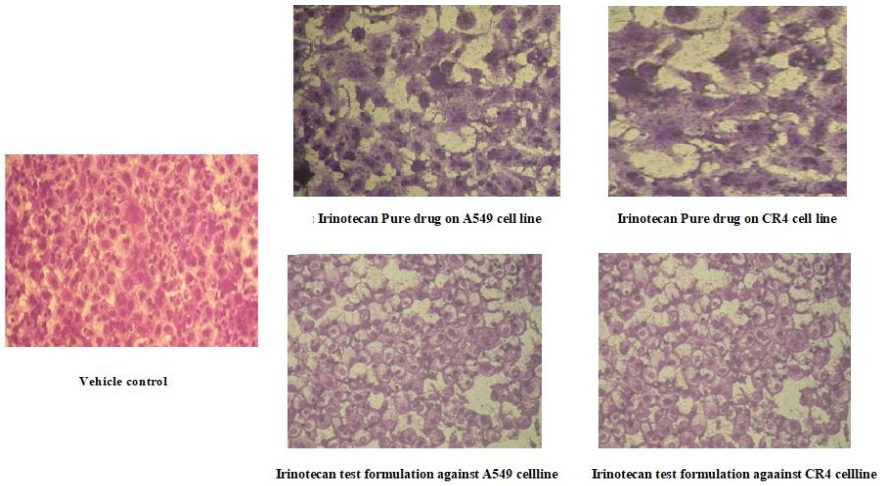

Fig. 3: Morphological changes of cancer cells when treated with irinotecan and irinotecan nanobubbles at 1000x

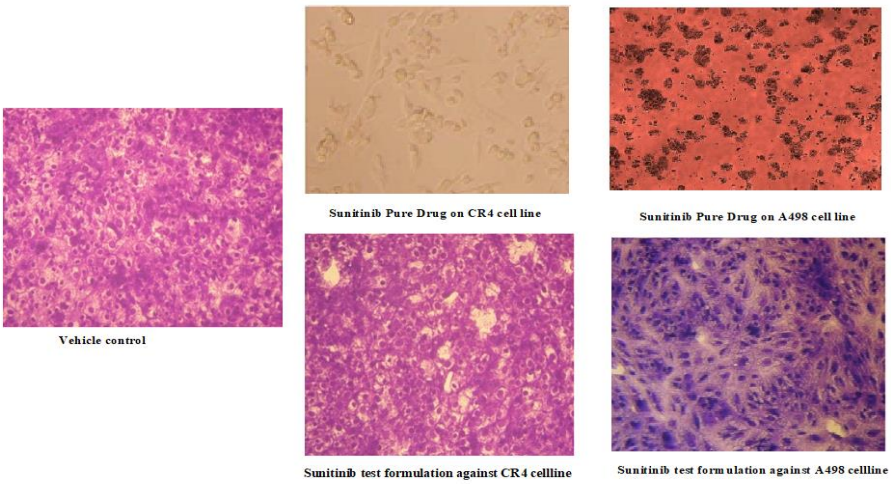

Fig. 4: Morphological changes of cancer cells when treated with sunitinib and sunitinib nanobubbles at 1000x

MTT assay is a colorimetric assay based on assessing the cell metabolic activity. CR4, A-498, and A-549 cell lines were used to see the cytotoxic potential of airinotecan and sunitinib for initial screening of apoptosis or necrosis. The biochemical mechanism behind the MTT assay involves NAD (P) H-dependent cellular oxidoreductase enzyme that converts the yellow tetrazolium MTT [3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] into insoluble (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenyl formazan formazan. Nanobubbles exhibited dose-dependent activity in comparison to free drugs [22]. A higher concentration of nanobubbles may be delivered to the intracellular space due to small size of the bubble. Sustained release of the drug from inner polymer matrix of nanobubble formulation resulted pronounced cytotoxic effect. Better efficacy and no cellular cytotoxic effects were observed

for nanobubble formulations, this confirms the safe nature of the nanoformulation [23]. In this study, the nanobubbles formulation containing irinotecan and sunitinib safe substances showed no cytotoxicity in a safety test using the MTT assay. We confirmed that the cell viability (%) of the pretreated media with a fabricated formulation of nanobubbles were over 94% (<4% cell death at all concentrations) for irinotecan, 95% (<3% cell death at all concentrations) for sunitinib. This result indicates that the prepared irinotecan and sunitinib nanobubbles have no cytotoxic effects on the CR4, A-498, and A-549 cell lines.

In this study, we have demonstrated the enhanced cytotoxicity of irinotecan and sunitinib loaded nanobubbles *in vitro*, showcasing their potential as effective drug delivery systems for cancer therapy. When compared to traditional nanocarrier-based systems, the results suggest that nanobubbles offer superior tumor penetration and drug release upon ultrasound activation. Our nanobubble approach may overcome these limitations by providing a more controlled, ultrasound-triggered release mechanism, offering targeted delivery and reduced systemic toxicity [8]. While the cytotoxicity results suggest that nanobubbles are relatively safe, potential off-target effects in *in vivo* applications should be considered. Nanobubbles may interact with plasma proteins, immune cells, or non-target tissues, leading to unintended biological effects. For instance, complement system activation, macrophage uptake, or off-target accumulation in organs such as the liver and spleen could influence safety profiles [9]. Despite promising *in vitro* results, several limitations must be considered. The stability of nanobubbles under physiological conditions is a known challenge, as the gaseous core may be prone to rapid degradation or collapse in the bloodstream [11]. Additionally, scalability remains a critical issue. The multi-step synthesis process used in this study may be difficult to scale up for large-scale production without compromising the uniformity and quality of the nanobubbles [18].

To build on these findings, in vivo studies are essential to assess the pharmacokinetics, biodistribution, and safety of irinotecan and sunitinib-loaded nanobubbles. Animal models, such as xenograft or transgenic mouse models of CRC, will be used to evaluate the therapeutic efficacy of these formulations in a more complex, physiological environment. In addition, clinical translation will require rigorous testing of the ultrasound-triggered release mechanism in humans, with careful consideration of ultrasound parameters, nanobubble stability, and the optimal drug dose for clinical efficacy.

CONCLUSION

Nanoparticulated drug delivery systems, such as nanobubbles, represent a promising approach for targeted cancer therapy by enhancing drug solubility, stability, and tumor-specific accumulation. The optimized formulations of irinotecan and sunitinib-loaded nanobubbles demonstrated favorable physicochemical properties, including uniform size distribution and stable zeta potential, ensuring the potential for effective drug delivery. Cytotoxicity assays confirmed the enhanced therapeutic efficacy of these formulations, showing substantial cell line-specific toxicity. These findings highlight the clinical significance of irinotecan and sunitinib nanobubbles in improving cancer treatment outcomes.

Beyond these *in vitro* results, the use of nanobubbles could have a substantial impact on clinical outcomes by enabling precise, ultrasound-triggered drug release, thereby minimizing systemic toxicity and enhancing therapeutic efficacy. Moreover, the ability to customize drug delivery based on individual tumor characteristics holds great promise for personalized medicine, offering a more tailored and effective approach to cancer therapy. Future *in vivo* studies will be essential to validate these findings and explore the clinical translation of these innovative drug delivery systems.

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AUTHORS CONTRIBUTIONS

AP completed the research work, execution, and writing, whereas KSG did the work plan, review, and corrections. Both authors agree with the submission and publication. Both authors have read and agreed to the published version of the manuscript.

CONFLICTS OF INTERESTS

No conflict of interest

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