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## ORIGINAL ARTICLE

# Cytotoxic Evaluation of Entrectinib -Loaded Nanobubbles on Cell Lines

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**ABSTRACT**
*For the purpose of treating cancer, the purpose of this research is to develop, test, and investigate nanobubbles that are loaded with Entrectinib and the cytotoxic effects that they have. The nanobubbles that were manufactured and contained entrectinib were evaluated using both quantitative and qualitative criteria. These nanobubbles were created by employing a water-in-oil-in-water (W/O/W) double emulsion, solvent-diffusion-evaporation process. Through the use of spectrophotometric analysis of mitochondrial activity in A 549 cell lines, the researchers were able to ascertain the cytotoxicity of the nanobubbles as well as their capacity to restrict cell growth in the MTT experiment. The nanobubbles that were made and contained Entrectinib were successfully finished, and all of the qualitative and quantitative parameters that were assessed were found to be within the acceptable range. When the in vitro anticancer activity was examined on A 549 cell lines using the nanobubbles that were generated, the IC50 value obtained was 63.05 µg/ml, respectively. This value suggested that the nanobubbles had a significant anticancer effectiveness. With regard to the A 549 cell lines, this data demonstrates that the nanobubbles loaded with Entrectinib were successfully described and exhibit minimal levels of cytotoxicity.*
***Keywords****: Entrectinib, nanobubbles, necrosis, cell lines, cytotoxicity, $IC_{50}$*

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## INTRODUCTION

Entrectinib (ENT) is an innovative and powerful inhibitor targeting tyrosine receptor kinases (TRKA, TRKB, and TRKC), ROS1, and anaplastic lymphoma kinase (ALK) with oral administration capabilities[1]. It is delivered orally and represents a significant advancement in the healthcare industry. It was licensed as an emerging treatment in Japan in 2019, with the intention of catering to the needs of patients of all ages, including those who are dealing with advanced or solid tumors that are positive for the neurotrophic tropomyosin receptor kinase (NTRK) blend. These two disorders include solid tumors and non-small cell lung cancer (NSCLC), both of which have been authorized for management by Food & Drug Administration (FDA) & European Union (EU). In addition to this, it is helpful in the treatment of disease that has spread to the brain [2]. This group includes an extensive variety of cancers, counting but not restricted to sarcoma, thyroid cancer, pancreatic cancer, breast cancer, cancer of the salivary glands, colon cancer, and a number of other types of cancer [3]. It is recommended that adults take 600 milligrams of the medication, while youngsters should take 400 milligrams. The larger the dose of the medication, the greater the risk of experiencing serious side effects [4]. Interactions between different medications have also been linked to ENT infections [5]. It is possible to lessen the dosage of the medication by increasing its solubility via the use of formulation techniques. This would result in the alleviation of the mentioned adverse effects. In addition, it is of the utmost importance to lessen the first-pass effect and the pharmacokinetic variation that are associated with the existing capsule formulation [6].

Concavities in an aqueous solution that are capable of transporting gas and have a size series of <1μm got referred to as nanobubbles [7]. These are globular, globular particle that have a gas-containing core & shell, both of which contribute to the distinctive dynamic properties that bubbles possess. While center may be stimulating by different gases (air, $CO_2$, sulfurhexafluoride, perfluorocarbon, & sulfurdioxide), shell is predominantly made up of lipids, polymers, proteins, and surfactants. Polyelectrolyte multilayers are also a component of the shell. It is possible for the composition of a bubble's shell to have a considerable impact on the bubble's half-life. This is because the shell's composition controls the flow of gas from the shell core to the medium that surrounds it. Nanobubbles are characterized by a number of features that contribute to their overall stability [10]. These qualities include shell thickness and elasticity. These nanobubbles, which are at a size of less than one micron, are being manufactured in order to improve the biodistribution of the medicine at the sick site that is meant to be treated, as well as its bioavailability and stability [11]. They facilitate the extravasation of blood vessels into the tissues that are close to the blood vessels, which results in an improvement in both the efficiency and location of delivery [12].

At the location of a tumor, these nanoparticles tend to collect and aggregate, and it is there that they mix to form microbubbles [13]. As a consequence of the successful distortion of these microbubbles in the target location by powerful ultrasound, the release and accumulation of medication within the targeted cells are boosted, resulting in better effectiveness and lower toxicity [14]. This is because the microbubbles are distorted in a way that allows them to be more effective. It is necessary to do more study in order to determine whether or if these techniques are beneficial in the treatment of a wide variety of cancers. Nanobubbles have the potential to be used in both in vitro and in vivo settings for the delivery of anticancer therapies, such as Entrectinib [15].

Nanobubbles have the ability to affect the shape of cells, as well as the rate of cell development, cell mortality, and cell disintegration of cells that are exposed to them. As a consequence of this, it is of the utmost importance to carry out cell viability monitoring for any chemical that has the potential to demonstrate interest in the field of experimental pharmacology. Through the usage of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide), it is possible to see and track any changes that occur in the activity of mitochondria. An *in vitro* cell toxicity investigation is being conducted on nanobubbles that have been loaded with Entrectinib [16]. As part of the ongoing research project, the objective is to prepare, assess, & analyze cellular toxicity of nanobubbles having Entrectinib at the conduct of cancer. Another proven technique for increasing drug solubility, dissolving rate, and enhancing the bioavailability of medications with limited water solubility is the production of nano formulations (17). Targeted cancer therapies aim to minimize damage to healthy cells, offering a more precise and effective approach compared to conventional treatments like chemotherapy and radiotherapy. Advancements in cancer-targeted nanotechnologies benefit clinical diagnosis and treatment (18). NBs are advanced drug carriers with a gas-filled core and a protein, polymeric, or phospholipid coating. When exposed to high-pressure ultrasound, NBs undergo inertia cavitation, causing shock waves and microjets. This process has significant bioeffects, exerting mechanical impacts on nearby tissues or cells.

## MATERIAL AND METHODS

### Materials

Aelida Pharmaceuticals in Haryana, India was gifted Entrectinib. The PLGA 50:50 had a natural viscidness of 0.22dl per g &mw 25,000. It came from Sigma Aldrich in the US. From Sigma Aldrich in St. Louis, MO, USA, polyvinyl alcohol (PVA; MW 30,000–70,000) was bought. S.D. Fine Chemicals in Hyderabad was used to get the solvents. Hi Media was used to get D-PBS (#TL1006), Fetal Bovine Serum (#RM10432), DMEM (#AL007A), and EMEM (#AL047S). Sigma sold both the MTT Reagent (#M5655) and the DMSO (#PHR1309). Corning in the USA made the 96-well plate that was used to grow cells. Cell lines came from National Center for Cell Sciences in Pune, India, & were grownup on Eagle's essential media.

### Methods

#### Drug loaded nanobubbles preparation

Entrectinib was loaded to poly D, L-lactic-co-glycolic acid (PLGA) nanobubbles by a altered W/O/W emulsion, solventdiffusion and evaporation procedure. Original emulsion was created by sonicating PLGA with dichloromethane. Ultrasonifying polyvinyl alcohol resulted in a twofold emulsion. Drug-loaded bubbles produced got filtered & freeze-dried for additional examination [10].

#### Drug loaded nanobubbles evaluation

Morphology of plain &Entrectinib-loaded nanobubbles got studied using transmission electron microscopy. Fourier transformed infrared spectroscopy & differential scanning calorimetry got used to investigate drug-excipient compatibility. The release kinetics in vitro were studied using the dialysis bag

method. Nanobubble stability was assessed over a one-month period, and the haemolytic capability of drug-loaded nanobubbles was tested using human blood cell suspension.

***In-vitro* cytotoxic assessment**

The MTT test got used to analyze cytotoxicity and potential of Entrectinib-loaded nanobubbles to spectrophotometrically decrease cell growth in live A 549 cell lines, as well as mitochondrial function [16].

***Preparing cell lines***

All cell lines were taken out from liquid nitrogen packing& quickly brought to room heat in a vial. Contents of receptacles got mixed with 9mL of whole media& centrifuged at 125g; 5 min. Post centrifugation, supernatant got removed, & the particle got mixed by 10mL of whole media. The mixture was then suspended in T-25 flask & incubated at 37°C (5% $CO_2$). When cells stretched about 80% confluence, they got centrifuged at 125g; 5min. The particle got then mixed by 15 milliliters of whole media&relocated to T-75 flasks. Cells in flask were used for the experiment when their confluence reached 80-90% [18, 19].

MTT ASSAY

A 96-well plate was seeded with 20,000 cells per well and a 200µl suspension in whole culture media containing 10% FBS. The test agent not present, and the cells were cultured for 24hrs. Following 24hrs of cultivation, the exhausted medium in wells of a 96-well plate got replaced with Entrectinib formulation at the proper doses. The plate was then nurtured at 37°C; 5% CO2 environment for 48hrs. After incubation time, plates were removed from the incubator, together with the depleted medium. MTT got added at closing concentration of 0.5mg per ml (0.2 µm filter sterilized). To evadeexperience to light, plates got covered in aluminum foil & placed in incubator for three hours. Postcultivation, removed the MTT & added 100µl DMSO. The absorbance measured at 570 nm by a Tecan™ Infinite 200Pro spectrophotometer [19, 20].

Negative control setgot believed to have 100% cell viability, while the treated groups were predicted to have cell vitality proportional to -ve control [16, 21]. Concentration got plotted against % viability, and dosage response was assessed. Based on dosage response relations, a suitable model got fitted to determine $I_{max}$& $IC_{50}$ values.

Percentage viability formula:

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

OD570e: Optical Density test dilution

OD570b: Optical Density of -ve control

## RESULTS AND DISCUSSION

### Drug loaded nanobubbles

Various researchers attempted to develop new drug delivery methods that use nanosized bubbles to minimalize harmfulness, improve stability & specificity, boost bioavailability, & provide continueddischarge of a wide range of hydrophilic and hydrophobic pharmaceuticals. Various ways have been used to create nanobubbles, contingent on physicochemical qualities of polymer & medicine to be encumbered. The W/O/W emulsion approach gotutilized for creation of ENT-loaded nanobubbles.

### Evaluation of drug loaded nanobubbles

Entrectinib-loaded nanobubbles were evaluated. Entrectinib-loaded nanobubbles with particle sizes ranging from 61.22 to 253.23nm, a polydispersity index of 0.196 ± 0.005, and a zeta potential of -25.3 ±2.98 mV. The drug payload was 29.27 ± 1.54. Size, zeta potential, & polydispersity index were all assessed. Microscopy measurements of Entrectinib-loaded nanobubbles are well linked with the dynamic light scattering technique. The lack of an endothermic peak in the thermogram of Entrectinib-loaded nanobubbles, as well as the absence of drug-specific peaks in the Fourier transformed infrared spectra, suggest that the drug may be encapsulated inside the PLGA. With acoustic aid, nanobubbles discharged more than 99.34% of their contents in 24 hours. The stability studies revealed that nanobubbles loaded with Entrectinib are a stable formulation. The aqueous solutions of PLGA nanobubbles showed no haemolytic activity, whilst drug-loaded nanobubbles were very safe when employed with erythrocytes.

### *In-vitro* cytotoxic assay

Itprovides important insights into the model's benefits and drawbacks for the mechanistic assessment and screening of potentially hazardous chemicals. A broad range of biochemical and morphological markers are available to gather data at cellular & molecular level in order to notice chemical-induced disturbance [23]. Under laboratory circumstances, in vitro cell line models are rather simple to apply.

Human tumor cell lines are the principal targets of cellular screening in oncology research. This method is the most effective for repeatability and management [17, 24]. Cell viability experiments were performed on the A 549 cell lines (Table 1; Figure 1) to determine the toxicity of Entrectinib-loaded nanobubbles. The $IC_{50}$ values were calculated using the MTT technique.

**Table 1: Cellular toxicity of Entrectinib pure drug &Entrectinib nanobubbles on A 549 cell line**

| Concentration (µg/ml) | Entrectinib Pure Drug | | Entrectinib Loaded Nanobubbles | |
|---|---|---|---|---|
| | % Mean Viability | IC50 value (µg/ml) | % Mean Viability | IC50 value (µg/ml) |
| **Blank** | - | 63.29 | - | 87.16 |
| **Vehicle Control** | 100.00±0.00 | | 100.00±0.00 | |
| **5.00** | 98.37±2.54 | | 95.46±2.17 | |
| **10.00** | 93.36±2.78 | | 75.54±2.46 | |
| **20.00** | 86.26±2.46 | | 57.32±2.47 | |
| **30.00** | 78.54±2.71 | | 44.42±1.24 | |
| **50.00** | 66.87±2.14 | | 37.18±1.47 | |
| **100.00** | 22.36±1.24 | | 24.17±1.65 | |

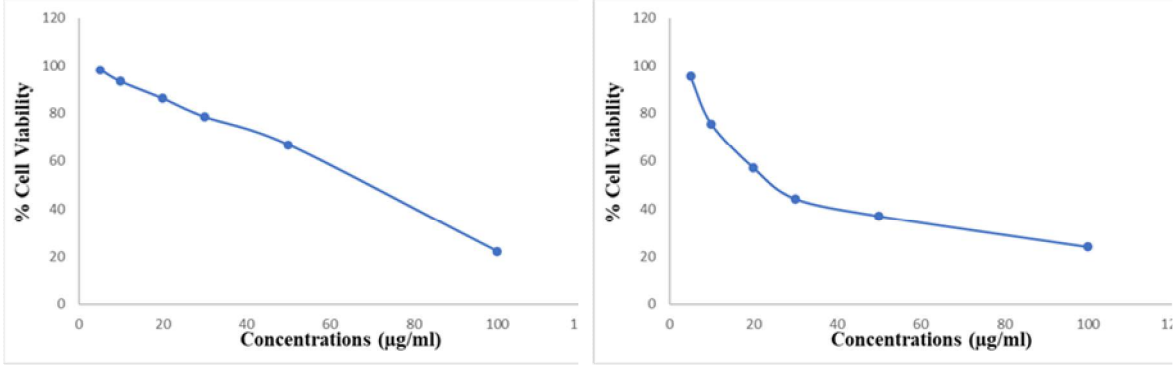
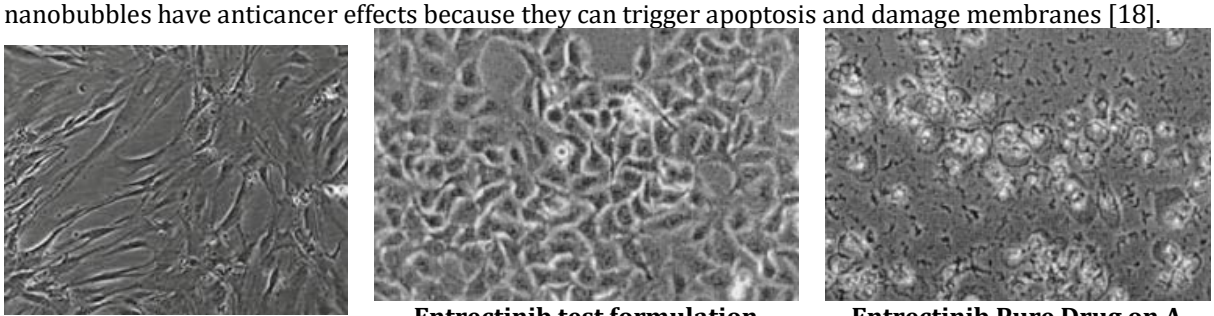



**Figure 1: Cytotoxicity of Entrectinib Pure Drug on A 549 cell line** **Cytotoxicity of Entrectinib nanobubbles on A 549 cell line**

The morphology of the A 549 cell lines was examined after treatment with various doses of Entrectinib and Entrectinib-loaded nanobubbles (Figure 2). Significant deformations & changes got seen on cell surface, that were most likely caused by the nanobubbles under investigation. The effect of pure medications on cell shape was dramatically diminished. The concentration of Entrectinib-loaded nanobubbles increased in response to changes in cell shape and apoptotic processes. Entrectinib-loaded nanobubbles have anticancer effects because they can trigger apoptosis and damage membranes [18].



**Vehicle control** **Entrectinib test formulation agaainst A 549 cellline** **Entrectinib Pure Drug on A 549 cellline**

**Figure 2: Morphological change of A 549tumour cells as treated with Entrectinib nanobubbles**

The MTT test is a colorimetric technique for determining cellular metabolic activity. The cytotoxic potential of a test medicine was evaluated using the A 549 cell lines to screen for necrosis or apoptosis [22]. The NAD(P)H-dependent cellular oxidoreductase enzyme alters yellow tetrazolium MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] into insoluble (E, Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan (formazan). In a safety study utilizing the MTT test, the formulation including the aforementioned safe substances showed little cytotoxicity. We verified that the modified formulation's pretreatment medium had a cell viability (%) of more than 97% (less than 3% cell death at all doses). This finding suggests that the microemulsion that was created has little cytotoxicity towards the A 549 cell lines.

## CONCLUSION

The targeted administration of a range of anticancer medications, including synthetic substances and natural materials, has lately sparked great interest in nanoparticulated drug delivery systems. Tumor accumulation was best suited for particle size in the improved formulation. The narrow size distribution of the nanobubbles revealed that the formulation was uniformly monodispersed. The MTT test indicated that the formulation had a considerable cytotoxic impact. Each of them demonstrated therapeutic implication of Entrectinib-containing nanobubbles. Data suggests that Entrectinib-loaded nanobubbles produced had low cytotoxic properties on A 549 cell lines.

## CONFLICTS OF INTEREST

No conflict of interest.

## AUTHOR CONTRIBUTION

The investigation work, execution, and writing were completed by P.N., and D.V.R.N.B, who also conducted the work plan, review, and corrections. The submission and publication are approved by all authors. The published form of manuscript was read &accepted by all writers.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