ORIGINAL ARTICLE

Evaluating the Antitumor Activity of Nintedanib by Solid Lipid Nanoparticle Drug Delivery System: *In vitro* and *In vivo*

Ramineni Sunitha[1], Praveen Sivadasu[1], P. Bharghava Bhushan Rao[2]

[1]Department of Pharmacy, Koneru Lakshmaiah Education Foundation, Vaddeswaram, Andhra Pradesh, India, [2]Department of Pharmaceutics, A. M. Reddy Memorial College of Pharmacy, Narasaraopet, Andhra Pradesh, India

Abstract

Introduction: Nintedanib was tested using the MTT assay on breast cancer cells and *in vivo* in mice bearing Ehrlich ascites carcinoma (EAC). Nintedanib (NTD)-solid lipid nanoparticles (SLNs) showed better drug loading, release, and bioavailability, enhancing anticancer activity *in vitro* and *in vivo*. **Materials and Methods:** Cytotoxicity of NTD and NTD-SLNs were evaluated on Michigan Cancer Foundation (MCF)-7 cancer cells using the MTT assay, determining cell viability. *In vivo* antitumor activity was assessed in EAC-bearing mice treated with NTD, NTD-SLNs, or standard 5-fluoro u , hematological parameters, and biochemical markers measured. Statistical analysis using one-way Analysis of Variance and Dunnett's test showed treatment effects compared to the toxic control, with significance at $P < 0.05$. **Results:** NTD and NTD-SLNs showed dose-dependent cytotoxicity in MCF-7 cancer cells using the MTT assay, with the highest inhibition at 200 µg/mL after 72 h. *In vivo* studies in EAC-bearing mice showed that NTD-SLNs improved tumor suppression and restored hematological parameters toward normal, without toxicity. Overall, NTD-SLNs demonstrated stronger antitumor activity than pure NTD and effects comparable to the standard drug 5-FU. **Conclusion:** *In vitro* studies showed that NTD-SLNs were more effective than free NTD due to improved solubility and dissolution, leading to stronger antitumor activity. *In vivo*, both NTD and NTD-SLNs demonstrated antitumor effects in EAC-bearing mice, though NTD-SLNs were not as effective as the standard 5-FU. Overall, drug-loaded SLNs offer a promising approach for enhancing the bioavailability and antitumor potential of poorly soluble drugs, such as NTD.

Key words: 5FU, MCF-7, Nintedanib, NTD-solid lipid nanoparticle

INTRODUCTION

Nintedanib is a medication that blocks three types of receptor tyrosine kinases, which are involved in cell growth and repair. These receptors include fibroblast growth factor receptor, vascular endothelial growth factor receptor, and platelet-derived growth factor receptor.[1]

Treating cells with nintedanib can lead to different outcomes for the cells. The cells might experience necrosis, which means they lose their membrane structure and die quickly because the cell breaks apart. Alternatively, the cells might stop growing and dividing, causing a reduction in their survival rate. Another possibility is that the cells trigger a programmed process called apoptosis, which is a controlled way of cell death. When cells undergo necrosis, they typically swell quickly, lose their membrane structure, stop using energy, and release their internal contents into the surrounding area.[2-5]

Apoptosis is marked by specific changes in the cell, such as a shift in how light reflects off the cell, shrinking of the cytoplasm, condensation of the nucleus, and breaking down of DNA into small, uniform pieces. Over time, cells that are undergoing apoptosis will eventually undergo secondary necrosis, where they stop using energy, lose their membrane structure, and break down.

Address for correspondence:
Ramineni Sunitha, Department of Pharmacy, K L College of Pharmacy, Koneru Lakshmaiah Education Foundation, Green Fields, Vaddeswaram, Andhra Pradesh, India.
Phone: 9739967534. E-mail: praveen.sivadasu399@gmail.com

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The anticancer effects of NTD and NTD-solid lipid nanoparticles (SLNs) were studied in an *ex vivo* setting by growing tumor cells (human breast cancer cells). This was done through the MTT assay to assess the toxicity of the drug and its nanoparticle form.

Ehrlich ascites carcinoma (EAC) was originally developed from a naturally occurring mouse breast cancer. The purpose of this study is to assess the *in vivo* anticancer impact of NTD and NTD-SLNs in mice that have been implanted with EAC. We used the ascites form of the EAC model as it is transplantable, specific to mice, and highly aggressive. For these reasons, EAC serves as an ideal model for assessment and tracking.

Experimental tumors play a significant role in cancer modeling, and EAC is one of the most commonly used tumors. EAC is described as an undifferentiated type of carcinoma that is originally hyperdiploid, with high transplantability, no tendency to regress, fast growth, a short lifespan, 100% malignancy, and no tumor-specific transplantation antigens. Often, as the tumors are passed through multiple generations, their virulence increases, and their growth rate gradually improves. However, the process of differentiation diminishes, and the cells lose control of their growth, gain the ability to be transplanted between different species, and eventually transform into the ascites form. EAC shares similarities with human tumors, which are particularly sensitive to chemotherapy because they are undifferentiated and have a high growth rate.

NTD-SLNs prepared were used due to their smaller and more uniform size, higher entrapment efficiency and drug loading, improved drug release *in vitro* dissolution studies; these characteristics lead to better drug absorption and increased oral bioavailability of NTD. Using these, antitumor activity was tested both *in vitro* by using various cell lines and *in vivo* using mice.

MATERIALS AND METHODS

Cytotoxicity studies on different human cancer cell lines

Cells and medium

The Dulbeco's modified eagle medium (DMEM) comprised of Incomplete Dulbecco's media with 10% fetal calf serum and 1% Penicillin, streptomycin, and amphotericin). Michigan Cancer Foundation (MCF)-7 cell line obtained from the National Centre for Cell Science, Pune, India, was used in the study. The cell lines were grown as monolayer cultures in DMEM in a humidified atmosphere of 5% CO_2 at 37°C in T-75 flasks and subcultured twice a week.[6]

Cytotoxicity assay of NTD and NTD-SLN son different cell lines

An *ex vivo* cytotoxic study for NTD was carried out using cell lines linked to solid tumors, specifically MCF-7. The extent of cell survival was determined using the MTT assay, which relies on the capacity of living cells to transform a tetrazolium compound called MTS into a colored product known as formazan. Initially, the cells were removed from the culture using trypsin, and then they were suspended at a concentration of 1×10^4 cells/mL in fresh culture medium.[7,8] These cells were allowed to grow for 72 h in a humidified atmosphere with 5% carbon dioxide and a temperature of 37°C, while being exposed to NTD and NTD-SLNs. To evaluate the cytotoxic impact, a combination of 100 µL of drug solution at various concentrations (ranging from 200 to 1.5 µg/mL) and 100 mL of culture medium was placed into each well. Following the incubation time, 20 µL of MTT solution (5.0 mg/mL in isopropyl alcohol) was added to each well, and the samples were incubated for an additional 4 h at 37°C in a CO_2 incubator. The percentage of cell viability and half maximal inhibitory concentration values for each cell line were determined from the outcomes of the MTT assay. The experiment was performed 3 times to obtain average values and standard deviations (SDs). The results are expressed as the percentage of viable cells relative to the control group.[7-9]

In vivo antitumor activity on mice

The *in vivo* study was conducted to evaluate the anticancer effects of NTD and its SLNs following oral administration to female mice, following established protocols. A total of thirty mice were divided into five groups, each consisting of six animals.[9,10] The study compared tumor regression and hematological parameters in mice after administering NTD in the form of a drug suspension in 1% CMC, NTD-SLNs, and subcutaneous injections of the commercially available formulation FIVOFLU® 500 mg/10 mL injection, produced by Dabur India Pvt. Ltd.

Maintenance of animals

The *in vivo* studies were performed on mice that were of mixed sex, aged between 5 and 6 weeks, and weighed between 20 and 25 g. These mice were housed in polycarbonate cages, with five mice per cage, under conditions that maintained an ambient temperature of 25 ± 2°C and a relative humidity of 55 ± 5%. The lighting cycle was set to 12 h of light followed by 12 h of darkness. The mice were provided with commercially available rodent chow and had free access to water. They were allowed to adjust to the laboratory environment before being randomly assigned to the experiment. Permission to conduct the *in vivo* anticancer study was granted by the Institutional Animal Ethics Committee, and all procedures followed the guidelines set by the committee. A total of 24 mice were approved for this study, with six mice assigned to each of the four groups (NTD and NTD-SLNs). It was also suggested to conduct the experiments concurrently, which had already received approval for the use of animals for control, toxic control, and market standard groups using the same model, so the study was carried out in accordance with these arrangements.

Transplant of tumor

The EAC cells were sourced from the Cell Culture Laboratory at INMAS, located in New Delhi, India. A suspension of EAC cells, containing 0.1 mL with 2×10^6 cells, was injected into each mouse through the intraperitoneal route.

Schedule of treatment

The mice were divided into five groups, with six animals in each group. Group I was the control group (non-tumor mice, untreated), and they received 1% CMC (0.1 mL orally, once daily for 10 days). Group II received EAC cells (2×10^6 cells/mouse, intraperitoneally), which was considered day 1 and served as the toxic control, that is, tumor-induced mice that were untreated. After 24 h of EAC transplantation, Group III, which consisted of tumor-induced mice, was treated with the standard marketed anticancer drug (5-FU, 20 mg/kg body weight, subcutaneously, once daily for 10 days). Animals in Group IV and Group V received pure drug (NTD suspension in 1% CMC) at a concentration of 10 mg/kg body weight orally (once daily for 10 days) and NTD-SLNs, respectively (AD equivalent to 10 mg/kg body weight orally, once daily for 10 days). After the administration of the last dose, mice from each group were kept fasting for 18 h, and blood was collected by cardiac puncture to estimate hematological and biochemical parameters. The animals were then sacrificed by cervical dislocation to study antitumor activity. The mice in each group were kept alive with a rodent diet and ad libitum access to food and water.

Tumor cell (viable/non-viable) count

The ascetic fluid was administered through a syringe and diluted 20 times using PBS. A single drop of the diluted cell suspension was placed on the Neubauer chamber, and the number of cells in the 64 small squares was determined. The viability and non-viability of the cells were assessed using the trypan blue assay. The cells were stained with trypan blue dye, which was prepared at a concentration of 0.4% in normal saline. Cells that did not absorb the dye were considered viable, while those that took up the dye were non-viable. The counts for both viable and non-viable cells were recorded.

$$\text{Cell count} = [\text{Number of cells} \times \text{dilution factor}]/\text{Area} \times \text{thickness of the liquid film}$$

Hematologic analysis

Blood samples were gathered through an intra-cardiac puncture after the animal was given anesthesia using ketamine and xylazine. The entire blood sample was collected right away into vials that had been coated with ethylene-diamine-tetra-acetic acid to check for any signs of hematologic toxicity. The hematology test involved measuring the levels of white blood cells, red blood cells, and platelets, as well as determining the mean corpuscular hemoglobin concentration and volume, all done with an automated hematologic analyzer.[10]

Statistical analysis

Values were presented as the average ± SD. To determine if the results were statistically significant, a one-way analysis of variance was performed, followed by Dunnett's test using GraphPad software. All groups that received treatment were compared against the toxic control group. A $P < 0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

To show that a substance has a cytotoxic effect, observing animal models is essential, but before that, an *in vitro* cytotoxic study of the drug and its SLNs should be conducted to evaluate both the drug and SLNs for their antitumor activity against solid tumors.[11] *In vitro* studies are less time-consuming and also help in understanding the mechanisms by which the tested substances work. One commonly used test for this is the MTT colorimetric assay. In this study, the MTT assay was used to evaluate the *in vitro* cytotoxicity of the drug NTD and its SLN formulation, NTD-SLNs, against various tumor cell lines. Overall, all the tested tumor cell lines showed a similar response to the drug, suggesting that the drug may act through a common mechanism in all these cell lines. The cell viability assessment for NTD and NTD-SLNs was carried out at 72 h using serial dilutions ranging from 200 µg/mL to 1.5 µg/mL.[12-15] Untreated cells were used as controls. The assay was stopped at 72 h, and the cell viability was determined using a colorimetric method. The MTT assay was employed to screen the response of the drug in cancer cell lines MCF-7. The image of the cancer cells are shown in Figure 1. The results are presented as the percentage of viable cells relative to the control.

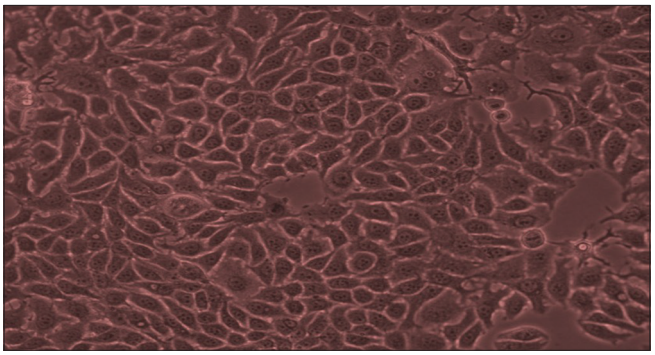

Figure 1: Microphotographs of Michigan Cancer Foundation-7 (human breast cancer cells)

The graph was created by plotting the percentage of cell proliferation against the concentration of the drug for all the cell lines. The experiment was conducted for 72 h, with measurements taken at 24, 48, and 72 h. Both NTD and NTD-SLNs demonstrated a relatively dose-dependent inhibitory effect on the proliferation of all tested cell lines within the concentration range of 200 µg/mL–1.5 µg/mL. The percentage of cell proliferation after 72 h of incubation for the tested cell line is shown in Figure 2. The highest level of cell inhibition was observed at a concentration of 200 µg/mL for both NTD and NTD-SLNs in the MCF-7 cell line at 72 h.

Hematologic analysis[16,17]

All the hematological parameters were determined and represented in Table 1. The parameters include:

- White blood cell (WBC) count: A total count of the number of white cells per liter of blood.
- Red blood cell (RBC) count: A total count of the number of red cells per liter of blood.
- Hemoglobin (HGB): The total amount of HGB in the blood (irrespective of the number of cells containing the HGB).
- Hematocrit: The total volume of the red cells in the blood.
- Mean corpuscular volume is an indication of the size of the red cells.
- Mean corpuscular hemoglobin (MCH) is a measure of the amount of HGB per red blood cell.
- MCH concentration (MCHC) is the amount of HGB per liter of fluid in each cell.
- Red blood cell distribution width is a measure of the variation of RBC volume.
- Platelet (PLT) count: A total count of the number of platelets per liter of blood.
- Platelet distribution width is a measure of the variation of PLT volume.

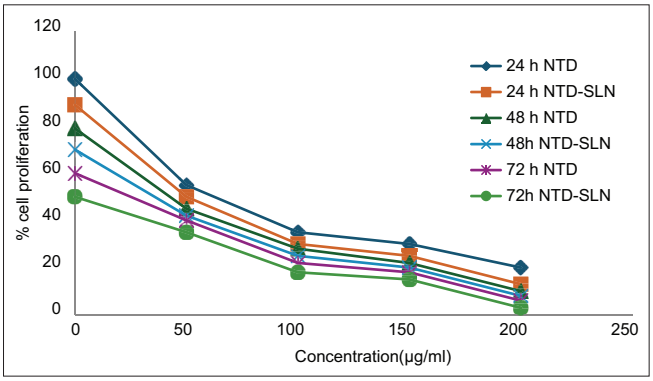

Figure 2: Cell viability assay of NTD and NTD-solid lipid nanoparticles at different concentration (from 200 to 1.5 µg/mL) and at different time intervals on Michigan Cancer Foundation-7 (Human breast cancer cell line)

The mice were checked every day for any changes in their physical appearance and behavior. All the mice lived through the entire experiment and did not show any unusual signs. They did not display any signs of poisoning, such as tiredness, loss of interest in food, changes in fur color, or weight loss.[18-20] When comparing different blood-related measurements in both the treated mice and the control group, it was clear that there were no major changes. Some minor differences were observed, but they were still within the normal range.

A comparison of various blood-related measurements, such as RBC, WBC, PLT, and HGB, was done in both the treated mice and the control group.

Table 1: Hematological profiles of different groups

Parameters	Control±SD	Ehrlich ascites carcinoma control±SD	5-FU±SD	NTD±SD	NTD-solid lipid nanoparticles±SD
White blood cell cell *10^4/µL	0.49±0.03	0.82±0.33	0.27±0.01	0.61±0.01	0.56±0.03
Red blood cell count *10^6/µL	8.59±0.13	6.31±0.14	7.32±0.16	8.45±0.63	8.5±0.11
Hemoglobin g/dL	11.33±033	9.17±0.22	11.3±0.44	11.5±0.41	12.3±0.61
Hematocrit %	42.80±3.1	32.5±0.47	41.00±0.63	43.33±2.19	44.3±2.16
Mean corpuscular volume fL	45.22±1.90	37.3±1.9	46.3±1.2	49.20±1.33	48.50±2.11
Mean corpuscular hemoglobin pg	12.1±0.36	13.3±0.22	12.23±0.36	12.2±0.55	13.5±0.19
Mean corpuscular hemoglobin Concentration g/dL	25.30±0.63	27.2±0.66	27.3±1.22	29.33±0.67	29.12±0.31
Platelet count * 10^4/µL	65.80±1.99	110.3±3.21	65.3±1.74	84.3±1.92	75.9±1.93
Red blood cell distribution width fL	23.20±0.74	28.3±1.1	27.2±0.33	29.12±0.99	29.30±1.12
Platelet distribution width fL	9.10±1.33	9.7±0.31	8.2±0.21	9.65±0.33	9.10±0.19
MPV fL	6.9±0.0.25	6.33±0.19	6.11±0.22	7.15±0.31	7.37±0.19
P-LCR%	9.1±0.49	10.7±0.41	5.9±0.30	9.33±0.33	9.12±0.12

* is used to denote a multiplication factor (scientific notation) in the units. SD: Standard deviation; MPV: Mean platelet volume; P-LCR: Platelet large cell ratio

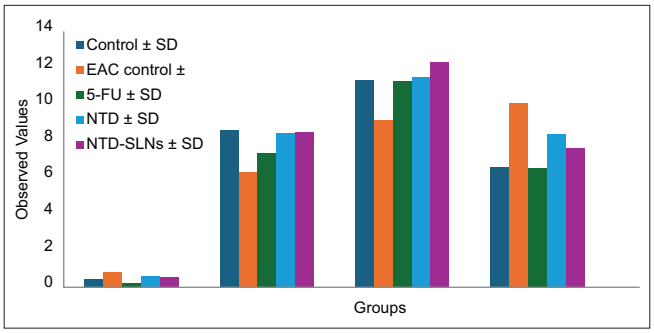

Figure 3: Impact of different treatments on the main parameters of the hemopoietic system (mean values±standard deviation, $n = 6$)

The findings showed that there were no significant changes in these blood-related measurements in either group. However, some small variations were seen, which were still within the normal range.

One of the main issues with cancer treatment is that it can affect the bone marrow, leading to reduced blood cell production and anemia. The anemia seen in mice with tumors might be caused by a lack of iron or by conditions that destroy red blood cells or affect bone marrow function, which results in lower levels of red blood cells or hemoglobin. When mice with tumors were treated with NTD-SLNs, their blood measurements returned to normal levels. The results suggest that the drug and its nanoformulation have a protective effect on the blood-forming system. The effects of different treatments on key blood-related parameters are shown in Figure 3.

In conclusion, it was found that NTD-SLNs inhibited the growth of the tumor (which was comparable to 5-FU) and it was more active than NTD.

CONCLUSION

In vitro cytotoxicity study showed that NTD-SLNs were as effective as free NTD in arresting cell growth in all the tested cell lines. The results showed that NTD-SLNs possessed more antitumor effect than NTD because of increased solubility and dissolution rate of drug-loaded SLNs.

In vivo antitumor study reveals that the NTD and NTD-SLNs have remarkable anti-tumor activity against EAC cells treated mice. In EAC tumor bearing mice, a regular, rapid increase in the ascetic tumor volume was observed. The NTD-SLNs showed not good results to 5-FU, as it is the standard established drug in the market for different types of tumors. Thus, the AD- loaded SLNs would be useful for the delivery of poorly water-soluble AD with enhanced bioavailability and improved antitumor activity.

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