

PARALLEL MTT AND SRB PROFILING OF FREE AND CUBOSOME-ENCAPSULATED NERATINIB AND PACLITAXEL IN MCF-7 CELLS

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ABSTRACT

Breast cancer is one of the most commonly diagnosed malignancies among women worldwide, and the development of effective and targeted therapeutic strategies remains an important research objective. Nanostructured drug delivery systems such as cubosomes have attracted considerable attention because of their potential to modify drug release and improve drug delivery. The present study comparatively evaluated the in vitro anticancer activity of Neratinib, Paclitaxel, Neratinib-loaded cubosomes, Paclitaxel-loaded cubosomes, and a drug combination against MCF-7 human breast cancer cells using MTT and Sulforhodamine B (SRB) assays. MCF-7 cells were cultured in DMEM supplemented with fetal bovine serum and antibiotics and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Cells were exposed to test samples at concentrations of 6.25, 12.5, 25, 50, and 100 µg/mL for 24 h. Cytotoxicity was evaluated by direct microscopic observation, MTT assay, and SRB assay. All treatments demonstrated concentration-dependent effects on cell viability. Paclitaxel exhibited the greatest cytotoxic activity, producing IC₅₀ values of 5.236 µg/mL and 5.20 µg/mL in the MTT and SRB assays, respectively. Neratinib demonstrated IC₅₀ values of 23.774 µg/mL and 24.20 µg/mL in the respective assays. The estimated IC₅₀ values for Neratinib cubosomes, Paclitaxel cubosomes, and the combination were substantially higher than those of the corresponding free drugs under the present experimental conditions. Microscopic examination showed concentration-dependent cellular shrinkage, rounding, detachment, and reduced cell density following treatment. Overall, Paclitaxel showed the highest in vitro cytotoxic activity against MCF-7 cells, followed by Neratinib, while the cubosomal formulations exhibited a slower apparent cytotoxic response during the 24-h exposure period.

Keywords: MCF-7; breast cancer; neratinib; paclitaxel; cubosomes; cytotoxicity

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INTRODUCTION

Breast cancer encompasses biologically distinct diseases rather than a single uniform malignancy. Its worldwide incidence remains high, and treatment failure may arise from toxicity, resistance, or inadequate drug exposure even when chemotherapy and receptor-directed agents are available ^{1,2}. These limitations provide the clinical rationale for examining both active drugs and their delivery systems.

Paclitaxel disrupts cell division by binding β -tubulin and suppressing normal microtubule dynamics. Prolonged mitotic disturbance can culminate in cell death, which explains its established role in breast-cancer therapy and its use as a benchmark cytotoxic drug in laboratory studies ³⁻⁷.

Neratinib acts through a different target. It forms an irreversible bond with members of the ErbB receptor family, including human epidermal growth factor receptor 2 and epidermal growth factor receptor, and thereby restricts signaling that supports tumor-cell growth and survival ⁸⁻¹⁰.

Formulation remains relevant for both drugs because solubility, systemic exposure, distribution, and vehicle-related toxicity can influence the amount of active compound that reaches tumor cells. Lipid nanocarriers are one approach to modifying these delivery constraints ^{7,11}.

Cubosomes are dispersed particles derived from bicontinuous cubic lipid phases. Their interconnected aqueous channels and lipid domains provide space for compounds with differing solubility characteristics. Particle composition and internal structure may also change drug release and contact with cellular membranes ¹²⁻¹⁶.

No single colorimetric assay describes every aspect of cell survival. MTT reduction reflects cellular metabolic activity, whereas SRB staining measures the protein retained by fixed cells. Applying both methods to the same treatment series allows the concentration-response pattern to be checked using two different biological readouts ^{17,18}.

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Accordingly, free neratinib, free paclitaxel, neratinib cubosomes, paclitaxel cubosomes, and a combined treatment were tested in MCF-7 cells. Morphological findings were considered alongside MTT and SRB viability data, with particular attention to whether the two assays produced consistent IC50 rankings.

MATERIALS AND METHODS

Materials

MCF-7 human breast adenocarcinoma cells were obtained from the National Centre for Cell Science, Pune, India. Dulbecco's modified Eagle medium and MTT were sourced commercially, as were dimethyl sulfoxide and the remaining analytical reagents. Fetal bovine serum was supplied by Gibco (USA), and trypsin by Invitrogen (USA). Cell-culture-grade materials were used where applicable.

Cell culture and maintenance

Cells were propagated in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, L-glutamine, sodium bicarbonate, penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin B (2.5 µg/mL). Cultures were held at 37 °C under humidified 5% CO₂ and examined routinely with an inverted phase-contrast microscope.

Cell seeding

A confluent culture approximately 2 days old was detached with trypsin and resuspended in growth medium. The suspension was adjusted to approximately 5×10^3 cells per well. Aliquots of 100 µL were dispensed into sterile 96-well plates and left for 24 h under the stated culture conditions to permit attachment.

Preparation of test samples

For each treatment, 1 mg of sample was dispersed in 1 mL of 0.1% dimethyl sulfoxide using a cyclomixer. The stock was passed through a 0.22-µm syringe filter, after which working concentrations were prepared by dilution with culture medium.

Treatment of MCF-7 cells

At 24 h after seeding, the medium was exchanged for medium containing one of the five test groups at 6.25, 12.5, 25, 50, or 100 µg/mL. Three wells were assigned to each concentration. Control wells underwent the same incubation without a test sample.

Direct microscopic observation

Cell fields were photographed after the 24-h exposure using an inverted phase-contrast microscope fitted with an Optika Pro5 charge-coupled device camera. Images acquired at 10× were assessed for loss of confluence, rounding, shrinkage, cytoplasmic granulation or vacuolation, membrane alteration, and detachment.

MTT assay

MTT analysis followed Mosmann¹⁷. A sterile working solution was prepared by dissolving 15 mg MTT in 3 mL phosphate-buffered saline and passing the solution through a membrane filter. Treatment medium was aspirated after 24 h, and 30 µL of MTT

solution was placed in every treated and control well. Plates were mixed gently and returned to the incubator for 4 h. The supernatant was discarded, 100 µL dimethyl sulfoxide was used to dissolve the formazan, and absorbance was read at 540 nm. Cell viability (%) was calculated as mean optical density of treated wells divided by mean optical density of untreated control wells $\times 100$. Growth inhibition (%) was calculated as $100 - \text{cell viability}$. Three replicate wells contributed to each result.

SRB assay

For the SRB procedure, treated monolayers were fixed with chilled 10% trichloroacetic acid for approximately 1 h at 4 °C. Fixative was removed by washing, and plates were dried before staining according to Skehan et al.¹⁸. Fixed cellular protein was stained for approximately 30 min with 0.057% SRB. Repeated washing with 1% acetic acid removed unbound stain. Once dry, retained dye was released with 10 mM Tris base at approximately pH 10.5, and absorbance was recorded at 510 nm. SRB viability was expressed as a percentage of untreated-cell absorbance.

Determination of IC50

ED50 PLUS version 1.0 was used to estimate the concentration associated with 50% viability from each concentration-response series. Free-drug curves crossed the 50% level within the experimental range. Curves for both cubosomal preparations and the combined treatment did not; their software-derived IC50 values extend beyond 100 µg/mL and are reported only as extrapolated estimates pending experiments at higher concentrations.

Statistical analysis

Each concentration was tested in three replicate wells. The available dataset contains mean percentage-viability values but does not provide replicate-level numerical values, standard errors, or exact p values. Therefore, inferential statistical comparisons are not reported in the present manuscript; interpretation is based on descriptive concentration-response patterns and IC50 estimates.

Ethical statement

Ethics committee approval was not applicable because this in-vitro study used an established commercially available human cell line and did not involve human participants, identifiable human material, or experimental animals.

RESULTS

Morphological changes

Control cultures retained the closely packed epithelial appearance expected for MCF-7 cells. Exposure to the free drugs progressively reduced confluence as concentration increased. Rounding, shrinkage, sparse cell fields, and detachment were most evident after paclitaxel exposure and were also clear with neratinib. Cells exposed to either cubosomal preparation changed less markedly during the 24-h observation window. The complete

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concentration-ordered microscopy panels are shown for the MTT and SRB experiments in Figs. 1 and 2, respectively.

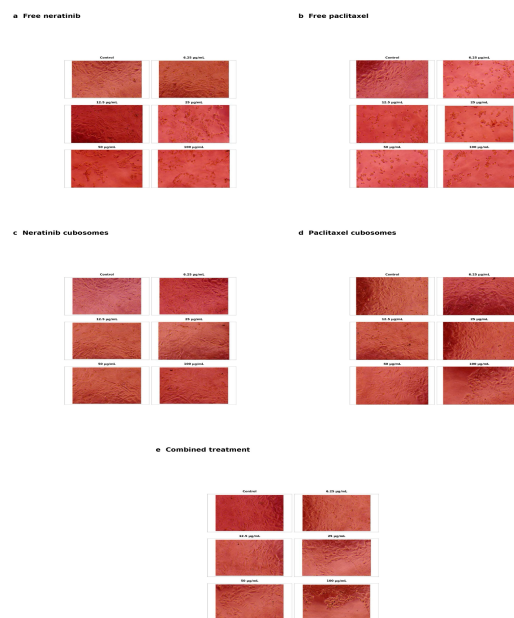


Figure 1. Phase-contrast morphology of MCF-7 cells in the MTT experiment after 24 h exposure to (a) free neratinib, (b) free paclitaxel, (c) neratinib cubosomes, (d) paclitaxel cubosomes, and (e) the combined treatment. Each panel is arranged from untreated control through 6.25, 12.5, 25, 50, and 100 µg/mL; original magnification 10 $\times$.

Interpretation of Fig. 1. In the MTT experimental series, untreated cells retained a dense, adherent epithelial monolayer. Increasing concentrations of free paclitaxel and free neratinib produced progressively greater cell rounding, shrinkage, loss of cell-to-cell contact, and detachment. These changes were most pronounced with free paclitaxel and corresponded to its steep reduction in metabolic viability. Cells treated with the cubosomal formulations retained comparatively greater confluence and normal morphology during the 24-h exposure, consistent with their higher MTT viability. The combined treatment produced an intermediate morphological response. This pattern supports slower short-term drug availability from the cubosomes, but morphology alone cannot distinguish delayed release from reduced cellular uptake.

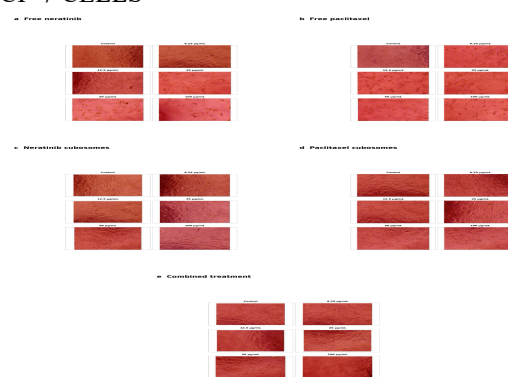


Figure 2. Phase-contrast morphology of MCF-7 cells in the SRB experiment after 24 h exposure to (a) free neratinib, (b) free paclitaxel, (c) neratinib cubosomes, (d) paclitaxel cubosomes, and (e) the combined treatment. Each panel is arranged from untreated control through 6.25, 12.5, 25, 50, and 100 µg/mL; original magnification 10 $\times$.

Interpretation of Fig. 2. The SRB experimental series reproduced the concentration-dependent pattern observed in the MTT plates. Free paclitaxel caused the clearest reduction in adherent cell density, followed by free neratinib, whereas both cubosomal formulations produced less extensive structural disruption at the same nominal concentrations. The combined treatment again showed an intermediate effect. Agreement between these morphological observations and SRB protein-based viability measurements strengthens the treatment ranking obtained by the two independent assays. However, these images are qualitative and should not be interpreted as evidence of apoptosis or treatment synergy without specific mechanistic and combination-index analyses.

MTT and SRB viability

Both assays separated the free-drug groups from the cubosomal preparations across the concentration series (Table 1; Fig. 3). Viability declined most steeply with free paclitaxel, followed by free neratinib. In the MTT assay, neratinib viability fell from 89.97% at 6.25 µg/mL to 20.24% at 100 µg/mL. Paclitaxel was below 50% at the lowest tested concentration (40.32%) and reached 14.33% at 100 µg/mL. The corresponding decline was smaller for the encapsulated drugs. The combination decreased from 93.33% to 52.78%. SRB measurements yielded the same overall ordering despite the different assay endpoint.

Table 1. Cell viability after 24 h exposure measured by MTT and SRB assays

Treatment	Concentration (µg/mL)	MTT viability (%)	SRB viability (%)
Neratinib	6.25	89.97	90.43
	12.5	76.99	76.66
	25	47.07	48.18
	50	29.95	30.58

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Treatment	Concentration (µg/mL)	MTT viability (%)	SRB viability (%)
	100	20.24	21.30
Paclitaxel	6.25	40.32	39.98
	12.5	35.37	34.38
	25	28.66	29.61
	50	19.21	18.77
	100	14.33	13.16
Neratinib cubosomes	6.25	98.01	98.60
	12.5	93.47	93.07
	25	89.20	87.00
	50	84.43	83.82
	100	77.96	76.72
Paclitaxel cubosomes	6.25	96.21	96.06
	12.5	88.43	88.69
	25	80.72	79.14
	50	73.82	72.28
	100	68.08	66.67
Combination	6.25	93.33	92.34
	12.5	79.93	78.67
	25	70.78	69.09
	50	61.88	59.15
	100	52.78	51.33

Note: Values are mean percentages from three replicate wells. Standard errors and exact p values were not available in the source dataset.

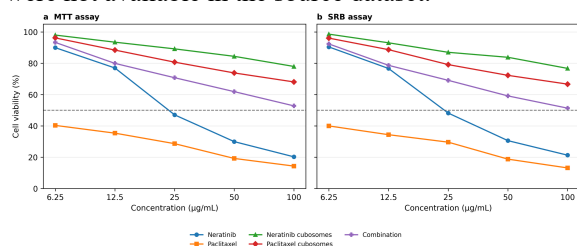


Figure 3. Concentration-response profiles for free drugs, cubosomal formulations, and the combined treatment measured by (a) MTT and (b) SRB after 24 h. Points represent reported mean viability values from three replicate wells; the dashed line indicates 50% viability. Error bars are omitted because numerical standard errors were not available in the source dataset.

IC₅₀ estimates

The MTT-derived potency order began with paclitaxel (IC₅₀ 5.236 µg/mL) and then neratinib (23.774 µg/mL). SRB values were closely matched at 5.20 and 24.20 µg/mL, respectively. Estimates for the combination and both cubosomal preparations exceeded the upper experimental limit in both assays and must be interpreted as extrapolations (Table 2).

Table 2. Comparative IC₅₀ estimates from MTT and SRB assays

Treatment	MTT IC ₅₀ (µg/mL)	SRB IC ₅₀ (µg/mL)	Interpretation
Neratinib	23.774	24.20	Within tested range
Paclitaxel	5.236	5.20	Within tested range
Neratinib cubosomes	235.574	221.78	Extrapolated
Paclitaxel cubosomes	156.096	146.96	Extrapolated
Combination	115.245	108.53	Extrapolated

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Paclitaxel cubosomes	156.096	146.96	Extrapolated
Combination	115.245	108.53	Extrapolated

IC₅₀ values above 100 µg/mL lie outside the tested interval and are not experimentally bracketed.

DISCUSSION

The present study investigated the comparative in vitro cytotoxicity of Neratinib, Paclitaxel, their respective cubosomal formulations, and a combination treatment against MCF-7 breast cancer cells. Cytotoxicity was evaluated using direct microscopic examination together with two colorimetric assays, namely MTT and SRB.

Both MTT and SRB assays demonstrated concentration-dependent changes in MCF-7 cell viability. The reduction in cell viability with increasing drug concentration indicates a concentration-dependent cytotoxic response. Among the tested treatments, Paclitaxel demonstrated the greatest cytotoxic activity, as indicated by the lowest IC₅₀ values in both assays.

The MTT assay showed an IC₅₀ of 5.236 µg/mL for Paclitaxel, while the SRB assay produced a closely comparable value of 5.20 µg/mL. This strong agreement indicates that the cytotoxic effect of Paclitaxel was consistently detected by both metabolic and cellular-protein-based assay systems. The high activity of Paclitaxel may be associated with its established mechanism of action involving microtubule stabilization, mitotic disruption, and subsequent induction of cell death.

Neratinib also produced substantial cytotoxic activity against MCF-7 cells. The IC₅₀ values were 23.774 µg/mL by MTT and 24.20 µg/mL by SRB. Although its cytotoxic potency was lower than that of Paclitaxel under the experimental conditions, Neratinib produced a marked concentration-dependent reduction in cell viability. Its activity may be associated with inhibition of HER2/EGFR-associated signaling pathways involved in cellular proliferation and survival.

Interestingly, the cubosomal formulations demonstrated higher cell viability compared with their respective free drugs following 24 h exposure.

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Neratinib cubosomes produced 77.96% viability at 100 $\mu\text{g/mL}$ in the MTT assay, compared with 20.24% for free Neratinib. Similarly, Paclitaxel cubosomes produced 68.08% viability compared with 14.33% for free Paclitaxel.

This finding does not necessarily indicate that the cubosomal formulations are therapeutically inferior. Rather, the observed response may reflect the release and cellular uptake characteristics of the formulation during the selected 24-h incubation period. Encapsulation of drugs within a lipidic nanostructured carrier may modify the rate at which the drug becomes available to cells. Consequently, a slower apparent cytotoxic response may be observed during a short in vitro exposure period.

The combination treatment demonstrated greater cytotoxicity than the individual cubosomal formulations but was less cytotoxic than free Paclitaxel and Neratinib at most tested concentrations. The combination produced 52.78% viability at 100 $\mu\text{g/mL}$ in the MTT assay and 51.33% in the SRB assay. Although combination therapy can potentially provide enhanced therapeutic effects through simultaneous modulation of different molecular pathways, the present data alone are insufficient to establish synergism. Formal combination-index analysis using appropriate dose-response designs would be required to demonstrate synergistic, additive, or antagonistic interactions.

The morphological observations supported the quantitative cytotoxicity results. Untreated MCF-7 cells exhibited relatively normal epithelial morphology, whereas treated cells showed shrinkage, rounding, detachment, decreased cell density, and other morphological alterations. These changes were more apparent following treatment with free Paclitaxel and Neratinib and were comparatively less pronounced following treatment with the cubosomal formulations.

The SRB results closely followed the MTT findings. The MTT assay evaluates metabolic activity, whereas the SRB assay provides an estimate of cellular biomass through measurement of cellular protein content. The comparable concentration-response patterns observed in the two assays strengthen the consistency of the cytotoxicity findings.

A limitation of the present study is that the calculated IC_{50} values for the cubosomal formulations and combination were higher than the maximum experimental concentration of 100 $\mu\text{g/mL}$. Therefore, these values represent extrapolated estimates and should not be interpreted with the same confidence as the IC_{50} values obtained for free Neratinib and

Paclitaxel. Additional experiments using a broader concentration range extending beyond 100 $\mu\text{g/mL}$ would be necessary to experimentally establish these IC_{50} values.

Another limitation is that the present study was performed using a single breast cancer cell line and a 24-h exposure period. Further studies involving longer incubation periods, additional breast cancer cell lines, appropriate normal-cell controls, apoptosis assays, cellular uptake studies, and mechanistic investigations would provide a more comprehensive evaluation of the formulations.

Overall, the results demonstrate that Paclitaxel showed the strongest short-term cytotoxic effect against MCF-7 cells, followed by Neratinib. The cubosomal formulations produced a slower apparent cytotoxic response under the present experimental conditions, which may be associated with controlled drug release and altered cellular drug availability.

CONCLUSION

The present study successfully compared the in vitro anticancer effects of Neratinib, Paclitaxel, Neratinib cubosomes, Paclitaxel cubosomes, and a combination treatment against MCF-7 breast cancer cells using MTT and SRB assays.

Paclitaxel demonstrated the highest cytotoxic potency, with IC_{50} values of 5.236 $\mu\text{g/mL}$ and 5.20 $\mu\text{g/mL}$ in the MTT and SRB assays, respectively. Neratinib exhibited substantial cytotoxic activity with IC_{50} values of 23.774 $\mu\text{g/mL}$ and 24.20 $\mu\text{g/mL}$. The cubosomal formulations exhibited comparatively higher cell viability during the 24-h treatment period, suggesting slower apparent drug availability under the experimental conditions.

The close agreement between the MTT and SRB assay results supports the reproducibility of the observed cytotoxicity pattern. However, the IC_{50} values calculated for the cubosomal formulations and combination exceeded the highest tested concentration and should therefore be regarded as extrapolated estimates requiring experimental confirmation.

Further studies involving extended concentration ranges, longer treatment periods, normal-cell controls, cellular uptake studies, apoptosis investigations, and in vivo evaluation are recommended to determine the therapeutic potential of the developed cubosomal formulations.

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Author contributions: **Ayesha Farhath Fatima:** conceptualization, methodology, investigation, data collection, formal analysis, visualization, literature review, and writing-original draft. **Kumaraswamy Gandla:** conceptualization, methodology, supervision, validation, interpretation of results, project administration, and writing-review and editing. Both authors reviewed and approved the final manuscript and accept responsibility for the integrity of the work.

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