

# Efficiency and mechanism of intracellular paclitaxel delivery by novel nanopolymer-based tumor-targeted delivery system, Nanoxel™

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## Abstract

**Introduction** An increasing research interest has been directed toward nanoparticle-based drug delivery systems for their advantages. The appropriate amalgamation of pH sensitivity and tumor targeting is a promising strategy to fabricate drug delivery systems with high efficiency, high selectivity and low toxicity.

**Materials and Methods** A novel pH sensitive Cremophor-free paclitaxel formulation, Nanoxel™, was developed in which the drug is delivered as nanomicelles using a polymeric carrier that specifically targets tumors. The efficiency and mechanism of intracellular paclitaxel delivery by Nanoxel™ was compared with two other commercially available paclitaxel formulations: Abraxane™ and Intaxel™, using different cell lines representing target cancers [breast, ovary and non-small cell lung carcinoma (NSCLC)] by transmission electron microscopy and quantitative intracellular paclitaxel measurements by high performance liquid chromatography.

**Results** The data obtained from the present study revealed that the uptake of nanoparticle-based formulations Nanoxel™ and Abraxane™ is mediated by the process of

endocytosis and the uptake of paclitaxel was remarkably superior to Intaxel™ in all cell lines tested. Moreover, the intracellular uptake of paclitaxel in Nanoxel™- and Abraxane™-treated groups was comparable. Hence, the nanoparticle-based formulations of paclitaxel (Nanoxel™ and Abraxane™) are endowed with higher efficiency to deliver the drug to target cells as compared to the conventional Cremophor-based formulation.

**Conclusion** Nanoxel™ appears to be of great promise in tumor targeting and may provide an advantage for paclitaxel delivery into cancer cells.

**Keywords** Paclitaxel · Delivery system · Tumor · Nanoxel · Nanoparticle · Intaxel

## Introduction

Paclitaxel is a diterpenoid natural product derived from the bark of the Pacific Yew tree. It is an effective first-line drug against breast and ovarian cancer. It binds to the beta subunit of microtubules promoting their polymerization and stabilization which leads to mitotic arrest and cell death [1].

Paclitaxel is highly hydrophobic and virtually insoluble in water, limiting its practical use, despite its anti cancer activity [2]. Currently, paclitaxel is available in a formulation, which contains Cremophor EL as a solubilizer. This formulation is associated with serious side effects, such as severe hypersensitivity reactions, nephrotoxicity, and neurotoxicity [3, 4]. Hence, to improve its clinical administration, there is a need for an improved formulation of paclitaxel.

Many approaches, including micellar carriers, soluble polymers, soluble prodrugs, and polymeric nanocapsules, have been proposed in order to improve the therapeutic

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effects of paclitaxel and reduce its adverse effects [5, 6]. The nanoparticle carriers have the potential to improve the solubility of the drug and its delivery to the target cells or organs.

Nanoxel™ is a newly developed, pH sensitive, Cremophor-free, novel nanopolymer-based tumor-targeted delivery system for paclitaxel produced by Fresenius Kabi Oncology Limited, Himachal Pradesh, India. Nanoxel contains a co-polymer of *N*-isopropyl acrylamide (NIPAM) and vinyl pyrrolidone (VP). This polymer is biodegradable and amphiphilic in nature and it forms nanometer-sized micelles (mean particle size 80 nm) when exposed to water. Paclitaxel retains in the hydrophobic core region, which is surrounded by hydrophilic groups in the periphery. Finally, the hydrophobic paclitaxel is released slowly by surface erosion of the hydrophilic polymer (reference). The nanoparticle size of 80 nm permits selective entry into the tumor cells, utilizing the enhanced vascular permeability associated with tumorigenesis while sparing normal tissue. Nanoxel™ is shown to have minimized toxicity, along with an increase in the antitumor activity, due to selective accumulation of the drug in tumor [7].

Intaxel™ (Fresenius Kabi Oncology Ltd, Himachal Pradesh, India) and Abraxane™ (Abraxis BioScience, IL, USA) are commercially available forms of paclitaxel. Intaxel is a Cremophor-based formulation, which physicians prescribe in the treatment of breast, lung and ovarian cancer [8]. An earlier study conducted with 39 patients with NSCLC reported that 24-h infusion of Intaxel at a dose of 135 mg/m<sup>2</sup> is efficient and well-tolerated regimen in the treatment of advanced NSCLC patients [8].

Abraxane, nanoparticle albumin-bound paclitaxel, addresses the toxicities associated with excipients in taxane-based chemotherapy and minimizes the occurrence of severe anaphylactic reactions. In addition, it has been purported to improve drug delivery into tumor cells when compared to paclitaxel [9–11]. It has been approved for the treatment of cancer [12–15].

This study aimed to compare the efficiency and mechanism of intracellular paclitaxel delivery by Nanoxel with Abraxane and Intaxel using different cell lines representing target cancers (breast, ovary and NSCLC) by transmission electron microscopy and quantitative intracellular paclitaxel measurements by high performance liquid chromatography.

## Materials and methods

### Materials

Abraxane™ was obtained from Abraxis Bioscience, IL, USA. Nanoxel™ and Intaxel™ injection 100 were procured from Fresenius Kabi Oncology Ltd., Himachal

Pradesh, India. The vehicle/solvent (excipients) used for the preparation of Nanoxel were obtained from Fresenius Kabi Oncology Ltd. Himachal Pradesh, India. The cancer cell lines used in this study included HBL-100 (Human Breast carcinoma), obtained from National Centre for Cell Science, India; PA-1 (human ovarian carcinoma) and A549 (human lung carcinoma, NSCLC), procured from American Type Culture Collection, Virginia, USA.

### Preparation of Nanoxel, Intaxel and Abraxane

Nanoxel was prepared by adding 10 % dextrose with 0.03 % of excipients of Nanoxel and 20 mg/ml of paclitaxel. Intaxel was prepared by adding 0.6 mg/ml of paclitaxel in a solution containing 0.1 % of Intaxel in 10 % dextrose. 100 mg of Abraxane which contained the paclitaxel at a concentration of 5 mg/ml was reconstituted with 20 ml of sterile 0.9 % w/v sodium chloride injection (Clarix life sciences). Nanoxel, Intaxel and Abraxane stock solutions were further diluted using HBSS (Sigma) to obtain 100 μM of paclitaxel and were used for subsequent treatments.

### Cell culture

HBL-100 was cultured in media containing RPMI 1640 (Biowhittaker Inc, MD, USA), whereas, human ovarian carcinoma (PA-1) was cultured in minimum essential media (Lonza Walkersville, Inc., MD, USA) and human lung carcinoma (A549)–NSCLC in F-12K (Caisson laboratories) media supplemented with 10 % FBS (Himedia Laboratories Pvt. Ltd., Mumbai, India) that was incubated at 37 °C under 5 % CO<sub>2</sub>.

The cell lines were seeded at the density of 0.5 million cells/60 mm petri dish in the respective growth medium. Thereafter, the plates were kept for overnight incubation under appropriate growth conditions as mentioned above for each cell line and used in the latter experiments.

### In vitro cellular uptake of paclitaxel

Following overnight incubation, the medium of the plates containing the cells was replaced with fresh cell culture medium corresponding to each cell line. The cells were then treated with Nanoxel, Intaxel and Abraxane corresponding to a paclitaxel concentration of 10 μM. The vehicle control plates were treated only with the vehicle used in Nanoxel corresponding to volume of excipients in Nanoxel formulation. Untreated cells were used as control.

After treatment, the cells were subjected to transmission electron microscope (TEM) studies, in order to assess the uptake of drugs (qualitative assessment), and high performance liquid chromatography (HPLC) studies to quantify the paclitaxel released into the cells.

### Qualitative assessment by TEM

Cells were harvested after 30 min of treatment by gentle scraping and centrifuged at 2,000 rpm for 10 min. The cell pellets were fixed in the fixative solution for 1 h at room temperature. Fixed cells were washed three times with phosphate buffered saline (PBS), pH 7.4 by centrifugation at 2,000 rpm for 10 min and stored at 4 °C. Cells were stained with 2 % osmium tetroxide and dehydrated with a series of ethanol solutions. Cells were infiltrated using propylene oxide and spurr's resin. After polymerization of blocks, ultrathin sections were cut using ultramicrotome and collected on copper grids. Sections were stained with uranyl acetate and images were visualized using TEM.

### Quantitative assessment of by HPLC

After drug treatment, the cells were harvested at 15, 30 and 60 min time intervals and were used to determine intracellular concentrations of paclitaxel at each time point. Cells were harvested, washed as describe previously and suspended in 0.5 ml of 2 % SDS (S.D. Fine chemicals) solution (w/v). Thereafter, the cells were subjected to sonication for 3 min to obtain the cell homogenate and stored at –20 °C. The above cell homogenates were then thawed at room temperature and 100 µl of each was then processed using 100 µl of methanol as precipitation agent. Paclitaxel concentration in each processed sample was then determined by standard linearity HPLC (Shimadzu LC 2010) method.

HPLC system was equipped with YMC ODS-A, 3 µm, 150 × 4.6 mm column and the detector wavelength was set as 225 nm. Mixture of phosphate buffer and acetonitrile (50:50; v/v) was used as the mobile phase at a flow rate of 1 ml/min.

The cellular uptake efficiency was calculated as follows:

Cellular uptake efficiency = amount of paclitaxel in the cells after incubation × 100/amount of paclitaxel in the liposome added to the cells.

## Results

The cellular uptake of Nanoxel, Intaxel and Abraxane was studied both qualitatively and quantitatively.

### Visualization of uptake of Nanoxel, Intaxel and Abraxane

Nanoxel, Abraxane and Intaxel were prepared to have a final concentration of 10 µM of paclitaxel and cultured with various human cancer cell lines such as A549, HBL-100 and PA-1 cell lines. After 30 min, the cells were

processed and studied under TEM in order to study the uptake of drugs.

Nanoxel and Abraxane were successfully taken up by A549 cell lines used in this study. After 30 min, they penetrated into the plasma membrane and formed well-defined and intact endocytic vesicles (Fig. 1b, c). The length of the endocytic vesicles were 155.34 and 141.87 nm for Nanoxel- and Abraxane-treated cells, respectively. Similarly, the vehicle used for preparation of Nanoxel also formed endocytic vesicle (Fig. 1e). In a comparison, the untreated control cells showed a typical morphology of malignant cell and absence of any endocytic vesicle formation (Fig. 1a). On the other hand, the cells treated with Intaxel contained numerous swollen mitochondria and did not show any endocytic vesicle formation inside the cells 30 min after treatment with Intaxel. (Fig. 1d).

In order to confirm the efficient uptake of Nanoxel and Abraxane, the other human cancer cell lines, HBL-100 (Fig. 2) and PA-1 (Fig. 3), were treated. As in A549 cell line, Nanoxel and Abraxane entered into the HBL-100 and PA-1 cells, after 30 min of treatment, and formed endocytic vesicles. The untreated control cells and Intaxel-treated cells did not show any such phenomenon, when seen under TEM.

### Quantitative analysis of cellular uptake of Nanoxel, Abraxane and Intaxel

The encouraging qualitative results obtained using TEM studies prompted us to quantify the drug inside the human cancer cell line.

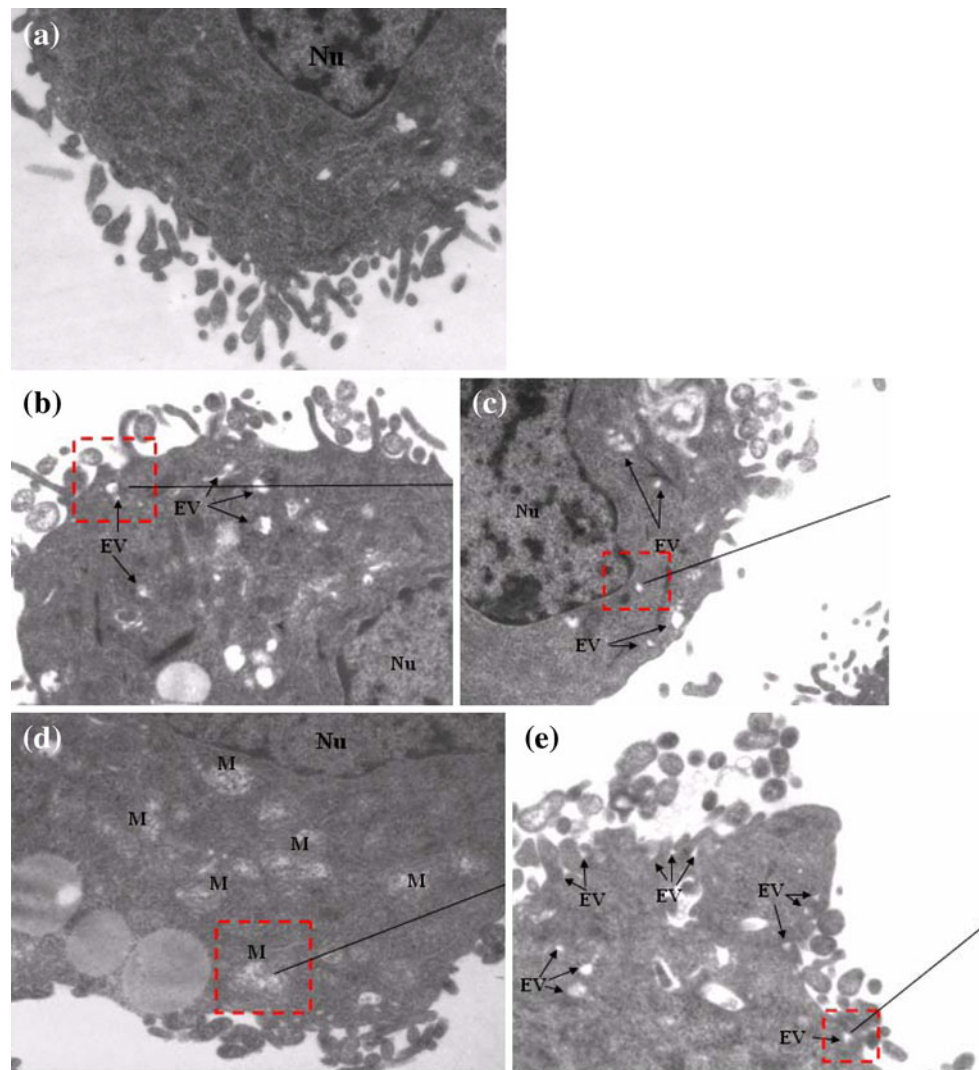
The PA-1, HBL-100 and A549 cell lines were treated with Nanoxel, Abraxane and Intaxel and subjected to HPLC studies in order to quantify the level of paclitaxel at time points of 15, 30 and 60 min. The uptake of paclitaxel was observed in all cell lines treated with Nanoxel, Abraxane and Intaxel within 15 min. The quantity of paclitaxel was found to be significantly higher in Nanoxel- and Abraxane-treated cells when compared to Intaxel-treated cells at all time points tested.

In PA-1 cells, treatment with Nanoxel and Abraxane resulted in up to 3.5- and 4.3-fold higher intracellular uptake of paclitaxel ( $p < 0.001$ ), respectively, as compared to Intaxel after 30 min of treatment (Table 1c).

Similarly, the intracellular uptake of paclitaxel was found to be significantly higher ( $p < 0.001$ ) (3.27- and 3.31-fold) in Nanoxel and Abraxane-treated HBL-100 cells, as compared to Intaxel, after 30 min of treatment (Table 1b).

In A549 cells also, the intracellular accumulation of paclitaxel was found to be significantly higher ( $p < 0.05$  and  $p < 0.001$ ) (2.5- and 3.3-fold) in Nanoxel- and

**Fig. 1** Visualization of uptake of Nanoxel, Intaxel and Abraxane™ by A549 cells. Intracellular uptake of paclitaxel in A549 cells treated with no drug (a), Nanoxel (b), Abraxane (c), Intaxel (d) and vehicle for Nanoxel (e) after 30 min. In b, d and e, the red mark indicates the formation of endocytic vesicles, whereas in c it indicates the swollen mitochondria. EV endocytic vesicles, M mitochondria



Abraxane-treated cells, respectively, as compared to Intaxel-treated cells after 30 min of treatment (Table 1a).

## Discussion

Paclitaxel is one of the most effective anticancer agents available clinically and it has a wide spectrum of activity against solid tumors [16]. However, its clinical use is impaired by its poor aqueous solubility. pH sensitive nanoparticles offer great potential to deliver therapeutic agents as they have higher efficiency, high selectivity and low toxicity [17]. Nanocarriers encapsulate, covalently attach, or adsorb the therapeutic agents thus overcoming drug solubility problems.

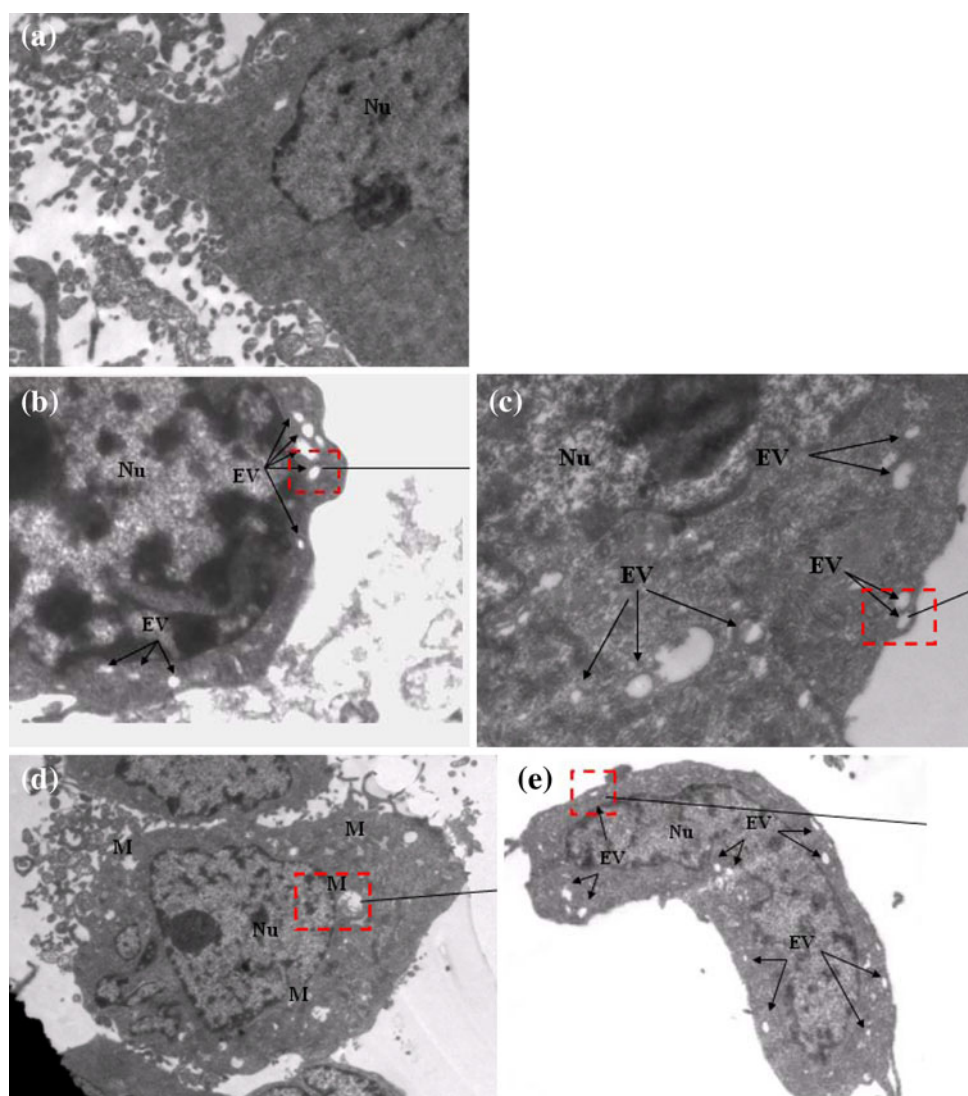
The present study was undertaken to compare the efficiency, transport and intracellular accumulation of paclitaxel in target cancer cells treated with Nanoxel, Intaxel and Abraxane at the clinically relevant dose of paclitaxel. It is

noteworthy to mention that Nanoxel is approved in India by drug controller general of India (DCGI) for clinical use.

Earlier studies indicated that nanoparticles are transported through endocytosis into cells [18]. Thus, we first studied the formation of endocytic vesicles using TEM, an advanced and accurate technique to assess intra cellular organelles and processes [19].

In this study, three different human cancer cell lines were used: A549, HBL-100 and PA-1, which represent NSCLC, breast cancer and ovarian cancer, respectively. The formation of well defined and intact endocytic vesicles in cells treated with Nanoxel or Abraxane indicated the transport of paclitaxel into the cells. The cells treated with vehicles used to prepare Nanoxel also formed endocytic vesicles after 30 min of treatment. In addition, no formation of endocytic vesicles in untreated control cells confirmed the formation of endocytic vesicles was due to the treatment with drug. The formation of endocytic vesicles in three types of human cancer cell lines confirmed that

**Fig. 2** Visualization of uptake of Nanoxel, Intaxel and Abraxane by HBL-100 cells. Intracellular uptake of paclitaxel in HBL-100 cells treated with none of the drug (a) Nanoxel (b), Abraxane (c), Intaxel (d) and vehicle for Nanoxel (e) after 30 min. In b, d and e, the red mark indicates the formation of endocytic vesicles, where as in c it indicates the swollen mitochondria. EV endocytic vesicles, M mitochondria



Nanoxel and Abraxane can transport into a wide range of tumor cells.

Another form of paclitaxel, Intaxel, failed to form endocytic vesicles and formed only numerous swollen mitochondria strongly indicating oxidative stress. It has been postulated that direct perturbation of cell membrane and/or formation of reactive oxygen species by peroxidation of polyunsaturated fatty acids could contribute to this oxidative stress [20].

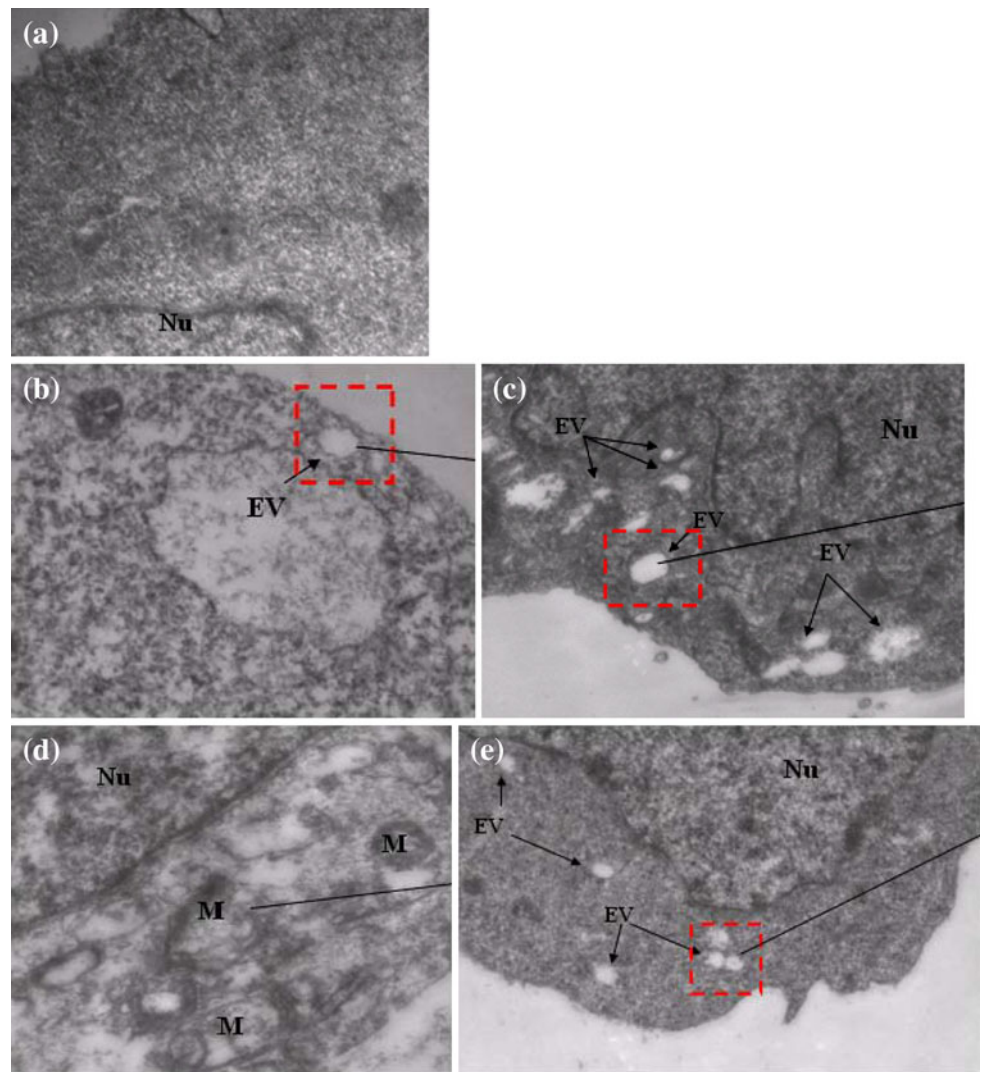
Overall, the visual data obtained from this study suggests that both Nanoxel and Abraxane are internalized into cancer cells by a process of endocytosis as compared to Intaxel, which is internalized by a non-endocytic mechanism.

In addition to a targeted drug disposition, the duration of drug retention at the target site could be critical to achieve the desired therapeutic outcome in cancer treatment [21]. Thus, we were further interested to assess the intracellular

quantity of paclitaxel upon treatment with all the three drugs. As expected, in the Nanoxel- or Abraxane-treated cells, the quantity of paclitaxel was significantly higher even after 15 min of the drug treatment and it was retained until 60 min. This difference is likely due to the different modes of transportation of drug into cells.

In general, fast-growing cancer cells require the recruitment of new vessels or rerouting of existing vessels near the tumor mass to supply them with oxygen and nutrients. The resulting imbalance of angiogenic regulators such as growth factors and matrix metalloproteinases makes tumor vessels highly disorganized and dilated with numerous pores showing enlarged gap junctions between endothelial cells and compromised lymphatic drainage [22]. Using this unique pathophysiology of tumor cells, called enhanced permeability and retention (EPR) effect, macromolecules such as nanoparticles accumulate in greater quantity in tumor tissue [23]. Smaller molecules,

**Fig. 3** Visualization of uptake of Nanoxel, Intaxel and Abraxane by PA-1 cells. Intracellular uptake of paclitaxel in PA-1 cells treated with none of the drug (a), Nanoxel (b), Abraxane (c), Intaxel (d) and vehicle for Nanoxel (e) for 30 min. In b, d and e, the red mark indicates the formation of endocytic vesicles, where as in c it indicates the swollen mitochondria. EV endocytic vesicles; M mitochondria



**Table 1** Kinetics of uptake of paclitaxel in Abraxane-, Nanoxel- and Intaxel-treated cells: (a) PA-1, (b) HBL-100, (c) A549

Time points (min)	% of paclitaxel uptake		
	Intaxel	Abraxane	Nanoxel
<b>a</b>			
15	3.39	9.18*	8.81*
30	2.95	9.79*	9.67*
60	3.67	9.88*	10.95*
<b>b</b>			
15	3.63	9.20*	10.28*
30	2.48	10.77*	8.76*
60	3.95	9.88*	9.03*
<b>c</b>			
15	2.06	5.09*	5.22*
30	2.31	7.73*	5.79*
60	4.76	8.78*	5.75*

% of paclitaxel uptake was significantly higher than Intaxel (\*  $p < 0.05$ )

such as Intaxel, do not exhibit this EPR effect. Thus, the cancer cells may take up higher quantity of paclitaxel in the form of Nanoxel and Abraxane when compared Intaxel.

The HPLC studies indicated the presence of free paclitaxel inside the drug-treated cancer cells. Following the entry of Nanoxel and Abraxane into the cells through endocytosis, the low pH in the endolysosome would presumably degrade the pH sensitive carrier of paclitaxel in the Nanoxel form and release the free paclitaxel inside the cell. Of note, release of intracellular paclitaxel from nanomicelles in the lysosomal compartment was correlated with in vitro release of the drug at varying pH conditions. This previous study revealed that tubulin polymerization of paclitaxel was retained in the form of Nanoxel [24].

Overall observation leads to a hypothesis that the macromolecule carrier in the Nanoxel helps to accumulate in the tumor cell in high quantity. Following the entry of Nanoxel into the cell through endocytosis, free paclitaxel is released in the endolysosomal fusion due to the low pH.

The released free paclitaxel stabilizes the tubulin formation that leads to apoptosis of cancer cells.

Altogether, the results obtained from the present study suggest that the nanoparticle-based formulations of paclitaxel (Nanoxel and Abraxane) deliver the drug to tumor cells with higher efficiency than a conventional Cremophor-based formulation. In conclusion, Nanoxel appears to be of promise in tumor targeting and may provide advantages for paclitaxel delivery in cancer cells.

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**Conflict of interest** This project was financially supported by Fresenius Kabi. Dr. Hrishikesh Kulkarni, Dr. Sadanand Kulkarni and Dr. Shiva Kant Mishra are employees of Fresenius Kabi.

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