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In vitro evaluation of Aurora kinase inhibitor —VX680—in formulation of PLA-TPGS nanoparticles

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Abstract

Polymeric nanoparticles prepared from poly(lactide)-tocopheryl polyethylene glycol succinate (PLA-TPGS) were used as potential drug carries with many advantages to overcome the disadvantages of insoluble anticancer drugs and enhance blood circulation time and tissues. VX680 is an Aurora kinase inhibitor and is also the foremost Aurora kinase inhibitor to be studied in clinical trials. In this study, we aimed to investigate whether VX680-loaded PLA-TPGS nanoparticles (VX680-NPs) are able to effectively increase the toxicity of chemotherapy. Accordingly, we first synthesized VX680-loaded nanoparticles and NP characterizations of morphology, mean size, zeta potential, and encapsulation efficiency were spherical shape, 63 nm, −30 mV and 76%, respectively. Then, we investigated the effects on HeLa cells. The cell cytotoxicity was evaluated by the xCELLigence real-time cell analyzer allowing measurement of changes in electrical impedance on the surface of the E-plate. Analysis of nucleus morphology and level of histone H3 phosphorylation was observed by confocal fluorescence scanning microscopy. Cell cycle distribution and apoptosis were analyzed by flow cytometry. Our results showed that VX680-NPs reduced cell viability with IC_{50} value lower 3.4 times compared to free VX680. Cell proliferation was inhibited by VX680-NPs accompanied by other effects such as high abnormal changes of nucleus, a decrease of phospho-histone H3 at Ser10 level, an increase of polyploid cells and resulted in higher apoptotic cells. These results demonstrated that VX680-NPs had more cytotoxicity than as treated with VX680 alone. Thus, VX680-NPs may be considered as promising drug delivery system for cancer treatment.

Keywords: nanoparticle, VX680, PLA-TPGS, xCELLigence, Aurora kinase inhibitors
Classification numbers: 2.04, 2.05

1. Introduction

Aurora kinases are serine/threonin kinases that are essential in multiple functions of mitosis. In mammals, the Aurora kinases consist of A, B, and C and they are involved in spindle apparatus organization during cell division [1, 2]. Studies have indicated that Aurora A and B are overexpressed in lung cancer [3], cervical cancer [4], colorectal cancer [5], prostate cancer [6], renal carcinoma [7], hepatocellular carcinoma [8], ovarian cancer [9] and bladder cancer [10]. As one of the main reagents of the chromosomal passenger complex which is a functional mitotic structure, Aurora B plays an important role in chromosomal biorientation, regulating the association of kinetochores and microtubules as well as cytokinesis [2]. Particularly, the role of Aurora B in phosphorylating histone H3 which is believed to aid in chromatin condensation and separation has also been explored. When Aurora B localizes at the centromere, it can control the activity of other kinases positioned along the chromosome arm [11]. In recent years, Aurora kinases are promising targets for cancer treatment. This led to development of new anticancer drugs that could target Aurora kinases. Currently, inhibitors of Aurora kinase have been discovered as a novel class of anti-cancer drugs under preclinical and clinical testing [1, 2, 12, 13] including VX680, MLN8237, AT9283, AZD1152 and ZM447439. Among these inhibitors, VX680 can associate with the ATP-binding site of Aurora kinases and inhibit all three isoforms, specially, Aurora kinases A and B. In its mechanism of action, VX680 delays cytokinesis, disrupts bipolar spindle formation but allows the cells to entry through the other stages of mitosis, which results in polyploidy or massive apoptosis in some cancer cell lines. VX680 was also the foremost Aurora kinase inhibitor to be studied in clinical trials. However, as the other anti-cancer drugs, VX680 is limited by its water-insoluble property [2, 14, 15].

The use of non-toxic biodegradable polymers for synthesizing nanoparticles can improve the solubility, the biocompatibility, and treatment efficiency and control the drug release of anti-cancer drugs [16, 17]. Poly(lactic acid) (PLA), an aliphatic polyester, is one of most extensively investigated biodegradable polymers which approved by the US Food and Drug Administration [18], was chosen to entrap VX680 in nanoparticles. However, PLA was limited by some chemophysical characteristics such as hydrophobicity, low degradation rate [19, 20] as used for drug delivery. The PLA surface modifications with D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) can limit disadvantages [20, 21]. TPGS was demonstrated not only to improve the drug solubility and treatment effects, overcome multidrug resistance, enhance cellular uptake but also to exhibit anti-cancer effects by P-glycoprotein inhibition mediated multidrug resistance [22–24]. In this study we developed PLA-TPGS nanoparticles for loading VX680 and investigated some cytotoxic effects of this drug delivery system compared to free VX680 on HeLa cells.

2. Materials and methods

2.1. Cell and reagents

HeLa cell line was purchased from ATCC. The cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. After cell growth reached 70%–80% confluence, logarithmic phase cells were used for the experiment. VX680 (Tozasertib, MK-0457) was purchased from BioVision (USA) and was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA), stored at −80 °C and diluted in fresh medium immediately before use. Staining agents such as Hoechst and propidium iodide (PI) were obtained from Invitrogen. All other organic solvents were analytical grade from Fisher Scientific.

2.2. Preparation of VX680 loaded PLA-TPGS nanoparticles

VX680 loaded PLA-TPGS nanoparticles (VX680-NPs) were prepared using emulsion solvent evaporation/diffusion technique and PLA-TPGS copolymer used in this study was synthesized in our previous studies [25, 26]. Briefly, 10 mg of VX680 were dissolved in 10 ml DMSO and 30 mg of PLA-TPGS copolymer in 30 ml of distilled water and stirred for 3 h to ensure complete dissolution of PLA-TPGS. The mixture was closed and stirred for 24 h. After that, the obtained mixture was dialyzed against distilled water for 48 h to remove DMSO. Dialyzed product was centrifuged at 5600 rpm to remove un-encapsulated VX680. Transparent solution containing VX680/PLA-TPGS NPs was obtained and stored at 4 °C for further uses.

2.3. Characterization of VX680 loaded PLA-TPGS nanoparticles

Morphological characteristics of VX680-NPs were observed by field emission scanning electron microscopy (FESEM) (Hitachi S-4800 FE-SEM).The average size of VX680-NPs was analyzed by dynamic light scattering (DLS). The zeta potential of these NPs was evaluated in deionized water using the electrophoretic mode of Zetasizer 3000 HS (Malvern instruments Ltd, UK). Each sample was measured in triplicate.

The encapsulation efficiency (EE) of VX680 into PLA-TPGS nanoparticles was calculated by following formulation from the HPLC results provided by sample solutions:

$$\mathrm{EE}(\%) = \frac{\text{total drug content} - \text{free drug content}}{\text{total drug content}} \times 100\%.$$

2.4. Evaluation of cellular cytotoxicity

To evaluate the effects of VX680 and VX680-NPs, we developed a system using the xCELLigence real-time cell analyzer (RTCA). HeLa cells were seeded on 96-well E-plates. After 24 h seeding, cells were treated with VX680

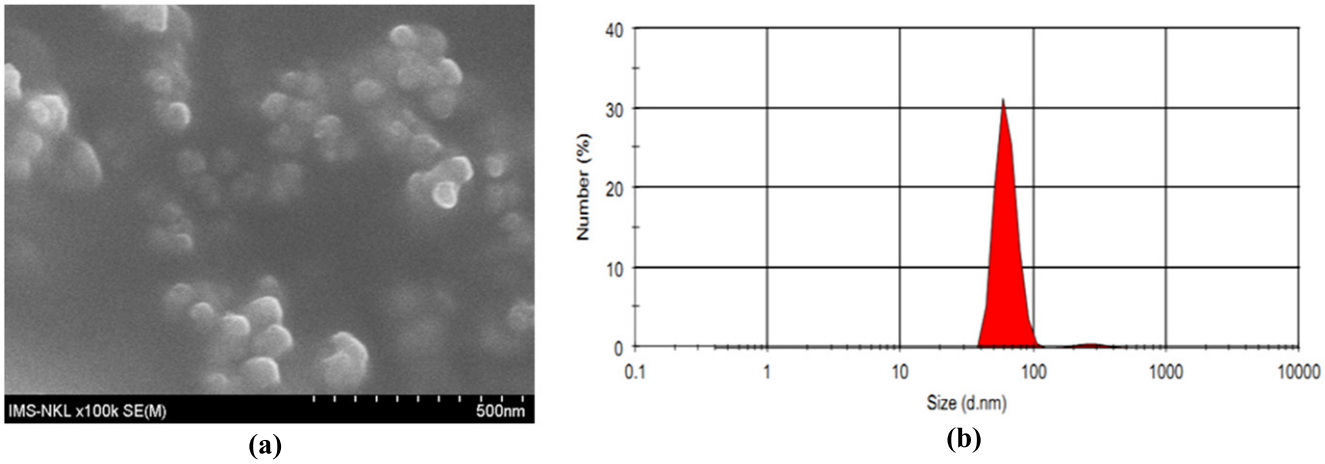

Figure 1. Characterization of VX680-loaded PLA-TPGS nanoparticles: (a) FESEM image of VX680-NPs and (b) size distribution of VX680-NPs.

and VX680-NPs and incubated at 37 °C, 5% CO_2 within 120 h. The normalize cell index (NCI) was evaluated and IC_{50} was determined by xCELLigence system (Roche).

2.5. Morphological examination

For the morphological examination of nuclear modification, cells were incubated with either VX680 or VX680-NPs at the concentration of 0.2 μM (calculated by VX680) for 24 h and then cells were stained with Hoechst 33342. Hoechst 33342 solution was added to the culture medium at a concentration of 1.6 μM. After incubating for 30 min, cells were collected and washed with phosphate buffered saline (PBS) and observed by confocal fluorescence scanning microscopy (Zeiss LMS 510 confocal microscopy).

2.6. Cell cycle analysis

Cells were seeded at 1.5×10^6 in 2 ml of DMEM per petri dish with diameter of 35 mm, allowed to grow for 24 h before being exposed to drug. Cells were treated with VX680 or VX680-NPs at the concentration of 0.2 μM (calculated by VX680) for 24 h and DNA content was determined by flow cytometry. Briefly, after incubation cells were collected to centrifuge tubes and fixed with ethanol 70%. Then, cells were stained with PI and treated with ribonuclease. Cell samples were diluted with PBS to appropriate concentration of 10^6 cells ml^{-1} to determine the DNA content by BD FACSCanto II Flow Cytometer.

2.7. Apoptosis analysis

Annexin V conjugated alexa fluor®488/PI apoptosis assay (Invitrogen) was employed to detect early apoptotic cells. After treatment at the concentration of 0.2 μM (calculated by VX680) for 48 h, cells were harvested and prepared for apoptosis analysis follow the instruction of the supplier. Briefly, cells were washed with PBS, then suspended in annexin-binding buffer to get the density at 10^6 cells ml^{-1}. Incubate cells solution with 5 μl of alexa fluor®488—annexin V and 100 μl of PI working solution for 15 min at room temperature. Gently mix with 400 μl of annexin-binding buffer and analyze cells solution on FACS Canto II system (BD).

2.8. Immunofluorescence

To investigate the effect of VX680 and VX680-NPs on phosphorylation level of histone H3 at Ser10, cells were grown on coverslips and treated with VX680 or VX680-NPs at the concentration of 0.2 μM (calculated by VX680) for 24 h. Then, cells were fixed in 2% formaldehyde (37 °C, 10 min), permeabilized in 90% ethanol (−20 °C, 15 min) and incubated with primary anti-phospho-Ser10-histone H3 mouse monoclonal antibody (Abcam, MA) for 1 h at room temperature (RT) and anti-mouse secondary antibodies conjugated with alexa fluor®488 (Santa Cruz, CA) for 30 min at RT. Fluorescently labeled cells were observed by confocal fluorescence scanning microscopy.

2.9. Statistical analysis

Statistical analyses were performed with GraphPad Prism 5 and RTCA software (Roche). The results were expressed as mean ± SD.

3. Results

3.1. Characteristics of VX680-NPs

The morphology and size of VX680-NPs were examined using FESEM (figure 1(a)) and DLS (figure 1(b)). The particles appeared to be spherical shape and have size range from 50 to 100 nm with the mean size of 63 nm.

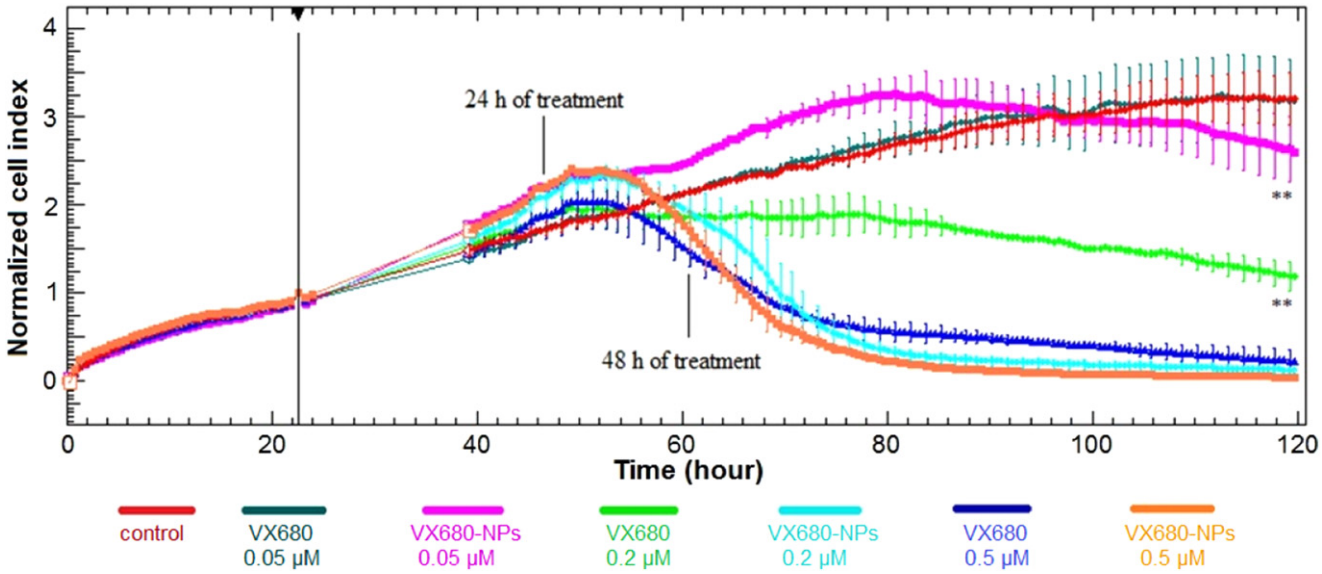

Figure 2. xCELLigence RTCA biosensor technology revealed the profile of cells treated with VX680 and VX680-NPs.

VX680-loaded PLA-TPGS nanoparticles prepared with emulsion solvent evaporation/diffusion technique resulted in a high drug EE of 76%. EE was calculated by measuring the concentration of free VX680 in supernatant from the ultracentrifugation of nanoparticles. The concentration of VX680 in the supernatant was determined by comparing the concentration to a constructed standard calibration curve. A standard calibration curve of VX680 with the absorbance was studied at 253 nm.

3.2. Evaluation of cellular cytotoxicity using xCELLigence biosensor technology

Cell indexes (CIs) were normalized at the time point of adding compound. The NCI values were used to create the cellular profiling upon drug treatment.

The comparison of HeLa cellular profiles between VX680 and VX680-NPs showed similar results at the higher doses: NCI values were higher than the control at the beginning and quickly dropped at the early stage of treatment (figure 2). RTCA profiles generated with two drugs treated on HeLa cells with indicated concentrations for 5 d. The CI profiles reflected the initial cell attachment, logarithmic growth phase, and responded to the compound treatment. CIs were normalized at the time point of addition compound. The normalized time point is indicated by the vertical line. Cell profiles at concentration of 0.05 and 0.2 μM showed significant difference between VX680 and VX680-NPs ($^{**}p < 0.01$). Each data point was calculated from triplicate values. Data represent the average $\pm$ standard deviation ($n = 4$).

At the dose of 0.05 μM the NCI values of VX680-NPs treated-cells were higher than the control and gradually decreased after 60 h of incubation; meanwhile the VX680-treated NCI values kept equal to the control during whole time of experiment. At the dose of 0.2 μM after 30 h of incubation, cells treated with VX680-NPs showed a dramatic decrease in cell growth. In the meantime, VX680-treated cell growth curve kept at a constant value from time point of 24–80 h of treatment then slightly decreased (figure 2). Using RTCA software, we got the time-dependent half maximal inhibitory concentration (IC_{50}) values of HeLa cells treated with either VX680 or VX680-NPs for 24, 48, 72 and 96 h (figure 3(a)), and the dose-response curve of these drugs on day 4 of treatment (figure 3(b)). Notably, the IC_{50} values of VX680-NPs were always lower than the VX680's one follow time of incubation. At time points of 72 h and 96 h treatment, the IC_{50} values of VX680-NPs were both smaller 3.2 and 4.3 times (respectively) than VX680 (figure 3(a)). The results indicated that nanoparticle form not only conserved the activity but also increased the effect of drug on HeLa cells.

3.3. The effect of VX680-NPs on cell cycle

The DNA content was assessed by flow cytometry analysis of cells stained with PI. Compared with the control group, the VX680 group showed change in DNA content after incubation of 24 h (figure 4). However, this change was even more significant in VX680-NPs treated samples with cells contained almost DNA content of 4N (3.1 folds higher) and >4N (1.2 folds higher) than the VX680. Most of cells treated with VX680-NPs were arrested at diploid and polyploid state $\geqslant$4N, accounted for 66.5%, meanwhile this number in VX680-treated cells was 33.3%. Thus, VX680 alone caused less polyploidy than VX680-NPs at the same time and concentration treatment.

These results were consistent with nucleus morphology analysis. The fluorescent images reflected the abnormal in the nucleus shape and size. The nucleus turned into multi-lobed morphology associated with appearing several restitution

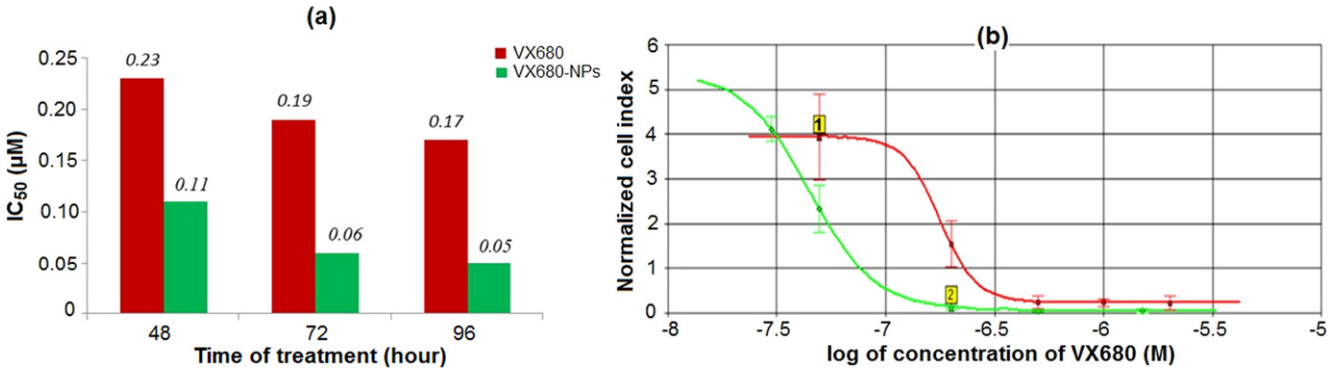

Figure 3. Cytotoxic effects of VX680 and VX680-NPs in HeLa cells. (a)Time dependent IC_{50} values of VX680 and VX680-NPs during 96 h of treatment; (b) dose-dependent curves with 5 concentrations of VX680 (0.05, 0.2, 0.5, 1 and 2 μM) in the form of VX680 alone (curve 1) and VX680-NPs (curve 2) after 96 h of treatment.

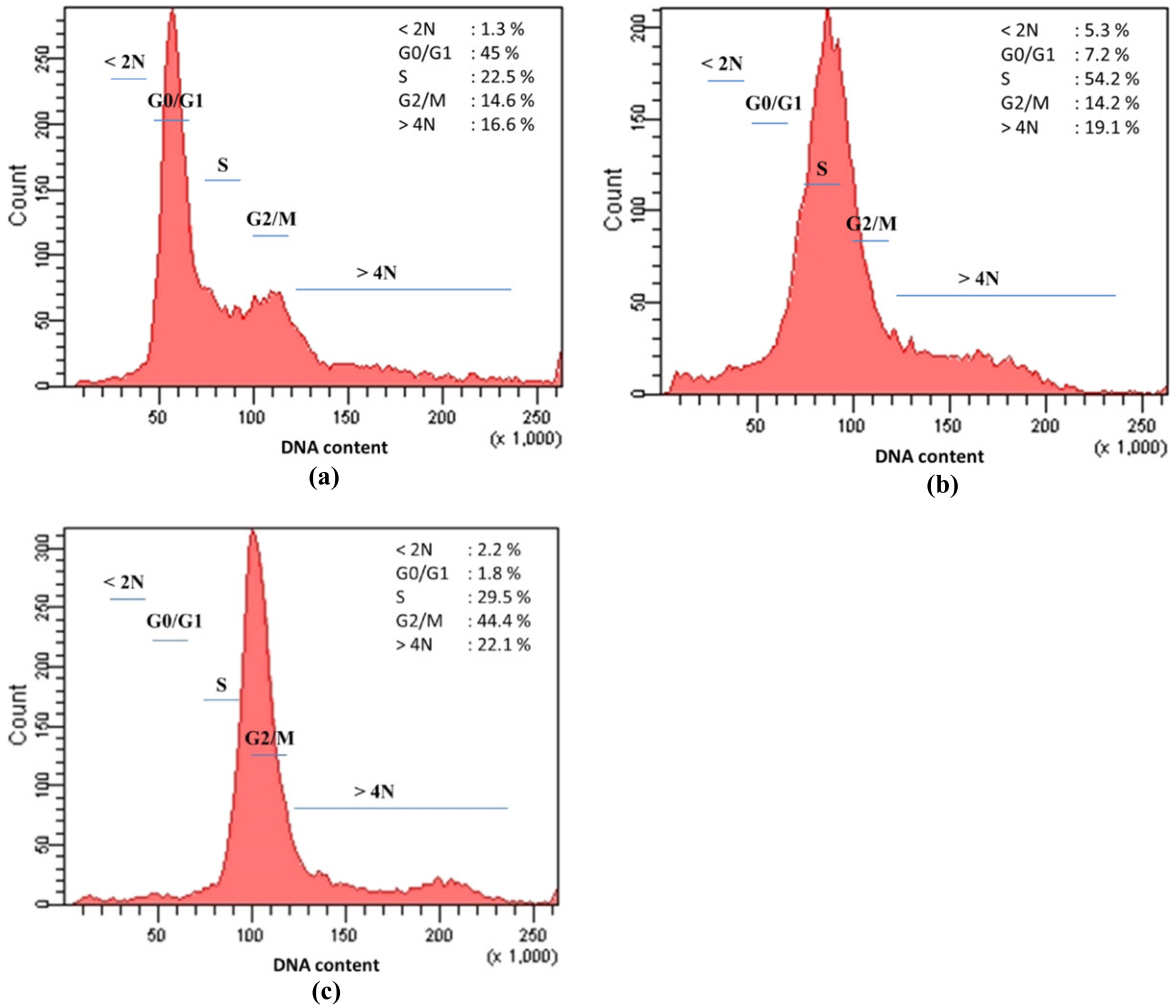

Figure 4. Effect of VX680-NPs on HeLa cell cycle. In the untreated sample (control), most cells accumulated on the G1 phase, which has chromosome set 2N, and least cells possessed chromosomes set >4N (a); in sample treated with VX680, cells occupied the abnormal DNA content with high percentage of >2N (b); however, in VX680-NPs treated sample, the most cells contained 4N and >4N DNA contents (c).

nuclei as treated with VX680 or VX680-NPs (figure 5). Simultaneously, we found that the nuclei of cells treated with VX680-NPs were larger and the number of multi-lobed morphology was higher than free VX680 after treatment of 24 h. Together with the results of cellular cytotoxicity, VX680-NPs showed that it had more efficient in term of time and concentration inhibiting HeLa cell growth and impairing cell cycle compare with normal VX680.

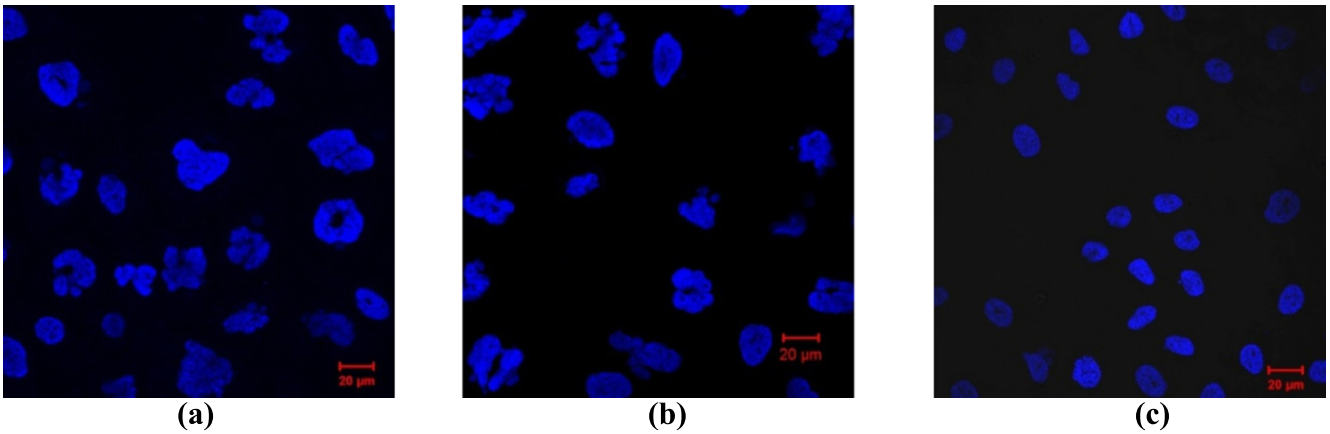

Figure 5. Fluorescent images of nucleus morphology stained with Hoechst. HeLa cells in VX680-NPs (a) treated sample showed largest and most multi-lobed nuclear in compare with the VX680-treated (b) and control (c) samples.

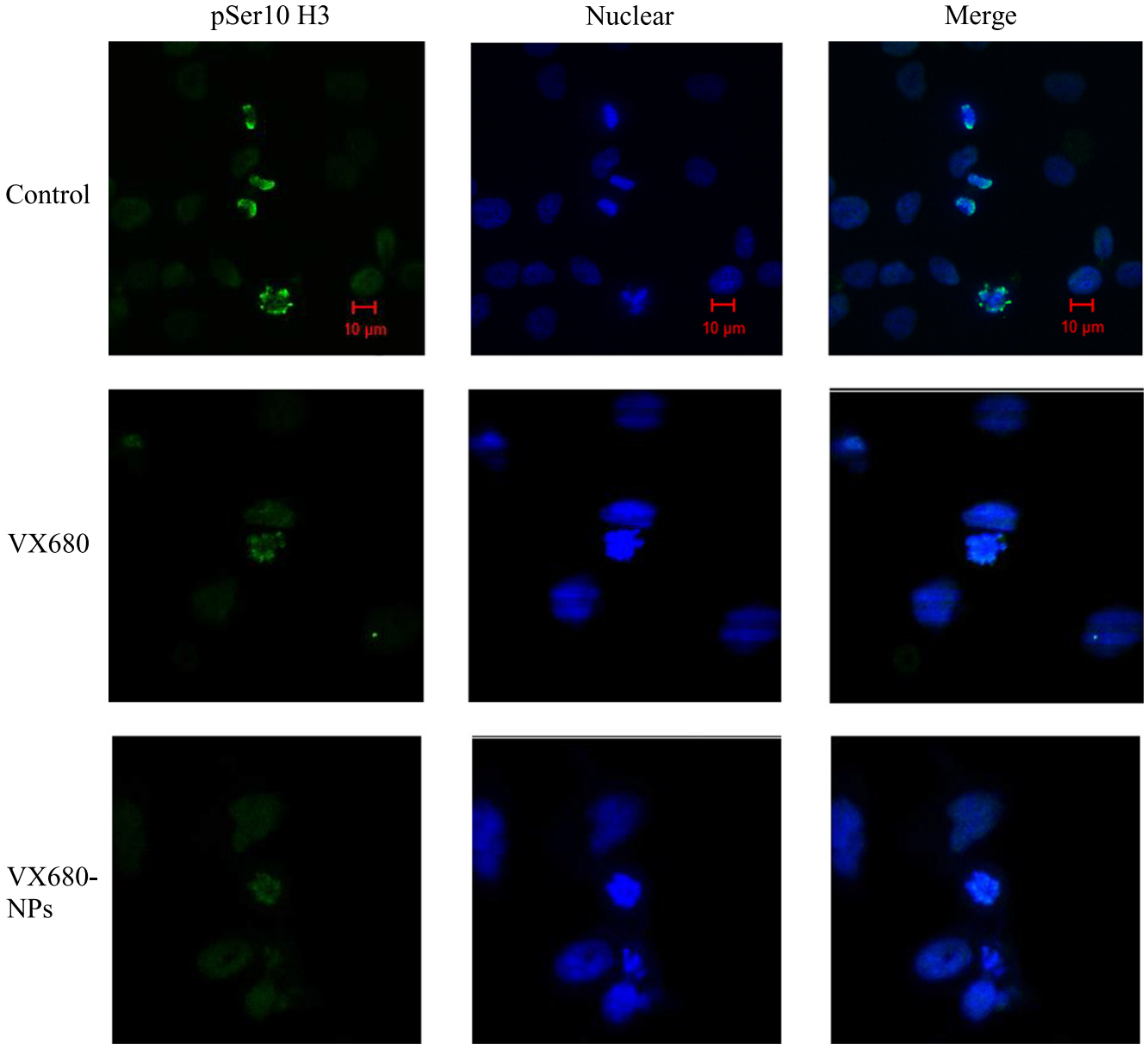

Figure 6. Phosphorylation of histone H3 at Ser10 detected by immunofluorescence staining. Scale bar: 10 μm.

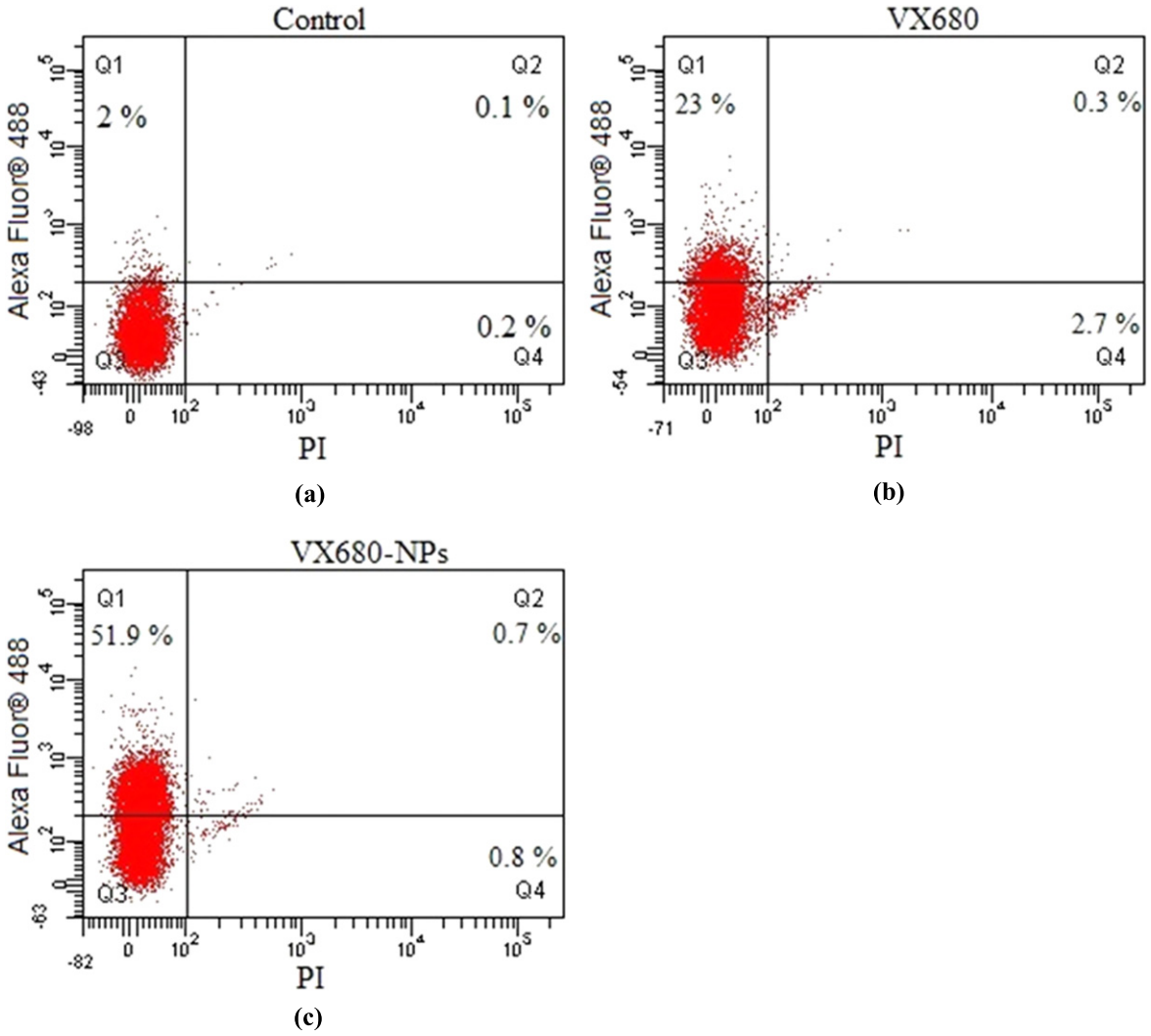

Figure 7. Apoptotic population in HeLa cells analyzed by flow cytometry after treatment of 48 h with (a) control sample, (b) VX680 and (c) VX680-NPs.

3.4. The effect of VX680-NPs on the level of phospho-histone H3 at serine 10

The ability of VX680 and VX680-NPs in inhibiting Aurora kinase B activities was examined via detection of phospho-histone H3 at serine 10 (pSer10 histone H3) immuno-fluorescent staining. The results showed that control sample had a clear and strong fluorescent signal of pSer10 histone H3 whereas samples treated with VX680, VX680-NPs had a decrease in the expression level of pSer10 histone H3 (figure 6). The fluorescent images showed a decrease in the expression level of histone H3 phosphorylation at Ser10 in samples treated with VX680 and VX680-NPs at concentration of 0.2 μm for 24 h. However, cells suffered from VX680-NPs treatment had the lowest expression of pSer10 histone H3. Therefore, we suggested that VX680-NPs inhibited the phosphorylation of H3 at Ser10 stronger than VX680 alone.

3.5. The effect of VX680-NPs on cell apoptosis

Most chemotherapeutic anti-cancer drugs used in the clinic today include agents that target the cell cycle in order to inhibit the hyper-proliferation state of tumor cells and subsequently to induce apoptosis, which is the desired outcome of chemotherapy. Checking the ability of VX680-NPs in increasing VX680—induced apoptosis is one of major aims of our work.

At 48 h post-treatment of drugs, cells treated with VX680-NPs and VX680 were collected and analyzed in FACS Canto II (BD). Dot plot map reflected the highest number of cells induced apoptosis in samples treated with VX680-NPs, 2.3 folds higher than VX680 alone. Obviously, the nanoparticulate formulation increased the drug-induced apoptosis in HeLa cells. Figure 7 shows the apoptotic population in HeLa cells analyzed by flow cytometry after treatment of 48 h, in which the quadrant Q1 represented for cells in early stage of apoptosis with high intensity of Alexa Fluor® 488 and low intensity of PI. In comparison with sample treated with VX680 alone (23%), cells suffered from treatment of VX680-NPs were forced to enter program cell death at higher level, accounting for 51.9% of all events.

4. Discussion

In published researches, nanoparticles smaller than 10 nm can be rapidly cleared by kidneys or through extravasation in contrast to larger nanoparticles which can be rejected by the immune system [27]. Nanoparticles smaller than 100 nm were demonstrated to be ideal size to enhance circulation time in the blood and reduce hepatic clearance [28]. This suggests that nanoparticles we designed have suitable size for use in cancer treatment with the zeta potential of VX680-NPs of −30 mV.

In the form of nanoparticles, VX680 was first checked its ability in inhibition of H3 phosphorylation at Ser 10, a natural substrate of Aurora B that is used as an indicator of Aurora B inhibition [11, 29–31]. The results revealed that the nanoparticle form not only kept the activity but also had more effect on this substrate of Aurora kinase B.

Previous studies on anti-mitotic drugs, such like VX680, have shown that treating cells at low concentrations can firstly cause mitotic slippage. This means cells escape the mitotic checkpoint and turn back to G1 phase without nuclear dividing. Some of cells die because of apoptosis, but other cells can continue to the next M phase with the second time of doubling the DNA content. After two or more rounds of mitotic slippage, most of cells will eventually go to apoptosis [29, 30, 32]. Possibility, in our research, VX680-NPs with higher solubility and smaller size could come inside cells easier and reach the adequate amount to induce apoptosis earlier than VX680.

xCELLigence RTCA equipment was used to evaluate cell viability and cytotoxicity based on changes in electrical impedance on the surface of a plate with electric nodes. CI values were relative to cellular changes including viability, morphology and adhesion degree. A normalized cell index (NCI) is introduced to reduce the influence of inter-experimental variations [33–35]. The higher NCI values of treated cells in compare to control could be explained by the mechanism of VX680 as an anti-mitotic drug. At the lower dose, the compound cause mitotic slippage several rounds before cells died. Mitotic slippage leads to the polyploidy in cells. At high doses, this phenomenon was shortened and cell died sooner. VX680 is a potent inhibitor of Aurora kinases that induces the accumulation of cells with >4N DNA content, followed by cell death. In cells lacking p53, endoreduplication and apoptosis in response to VX680 are markedly enhanced [15, 30, 36]. HeLa cell is reported to have in-activated p53 [37]. We suggested that both treated-cell profiles showed an increase in NCI value due to the increase in the size but not the number of cells. The expansion of site attachment probably led to elevation of the CI of treated cells, whereas the increase in cell number, cell viability, or attach degree is accountable for increasing CI values of the untreated sample [34]. Treatment with VX680-NPs showed that these cells were more likely to undergo endoreduplication, and resulted in higher NCI values, sooner and stronger than cells treated with VX680 alone lead to more polyploidy and multi-lobed nuclear. The results of real-time analysis experiment were coincidence with cell cycle and apoptosis analysis profile. Clearly, VX680-NPs showed more effective on HeLa cells than VX680.

5. Conclusion

VX680-loaded PLA-TPGS nanoparticles showed effectiveness as a pan—Aurora kinase inhibitor by inhibiting pSer10—H3, perturbed cell cycle, lead to polyploidy and induced apoptosis in cervical HeLa cancer cells. Moreover, our studies revealed that VX680-loaded PLA-TPGS nanoparticles not only conserved but also enhanced the activity of VX680. Thus, VX680-NPs may be considered as promising drug delivery system for cancer treatment.

Acknowledgments

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