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ORIGINAL RESEARCH

Nanodelivery and anticancer effect of a limonoid, nimbolide, in breast and pancreatic cancer cells

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Introduction: Nimbolide (Nim), a limonoid obtained from the neem tree, *Azadirachta indica*, has several pharmacological properties, including anticancer effects in different type of cancers. No drug-delivery system has been reported for enhancing the therapeutic application of this novel hydrophobic molecule.

Methods: In the present research, poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles of Nim (Nim-nano) were formulated by nanoprecipitation, characterized for physicochemical properties, and screened for anticancer potential in breast (MCF-7 and MDA-MB-231) and pancreatic (AsPC-1) cancer cell lines.

Results: The Nim-nano had a particle size of 183.73±2.22 nm and 221.20±11.03 nm before and after lyophilization, respectively. Cryoprotectants (mannitol and sucrose) significantly inhibited growth in particle size due to lyophilization. The ζ-potential of the Nim-nano was −22.40±4.40 mV. Drug loading and encapsulation efficiency of Nim-nano were 5.25%±1.12% and 55.67% ±12.42%, respectively. The Nim-nano exhibited sustained release of Nim for more than 6 days in PBS (pH 7.4) and showed two- to three-fold enhanced cytotoxicity in breast and pancreatic cancer cell lines compared with free Nim.

Conclusion: The Nim-nano formulation has great potential for treatment of cancers, such as pancreatic and breast cancer. Further, the PLGA-polymer surface can be modified by conjugation with polyethylene glycol, receptor-binding ligands (eg, folic acid), and other that which may lead to targeted delivery of Nim in the treatment of cancer.

Keywords: nimbolide, PLGA, nanoparticles, breast cancer, pancreatic cancer

Introduction

Cancer is the most dreadful disease worldwide and the primary cause of death in developed and developing countries.[1,2] Globocan 2012 data estimated 14.1 million cancer-diagnosed people worldwide and 8.2 million deaths due to the disease.[3] If the current trend continues, the estimated number of new cases would be 22 million and deaths of 13.2 million every year across the world by 2030.[4] The current concept of cancer treatment is to use drug combinations or a molecule that can modulate several targets, because cancer is a hyperproliferative disorder mediated through dysregulation of a number of genes and cell-signaling pathways.[5] Secondary plant metabolites have been used for a long time for management of various ailments, including cancer, because of their safety, efficacy, easy availability, and low cost.[6] Some phytochemicals used clinically as anticancer drugs are vinca alkaloids (vincristine and vinblastine), taxols (paclitaxel and docetaxel), etoposide, and camptothecin derivatives. Furthermore, phytoconstituents can target multiple signaling pathways without serious side effects or toxicity.[7]

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Nimbolide (Nim; 5,7,4'-trihydroxy-3',5'-diprenylflavanone, Figure 1), a tetranortriterpenoid, was first obtained from flowers and leaves of the neem tree. This bioactive compound belongs to the limonoid group and has a classic limonoid skeleton with an α,β-unsaturated ketone system and δ-lactone nucleus.[8] The α,β-unsaturated ketone system of Nim contributes to its anticancer potential.[9] Nim is a very hydrophobic drug (molecular weight 466.52) with solubility of ~50 mM in DMSO and ~8 mM in ethanol (product brochure; BioVision, Milpitas, CA, USA). The oral bioavailability of Nim has not been reported. Gupta et al reported only a single-point plasma concentration (2 hours from the last dose) of 222 and 409 ng/mL in mice after 10 days of repetitive intraperitoneal administration of 5 and 20 mg/kg/day of Nim, respectively.[6]

Nim has been used as an antimicrobial,[10,11] anti-HIV,[12] antifeedant,[13] antimalarial,[14] insecticidal,[15] antioxidant and free radical–scavenging,[16] anticolitis,[17] and anticancer agent. The anticancer nature of Nim has been widely screened against various types of cancer, such as lung carcinoma, melanoma, lymphoma, choriocarcinoma, colon carcinoma, promyleocytic leukemia, cervical carcinoma, hepatoma, hepatic carcinoma, fibrosarcoma, nasopharyngeal carcinoma, breast carcinoma, neuroblastoma, ovarian carcinoma, lymphocytic leukemia, prostrate carcinoma, monocytic leukemia, histiocytic lymphoma, glioblastoma, osteosarcoma, and pancreatic cancer.[7,18–22] Besides the anticancer activities of Nim as a single agent, it also shows additive or synergistic properties against cancer in combination with other anticancer agents (5-fluorouracil, thalidomide), various cytotoxic stimuli like TNFα, and TRAIL.[6,23] The cellular and molecular mechanism by which Nim produces cytotoxicity includes inhibition of cell-cycle progression, cell survival, migration, invasion, and initiation of apoptosis. The different targets altered by Nim are transcription factors, cytokines, growth factors and their receptors, enzymes, and genes controlling cell propagation and apoptosis.[7,18,19]

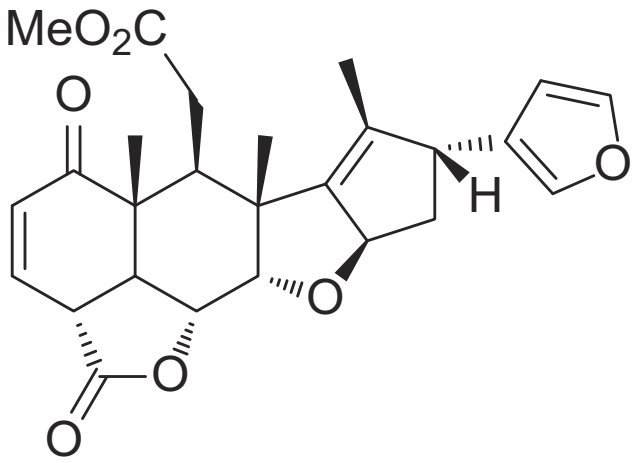

Figure 1 Chemical structure of nimbolide.

In addition to in vitro investigation in different cell lines, Nim has also been explored in vivo against colorectal cancer, lymphoma, brain cancer, Waldenström's macroglobulinemia tumor–xenografted mouse models, 7,12-dimethylbenz[a] anthracene-induced buccal pouch carcinogenesis, and oral carcinogenesis.[6,16,24–26] The LD_{50} of Nim administered intraperitoneally in adult male mice is 225 mg/kg and intravenously 24 mg/kg. Toxicity is markedly decreased when administered via oral, subcutaneous, and intramuscular routes, with the LD_{50} >600 mg/kg.[18]

Novel drug-delivery systems of drug molecules are at the center of pharmaceutical research and development for treatment of diseases like cancer. Recent developments in nanotechnology have radically changed the method of treatment, diagnosis, and prevention of cancers. Developing nanoparticle (NP) formulations have several advantages such as increased solubility and bioavailability, protection from physical and chemical degradation, improved tissue distribution, sustained delivery, enhancement of pharmacological activity, targeted delivery, and decreased toxicity.[27,28]

Polymeric NPs are useful nanocarriers for targeted delivery of drugs. Commonly used polymers for formulation of NPs include poly(lactic-*co*-glycolic acid) (PLGA), polylactic acid, dextran, and chitosan. The polymer should be easily metabolized and removed from the body after administration of polymeric formulations. In this regard, biodegradable polymers are most suitable for formulation development.[29–31] NP surfaces can also be sterically stabilized to decrease hepatic uptake and improve the enhanced permeability–retention effect.[32–34] PLGA is a US Food and Drug Administration (FDA) approved polymer for delivery of drugs, and is one of the most commonly used polymers because of its excellent biodegradability, biocompatibility, and mechanical strength.[35–37] PLGA NPs have also been explored for incorporation of single drug[38] and dual agents to decrease the toxicity and enhance the efficacy of a drug.[39]

The effects of Nim and its mechanisms of action have been studied extensively in breast (MCF-7 and MDA-MB-231)[40–42] and pancreatic cancer cells (PANC-1).[22] No drug-delivery system has been reported for enhancing the

therapeutic application of this novel hydrophobic molecule. NP formulation of Nim may help in delivery to cancer cells, sustained release, enhanced pharmacological activity, and decreased toxicity of the moiety. Also, PLGA is used in FDA approved drug products. In the current study, we formulated and characterized PLGA-based NPs of Nim (Nim-nano) to enhance its therapeutic potential. The formulation was evaluated for its cytotoxicity in breast (MCF-7 and MDA-MB-231), and pancreatic (AsPC-1) cancer cells.

Methods

Preparation of PLGA nanoparticles of Nim

NPs were prepared using nanoprecipitation.[33,43] Briefly, 1 mg Nim (BioVision Incorporation) and 9 mg PLGA (50:50, inherent viscosity range 0.15–0.25 dL/g; Durect, Pelham, AL, USA) were dissolved in 1 mL acetone. Ten mL of a 1.5% w:v solution of polyvinyl alcohol (Alfa Aesar, Ward Hill, MA, USA) was taken in a beaker and the drug–polymer solution was added in a controlled manner (0.1 mL/min). The mixture was stirred for 5 hours at 500 rpm on a magnetic stirrer to evaporate acetone. The resulting suspension of NPs was centrifuged at 25,000 *g* (Allegra 25R centrifuge; Beckman Coulter, Brea, CA, USA) for 1 hour at 8°C. The NP pellet was suspended in deionized (DI) water after discarding the supernatant and centrifuged again in the same conditions. The resulting pellet was suspended in 3 mL DI water by vortexing. The suspension was transferred to a clean and dry amber-colored bottle, kept at −80°C for 2 hours, and then lyophilized overnight (FreeZone Plus; Labconco). Blank NPs were also prepared similarly by adding 10 mg PLGA to 1 mL acetone without the drug. NP preparation was performed in triplicate under light-protected conditions.

Determination of particle size and ζ-potential

Mean particle size, size distribution and ζ-potential of NPs were determined using a NanoBrook 90 Plus PALS (Brookhaven Instruments, Holtsville, NY, USA). All measurements were performed in triplicate. Freeze-drying increases particle size, which can be reduced by addition of cryoprotectants before lyophilization. The effect of mannitol and sucrose as cryoprotectants on particle size of NPs was determined by taking a small volume of NP suspension in an amber vial, to which an equal volume of either sucrose or mannitol solution was added to make final concentrations of 5% or 10% w:v, respectively.[33] Suspensions were lyophilized as mentioned earlier. Sizes of the lyophilized NPs with or without cryoprotectant were determined by reconstitution in 3 mL DI water and sonication for a few seconds.

Determination of drug loading and encapsulation efficiency

For drug-loading and encapsulation-efficiency determination, 1 mg lyophilized NPs was dissolved in 1 mL acetone by sonication in an amber glass vial. The content was kept at room temperature for 1 hour and then filtered through a 0.22 μM PVDF membrane filter (Millex GV syringe-driven filter unit; Millipore, Bedford, MA, USA). Absorbance of the filtrate was measured by ultraviolet-visible spectrophotometry (Varioskan Flash; Thermo Fisher Scientific) at 207 nm against a blank (empty-NP solution prepared similarly in the same concentration). Encapsulation efficiency was calculated by measuring the amount of drug present in the NPs compared to the amount of drug used for preparation of the same amount of NPs. Drug loading was calculated by measuring the amount of drug present in the NPs compared to the total amount of polymer and drug taken for the preparation of the same amount of NP formulation.

$$\%\mathrm{EE} = \frac{\textit{Weight of drug in the nanoparticles}}{\textit{Weight of feeding drug}} \times 100$$

$$\%\mathrm{DL} = \frac{\textit{Weight of drug in the nanoparticles}}{\textit{Weight of polymer and drug taken}} \times 100$$

In vitro release

A dialysis-bag method was employed for determining in vitro release of drug from NPs. PBS (pH 7.4) was used as the release media. NPs containing 500 μg Nim in 0.5 mL PBS were put in a dialysis bag (molecular-weight cutoff 6,000–8,000 Da; Spectrum Laboratories) and sealed from both ends. PBS (30 mL) was added to an amber glass container, and then the sealed bag containing NPs was transferred into it. The glass container was allowed to shake horizontally at 37°C and 100 rpm on a horizontally shaking incubator (VWR). Release medium (1 mL) was taken out at predetermined time intervals (1, 2, 4, 8, 24, 48, 72, 96, 120, and 144 hours) and the amount of Nim in the media measured by ultraviolet-visible spectrophotometry. The same amount of fresh medium was added to the containers after each

sample withdrawal. The percentage of drug released was calculated from the equation:

% drug released = (amount of Nim in the medium [μg]/ amount of Nim loaded in the NPs [μg]) × 100

In vitro cytotoxicity

In vitro anticancer activity of Nim PLGA NPs (Nim-nano) was evaluated in AsPC-1 (pancreatic cancer cell line), and breast cancer cell lines (MCF-7 and MDA-MB-231) by MTT assay. All cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Pancreatic cells were grown in RPMI 1640 medium and breast cancer cells in DMEM (Mediatech, Manassas, VA). Media were supplemented with 10% FBS and 1% penicillin–streptomycin. Breast (3,000 cells/well) and pancreatic (4,000 cells/well) cancer cells were transferred to 96-well culture plates and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 hours. The culture medium was then taken out carefully and the cells treated with fresh medium (control) or different concentrations of pure Nim in medium or various concentrations of Nim-nano in medium. Plates were again incubated for 72 hours in similar conditions, then the medium was removed and the cells washed with PBS (pH 7.4). Fifty microliter of a 0.5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) prepared in respective media was added to each well and further incubated for 4 hours. Purple formazan was formed by reaction of MTT with mitochondrial succinate dehydrogenase enzymes of the live cells. This formazan complex was dissolved by adding 100 μL DMSO to each well after removing the medium carefully. Percentage cell viability with different treatments was calculated by measuring absorbance at 570 nm on a microplate reader (Varioskan Flash; Thermo Fisher Scientific, USA), considering absorbance of the blank (treatment with fresh medium only, control) as 100% growth. IC_{50} was calculated by GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA).

Statistical analysis

Data are presented as means ± SD/SEM. Student's *t*-test was used for statistical analysis. $P<0.05$ was considered statistically significant.

Results

Characterization of Nim-nano

The average particle size of Nim-nano was 183.73±2.22 nm and 221.20±11.03 nm before and after lyophilization, respectively (Figure 2). Lyophilization increased particle size by 20%. Polydispersity-index (PDI) values before and after lyophilization were 0.058±0.022 and 0.168±0.033, respectively. Lyophilized Nim-nano had negative ζ-potential (−22.40±4.40 mV) (Figure 3). This may improve the stability of the Nim-nano, as negative ζ-potential of particles cause electricals repulsion among them and prevents their aggregation.[44]

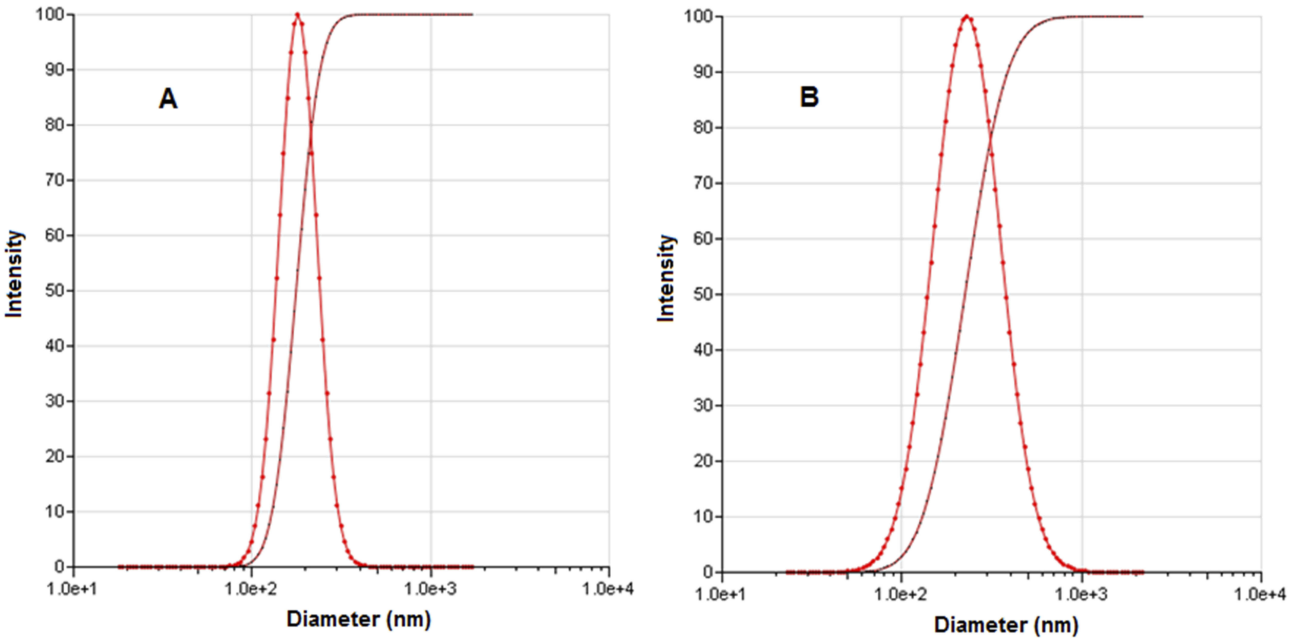

Figure 2 Particle size of Nim-nano before lyophilization (**A**) and after lyophilization (**B**).

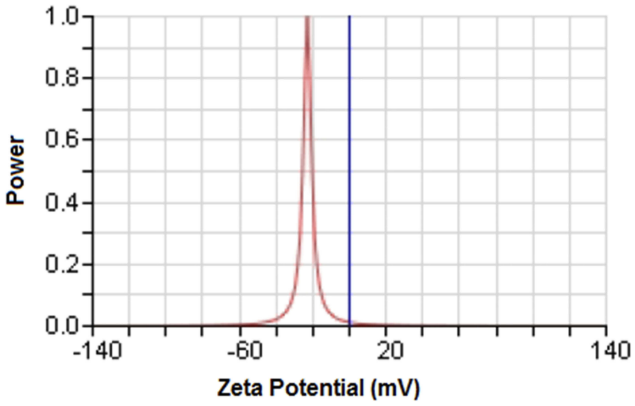

Figure 3 ζ-potential of Nim-nano after lyophilization.

Average drug loading and encapsulation efficiency of the lyophilized Nim-nano were 5.25%±1.12% w:w (0.0113 M Nim/100 g Nim-nano) and 55.67%±12.42%, respectively. Varied percentages of drug loading (5.43%–16.98%) and encapsulation efficiency (37.8%–88.4%) have been reported in the literature for different drugs.[34,45–47]

The effect of mannitol (10% w:v) and sucrose (5% w:v) as cryoprotectants on particle-size growth of lyophilized Nim-nano was assessed, and particle-size increase was significantly decreased by both cryoprotectants. Mannitol 10% was more effective in reducing the size of the NPs after lyophilization (Figure 4). Both cryoprotectants also reduced the PDI of the Nim-nano (0.083±0.050 and 0.143±0.045, respectively, for mannitol and sucrose) after lyophilization.

In vitro drug release

In vitro release of Nim from the Nim-nano exhibited an initial burst release of drug from the NPs: approximately 20% of drug was released after 1 hour, followed by sustained release (Figure 5). After 6 days, the percentage cumulative release of Nim was >80%. The initial fast release of Nim may have been because of the surface-bound Nim on the NPs, which released quickly by diffusion. The remaining drugs are embedded in the NP structure, and for release of these drugs, the polymer needs to be degraded by hydrolysis. This caused the drug to be released over a few days.

In vitro cytotoxicity

In vitro cytotoxic effects of free Nim, Nim-nano, and empty PLGA NPs (without drug) were evaluated in breast and pancreatic cancer cell lines by MTT assays and are illustrated in Figure 6. Nim-nano exhibited significantly higher cytotoxicity than pure Nim against all three cell lines tested. IC_{50} of Nim in MCF-7, MDA-MB-231, and AsPC-1 cell lines was 4.02, 2.24, and 2.30 μM, respectively. IC_{50} of Nim-nano was approximately three-fold, two-fold, and three-fold, respectively, less than the pure drug ($P<0.05$, Figure 7). Further, the empty PLGA NPs showed negligible cytotoxicity in all three cell lines (Figure 6).

Discussion

Physicochemical characteristics, such as size, size distribution, and surface charge, are functional performance features for NP-based delivery of drugs.[48,49] The observed particle size of the Nim-nano was in the acceptable size range for uptake in tumor cells by enhanced permeation–retention effects.[50] The non-solvent used in the preparation of NPs contributes to mean particle size.[38] In the present study, acetone–water (a commonly used pairing)[48] was employed for formulation of Nim-nano. We did not determine the morphology or shape of the NPs. PLGA NPs are usually spherical, as confirmed by SEM and TEM analyses in earlier studies.[34,46] ζ-Potential is an

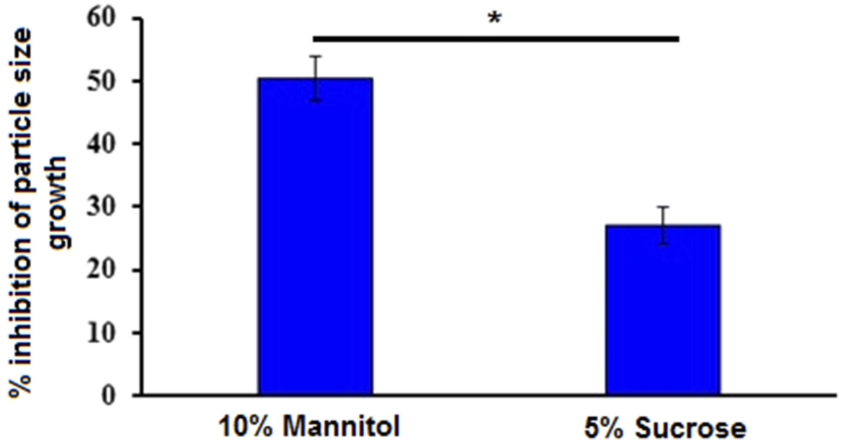

Figure 4 Effect of cryoprotectants on size of Nim-nano after lyophilization (means ± SD, n=3 each batch). *$P<0.05$ vs sucrose.

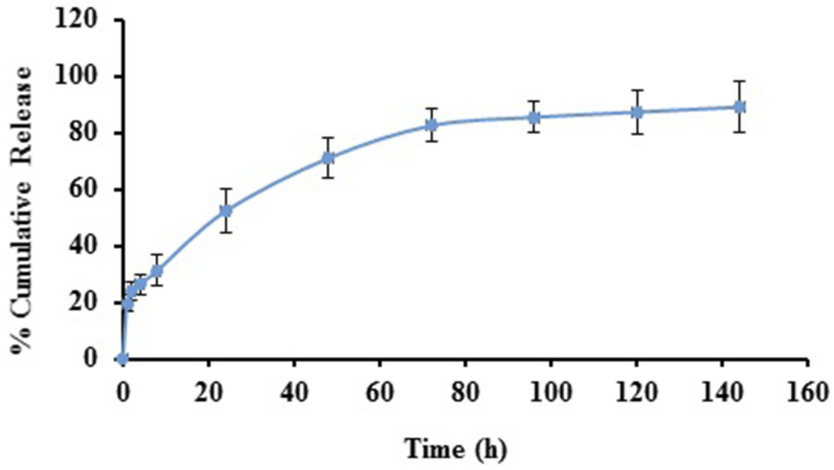

Figure 5 Release study of Nim-nano in PBS, pH 7.4 (means ± SEM, n=3 each batch).

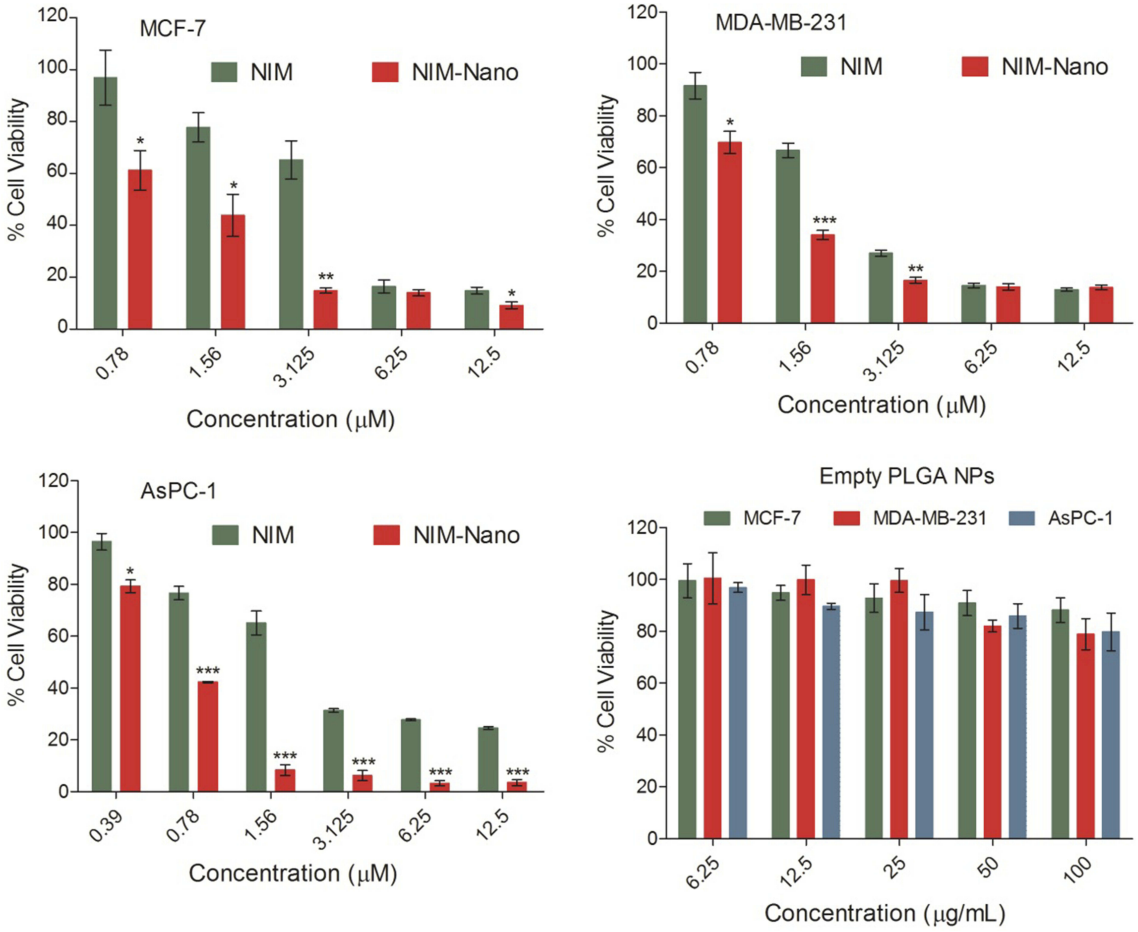

Figure 6 In vitro cytotoxicity of Nim, Nim-nano and empty PLGA NPs (drug-free blank PLGA nanoparticles) in different cancer cells after 72 hours of incubation (means ± SEM, n=3 each batch). *P<0.05, **P<0.01, ***P<0.001 versus Nim treatment at the same dose.

indicator of NP, stability and high ζ-potential causes electrostatic repulsion among NPs, preventing aggregation and increase in particle size.[44] Further, negative ζ-potential of formulations is beneficial for increasing circulation time and drug delivery.[51] During freeze-drying, the size of NPs increases by aggregation, due to considerable changes in

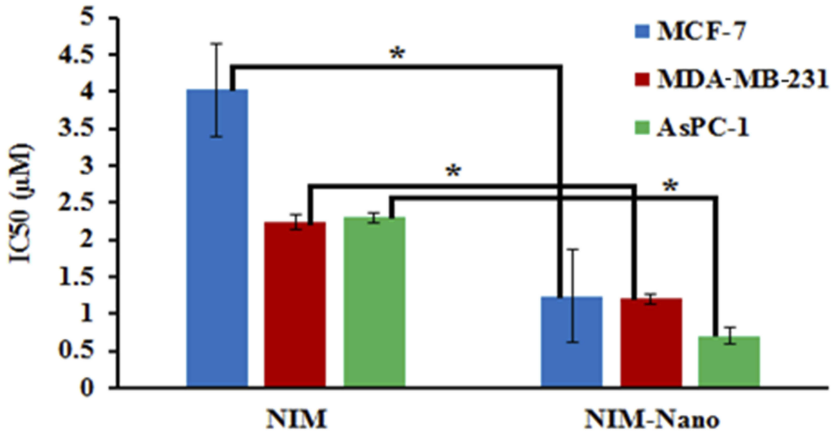

Figure 7 IC_{50} values of Nim and Nim-nano in MCF-7, MDA-MB-231, and AsPC-1 cells after 72 hours of incubation (means ± SD, n=3 different each batch). *$P<0.05$.

physical stress and introduction of freezing and drying.[33,52,53] Particle size plays an important role in the tissue distribution and cellular internalization of NPs.[54] The smaller the NPs, the better they will be on therapeutic efficacy, because of the enhanced permeability–retention effect. Cryoprotectant, normally a sugar, may prevent NPs from aggregation and extreme conditions of freezing by forming hydrogen bonds with the NPs and hence decreasing growth.[53,55] In the present study, the effect of mannitol (10% w:v) and sucrose (5% w:v) as cryoprotectants on particle-size growth was studied, and particle-size increase due to lyophilization was significantly inhibited by both cryoprotectants. Similarly to our earlier studies,[33] 10% mannitol was more effective in reducing the size of NPs after lyophilization. We did not study the role of cryoprotectants in cumulative release and cell viability. The NPs characterized and used for cell-viability assays were the lyophilized NPs without cryoprotectants. In the current study, we reported the role of cryoprotectants in controlling the increase in particle size due to lyophilization. This will be valuable for further development of the PLGA NPs for pharmaceutical development.

The release of Nim from Nim-nano was sustained, due to encapsulation of Nim inside the PLGA NPs (Figure 5). More than 90% of naïve Nim (dissolved in DMSO) was released within 2 hours from the dialysis bag to the release media (data not shown, as this period was very short compared to the days of sustained release of Nim from the Nim-nano). Drug release from the polymeric NPs can take place either by diffusion from the polymer surface and matrix or by diffusion and swelling of the polymer,[56] which are controlled by the nature of the polymer and physicochemical characteristics and surface-erosion properties of the NPs.[57] A biphasic release pattern of Nim from Nim-nano was observed in the present study, which has also been reported for other hydrophobic drugs.[33,58] The initial burst release may have been because of the release of weakly bound drugs on/near the surface of the NPs by diffusion from the surface layer. The remaining drug embedded in the core of Nim-nano showed controlled/slow release from NPs, most likely due to degradation of the polymer by hydrolysis and diffusion.[34]

The anticancer activity in different cell lines of Nim-nano was significantly higher than pristine Nim, as indicated in cell-viability and IC_{50} values. The enhanced cytotoxicity of Nim-nano may have been due to sustained release of the drug,[51] increased cellular uptake, and less efflux of the drug by Pgp pumps.[59] The effect of both Nim and Nim-nano was investigated only after 72 hours of treatment to compare the effect of Nim and Nim-nano. This time for in vitro anticancer activity was selected, as release of the drug from Nim-nano had been sustained at least for 72 hours in the drug-release study (around 80% Nim released, Figure 5). In future, we would like to investigate and compare the effects of Nim and Nim-nano earlier, such as at 24 and 48 hours.

In the presence of highest concentrations (6.25 and 12.5 μM), the cytotoxic effect was similar for Nim and Nim-nano in MDA-MB-231, whereas in MCF-7 and AsPC-1 cells Nim-nano was more active at these concentrations (Figure 6). Also, the reduction in IC_{50} of Nim-nano compared to Nim was more pronounced in MCF-7 and AsPC-1 cells than MDA-MB-231 cells (Figure 7). This may have been due to different potency and mechanism of action of Nim against different cell lines, which remains to be studied in future.

Earlier studies have demonstrated that Nim reduces the movement and invasive potential of MCF-7 and MDA-MB-231 cell lines by downregulation of uPA, uPAR, chemokines, pEGFR, VEGFR, NFκB, IKKα, IKKβ, MMP2, and MMP9 and upregulation of TIMP2.[40] Nim also inhibits cell propagation and cell survival by downregulation of IGF1R–Akt–ERK signaling, which accumulates G_0/G_1 cells and downregules cyclin protein expression,[41] causes apoptosis by both intrinsic and extrinsic pathways, increases levels of proapoptotic proteins Bax, Bad, FasL, TRAIL, FADDR, and cytochrome C, and downregulates, the anti-apoptotic proteins Bcl2, BclxL, Mcl1 and XIAP1.[42] In the present study, the enhanced effect of Nim-nano might have been due to increased cellular uptake and less efflux by Pgp pumps with the same mode of action.

The present research involved the formulation, characterization, and in vitro cytotoxicity of Nim-nano. No NP-based drug-delivery system has been reported for this novel molecule. Further in vitro studies such as cell-uptake studies, molecular mechanisms of anticancer effect, elucidation of the type of cell death (necrosis or apoptosis), and flow cytometry are warranted, in addition to in vivo investigations of Nim and Nim-nano in breast and pancreatic cancer.

Conclusion

We formulated and characterized a nanodelivery system of the potent natural anticancer molecule Nim. Nim-nano was more effective than pure Nim in breast and pancreatic cancer cell lines. Nim-nano exhibited sustained release of Nim in PBS. Although in vitro studies are reliable, correlating these studies with in vivo experiments would be of great value. The future scope of this project would be optimization of the PLGA-based nanoformulation of the drug and preclinical studies in animal models of cancer. The current NP formulation of Nim has potential for development into a pharmaceutical product. Also, this research will initiate further research into other drug-delivery systems for Nim and improvement of the current Nim-nano. In future, the PLGA-polymer surface can be modified by conjugation with polyethylene glycol, receptor-binding ligands (eg, folic acid), and other moieties. This will lead to targeted delivery of Nim and effective treatment of breast and pancreatic cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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