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VIP-targeted Cytotoxic Nanomedicine for Breast Cancer

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Abstract

Cancer chemotherapy is hampered by serious toxicity to healthy tissues. Conceivably, encapsulation of cytotoxic drugs in actively-targeted, biocompatible nanocarriers could overcome this problem. Accordingly, we used sterically stabilized mixed micelles (SSMM) composed of biocompatible and biodegradable phospholipids to solubilize paclitaxel (P), a hydrophobic model cytotoxic drug, and deliver it to breast cancer in rats. To achieve active targeting, the surface of SSMM was grafted with a ligand, human vasoactive intestinal peptide (VIP) that selectively interacts with its cognate receptors overexpressed on breast cancer cells. We found that even *in vitro* cytotoxicity of P-SSMM-VIP was 2-fold higher than that of free paclitaxel ($p < 0.05$). Given the unique attributes of P-SSMM and P-SSMM-VIP, most notable small hydrodynamic diameter (~15nm) and stealth properties, biodistribution of paclitaxel was significantly altered. Accumulation of paclitaxel in breast tumor was highest for P-SSMM-VIP, followed by P-SSMM and Cremophor based paclitaxel (PTX). Importantly, bone marrow accumulation of paclitaxel encapsulated in both SSMM-VIP and SSMM was significantly less than that of PTX. Administration of clinically-relevant dose of paclitaxel (5mg/kg) as P-SSMM-VIP and P-SSMM eradicated carcinogen-induced orthotopic breast cancer in rats, whereas PTX decreased tumor size by only 45%. In addition, a 5-fold lower dose (1mg/kg) of paclitaxel in actively targeted P-SSMM-VIP was associated with ~80% reduction in tumor size while the response to PTX and P-SSMM was significantly less. Hypotension was not observed when VIP was grafted onto SSMM. Based on our findings, we propose further development of effective and safe VIP-grafted phospholipid micelle nanomedicines of anti-cancer drugs for targeted treatment of solid tumors in humans.

Keywords

phospholipid mixed micelles; targeted drug delivery; human vasoactive intestinal peptide; breast cancer; paclitaxel; MNU-induced breast cancer

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Introduction

The efficacy of cancer chemotherapy is hampered by dose limiting toxicity to healthy tissues. Compounding this problem is the fact that potent cytotoxic drugs, such as paclitaxel, are sparsely water-soluble and are, therefore, formulated with cosolvents, such as Cremophor EL[®], which are toxic themselves [1]. Hence, developing alternate therapeutic modalities, that increase selective anti-cancer efficacy of cytotoxic drugs while minimizing their systemic toxicity, is urgently needed. Given these issues, targeted delivery of cytotoxic drugs specifically to the site of action using safe biocompatible materials could represent an important means to overcome this problem.

Phospholipid micelles, specifically sterically stabilized mixed micelles (SSMMs) and sterically stabilized micelles (SSMs) are self-assembled nanoparticles that are very attractive for drug delivery due to multiple reasons. Phospholipid molecules, used to prepare these micelles are biodegradable, relatively non-toxic and already FDA approved for human use as components of the marketed parenteral pharmaceuticals [2,3]. Structurally, the phospholipid micelles are a multi-compartment system that consists of an internal lipophilic core where water-insoluble drugs can reside and an outer polyethylene glycol (PEG) corona where semipolar molecules can be accommodated. Poor water solubility of many cytotoxic agents substantially limits their clinical use. We have demonstrated that encapsulation of hydrophobic drugs, including taxanes, in phospholipid micelles enhances their aqueous solubility in some cases up to thousand-fold without using any toxic solvents or detergents [4–7]. In contrast to many other surfactant micelles, phospholipid micelles are relatively stable against dilution due to extremely low critical micellar concentration ($\sim 1\mu\text{M}$) in aqueous media [8]. Moreover, our data suggest that phospholipid micelles can enhance stability of the drugs, protecting them from chemical or enzymatic degradation [4,9]. Nanoconstructs are thought to facilitate enhanced accumulation of entrapped drugs in areas with increased vascular permeability, such as in certain solid tumor malignancies [10]. More specifically, particles with size of $\sim 15\text{nm}$, similar to SSM and SSMM, were estimated to possess most favorable balance between systemic clearance and vascular extravasation, resulting in improved tumor accumulation [11].

Drug accumulation at the tumor site can be further improved by grafting the micelle surface with a targeting ligand specific to a cancer cell surface marker. In recent years the use of endogenous peptides, that are less immunogenic than large antibody fragments, for targeted drug delivery of therapeutics as well as imaging agents has received a lot of attention [12–14]. One of these attractive ligands is human vasoactive intestinal peptide (VIP) [15,16]. VIP receptors (VIP-R) have been shown to be overexpressed in many cancers, with about five times higher expression in all examined human breast cancer specimens [17,18], as well as in carcinogen-induced rat breast neoplasms [19]. Regardless of the histological site of origin, both ductal and lobular breast carcinomas overexpress VIP-Rs [20]. Furthermore, VIP-guided nanotherapy may also be useful for advanced cancers as evidenced by VIP-R expression in metastatic sites at the same or higher level than in primary neoplasms, including breast [21,22]. Moreover, the internalizing nature of VIP-R was shown to promote faster intracellular accumulation of the micelle cargo [23] and enhance sensitivity of resistant cells to anticancer agent by saturation or bypassing of the efflux pumps [24]. It is important to note, that VIP-Rs are only expressed in extravascular regions of microvasculature [25]. When VIP is associated with nanomicelles its extravasation to healthy tissues is minimized, hence off-target effect of the peptide is eliminated.

These attributes of phospholipid micelles as a drug delivery system and VIP as a specific ligand for cancer cell targeting, led us to test the cytotoxicity and safety of a novel, actively-

targeted paclitaxel nanomedicine, P-SSMM-VIP, against breast cancer both *in vitro* and *in vivo*.

Materials and Methods

Materials

Lipids, egg phosphatidylcholine (PC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol)2000] (DSPE-PEG₂₀₀₀), were purchased from Lipoid GmbH (Ludwigshafen, Germany) and Northern Lipids Inc. (Vancouver, Canada), respectively. Activated lipid for peptide conjugation 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-n-[poly(ethylene glycol)]-N-hydroxysuccinamide (PE-PEG₃₄₀₀-NHS) was obtained from Shearwater Polymers Inc. (Huntsville, AL). Paclitaxel (>99% purity) from Sigma-Aldrich Co. (St. Louis, MO), ³H-labeled paclitaxel for *in vivo* studies from Moravek Biochemicals (Brea, CA), and paclitaxel injection (generic equivalent of Taxol®) from Bedford Laboratories (Bedford, OH) were used. Vasoactive intestinal peptide (VIP), with common sequence for human and rat His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH₂, was synthesized by solid-phase synthesis and purified by Protein Research Laboratory at Research Resources Center, University of Illinois at Chicago. N-methyl nitrosourea (MNU) used for the induction of breast cancer was obtained from Ash Stevens Inc. (Detroit, MI). TS-2™ tissue solubilizer and Safety-Solve™ complete counting cocktail were obtained from Research Products International Corp. (Mount Prospect, IL). All other chemicals were of analytical grade from Fisher Scientific (Itasca, IL) or Sigma-Aldrich Co (St. Louis, MO).

Cell lines

MCF-7 human breast adenocarcinoma cells (ATCC # HTB-22) were kindly provided by Dr. William T. Beck (Department of Biopharmaceutical Sciences, University of Illinois at Chicago). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 50 IU/ml penicillin-50 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C.

Preparation of the formulations

Dispersions of paclitaxel solubilized in sterically stabilized mixed micelles (P-SSMM) and blank SSMM were prepared by coprecipitation and rehydration method, as described previously [7]. Optimal lipid composition for DSPE-PEG₂₀₀₀ to PC in a molar ratio of 90:10 was used in these studies as previously determined [7]. VIP was conjugated to the distal end of activated PEGylated lipid (PE-PEG₃₄₀₀-NHS) as previously described [26]. This reaction takes place between NHS-ester and a primary amine yielding predominantly a 1:1 PE-PEG-VIP conjugate. Incorporation of PE-PEG₃₄₀₀-VIP construct into preformed P-SSMM or SSMM was achieved by incubation for 1 h at 25°C to form P-SSMM-VIP or SSMM-VIP, respectively, alike our previously published protocols [4,23]. The saturation molar ratio of PE-PEG₃₄₀₀-VIP:SSMM was determined by isothermal titration calorimetry (ITC) by measuring total heat of the insertion as previously described [27,28], with modifications. Briefly, calorimetric analysis was carried out at 25°C in a 4200 ITC unit (Calorimetry Sciences Corporation, Provo, UT). Measuring cell was filled with 2mM SSMM in 0.01M HEPES buffer, pH 6.6 and PE-PEG₃₄₀₀-VIP (0.7mM) in same buffer was injected in 10 µl aliquots. Integrated heat change for each injection after subtracting the heat of dilution versus injection number was plotted and analyzed by Bindworks™ software (Calorimetry Sciences Corporation, Provo, UT). Average values for K (binding constant) and H (reaction enthalpy) for the titration were computed with an aid of Bindworks™ software based on the integrated modeling plots.

For *in vivo* tissue distribution studies, P-SSMM was prepared as described above containing 1mg/ml paclitaxel spiked with 34 μ Ci/ml 3 H-paclitaxel (specific activity 5.5Ci/mmol).

Formulation characterization

Particle size distribution and mean particle diameter was determined by dynamic light scattering using a Nicomp 380 particle size analyzer (Particle Sizing Systems, Santa Barbara, CA). Paclitaxel concentration was evaluated by reversed-phase high performance liquid chromatography (RP-HPLC) as described previously [7]. Phospholipid content was determined by a modified Bartlett's phospholipid assay [29]. VIP levels were assessed by enzyme-linked immunosorbent assay (ELISA) according to manufacturer instructions (Peninsula Laboratories, San Carlos, CA).

In vitro cytotoxicity

Human mammary adenocarcinoma MCF-7 cell line that was shown to overexpress VIP receptors [24,30] was used to evaluate cytotoxic activity of paclitaxel in SSMM-VIP and SSMM. A dimethyl sulfoxide (DMSO) solution of paclitaxel was used as a free drug control. Vehicle controls, SSMM-VIP and SSMM, were tested at same lipid concentration as the test solutions.

Samples were prepared as described above and serial dilutions were made to obtain paclitaxel concentrations ranging from 0.5–5 nM. Viable cells were seeded in 190 μ l of the complete medium at a density of 12,000 cells/well in a 96-well plate. After that, 10 μ l of test solutions and controls were added to each corresponding well. Final DMSO concentrations in each well did not exceed 0.5%. Each sample was evaluated in triplicate. The plates with test and control solutions were then incubated for 72 hours in a humidified atmosphere of 5% CO₂ at 37°C. After the incubation period, cell viability was determined by sulforhodamine B assay [6,7]. The values were expressed as percent of survival and paclitaxel effective dose (ED₅₀) was calculated using nonlinear regression analysis. Readings obtained for the solvent controls were used to define 100% growth.

Animals and breast cancer induction

All animal procedures were performed in compliance with UIC institutional guidelines and protocols approved by the Institutional Animal Care Committee.

Virgin female Sprague Dawley rats (45-day old, ~150 g) purchased from Harlan Laboratories (Indianapolis, IN) were housed and maintained in controlled temperature and humidity environment of Biological Recourse Laboratory of UIC on 12 hour light/dark cycle with free access to food and drinking water.

Breast cancer was induced by injection of carcinogen, MNU as previously described with some modifications [12,31]. Briefly, acclimatized virgin female Sprague Dawley rats were anesthetized with ketamine/xylazine (13.3/1.3 mg per 100g body weight, intraperitoneally). Each animal received a single intravenous injection of MNU (50 mg/kg body weight) in acidified saline (pH 5.0) via tail vein. Mammary tumors became palpable within 100–150 days after the injection. Once detected, two largest orthogonal dimensions were measured with electronic vernier calipers to estimate tumor size. The *in vivo* studies were initiated when the tumor size reached approximately 350 mm³.

In vivo biodistribution

These studies investigated the tissue distribution of paclitaxel delivered in SSMM-VIP in comparison with SSMM and commercial formulation paclitaxel for injection. The breast cancer bearing rats (16 animals per group) were anesthetized with ketamine/xylazine

(13.3/1.3 mg per 100g body weight, intraperitoneally) and received a single intravenous injection of 5mg/kg paclitaxel in SSMM-VIP, SSMM or PTX each spiked with ^3H -paclitaxel (34 $\mu\text{Ci/ml}$) through the tail vein. The rats (4 animals/time point) were sacrificed at 15 min, 60 min, 4h, and 24h post drug administration with an overdose of pentobarbital (200 mg/kg) intraperitoneally. Breast tumor, liver, spleen, kidneys, heart, lungs, and bone marrow were excised, homogenized in phosphate-buffered saline, and mixed with TS-2TM tissue and gel solubilized. Aliquots of solubilized tissue samples were mixed with Safety-SolveTM complete counting cocktail and total radioactivity were counted using a Beckman Coulter LS 6500 scintillation counter (Fullerton, CA). Concentration of paclitaxel per gram of tissue was determined based on known specific activity of the administered ^3H -paclitaxel.

***In vivo* efficacy**

These studies aimed to determine if paclitaxel administered in SSMM resulted in improved efficacy in comparison with PTX and if actively targeting P-SSMM-VIP was able to improve the efficacy further. Rats with qualifying breast tumor size were randomly divided into groups of six. The assigned rats were then treated intravenously with paclitaxel at 5mg/kg (an equivalent clinical human dose) and 1 mg/kg in one of the following formulations: P-SSMM-VIP, P-SSMM, and PTX. Vehicle controls, SSMM-VIP and SSMM, corresponding to the highest treatment group were also tested. The dosing schedule was once every three days for a total of 5 cycles. The tumor size was monitored daily with electronic vernier calipers for a total of 30 days post treatment initiation.

Effect of P-SSMM-VIP on systemic arterial blood pressure

Rats with qualifying tumor size were randomly assigned into two groups of four animals. Rats were acclimatized to a heated restrainer for 10 min daily for five consecutive days. Thereafter, systemic arterial pressure was monitored using a non-invasive tail-cuff blood pressure system RTBP2000 (Kent Scientific Corporation, Torrington, CT) and recorded with the aid of the software provided by the manufacturer. On the day of the experiment, animals were placed in the heated restrainer for 10 min and baseline systolic pressure recorded. Once the pressure stabilized, the tail was numbed with 10% lidocaine jelly and P-SSMM-VIP or VIP alone (4.5 $\mu\text{mol/kg}$ VIP; 5mg/kg paclitaxel corresponding to highest doses required for efficacy *in vivo* experiments) were injected intravenously through the tail vein. Systolic blood pressure of each animal was then monitored and recorded over 2 hours after the injection.

Data and statistical analyses

All *in vitro* experiments were performed in triplicate and data were expressed as mean $\pm$ standard deviation (SD). The data from each *in vivo* experiment were represented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey's test. A value of $p < 0.05$ was considered statistically significant.

Results and discussion

***In vitro* characterization and optimization of P-SSMM-VIP**

Previously we have shown that the optimum molar ratio of DSPE-PEG₂₀₀₀ and PC for SSMM was 90:10 [8,32] and at this ratio paclitaxel was solubilized at 1.5 fold higher concentrations when compared to simple DSPE-PEG₂₀₀₀ micelles [7].

One of the new aspects of this study was to covalently bind VIP on the surface of SSMM for active targeting. This was achieved using the same technique that we previously developed and reported for VIP conjugation on stealth liposomes for imaging [26]. The reaction

involved conjugation of the distal end of PEGylated lipid to the N-terminal of the peptide. This linking position does not interfere with the peptide receptor interaction [33]. A longer PEG spacer (PEG₃₄₀₀) was utilized to allow more flexibility to VIP when inserting into the PEG corona.

Isothermal titration calorimetry (ITC) was used to determine optimum ratio of the peptide-lipid conjugate (PE-PEG₃₄₀₀-VIP) insertion into SSMM. ITC has been widely used for the measurement of the binding equilibrium, which is directly computed from the heat evolved on association or physical insertion of ligands (e.g. peptides) with its binding partner (e.g. lipid surface) [28,34,35]. The heats of insertion of PE-PEG₃₄₀₀-VIP into SSMM after subtraction of the heat of dilution were integrated with the aid of Bindworks™ software (Fig. 1). Insertion of the conjugate into SSMM reached saturation at the molar ratio of ~17:1, total phospholipid: PE- PEG₃₄₀₀-VIP. Interaction between the binding partners was strong as indicated by the modeled binding constant (K), 45,583.73±4,605.70 and enthalpy (ΔH), -98.09±0.56kJ/mol. Based on these results, further SSMM-VIP formulations were prepared at the molar ratio corresponding to 15mM phospholipid to 0.9mM peptide.

The optimized formulation of P-SSMM-VIP was characterized and compared to P-SSMM (Table 1). P-SSMM average hydrodynamic diameter was 14.2±0.26nm which did not change significantly on insertion of VIP, 14.5±1.19 nm. Concentration of encapsulated paclitaxel in both SSMM and SSMM-VIP was found to be around 1mg/ml. The phospholipid content of P-SSMM and P-SSMM-VIP was not significantly different. The VIP content of P-SSMM-VIP was found to be 0.854±0.07mM, which translated to about five VIP molecules per micelle, computed based on the known micelle aggregation number of ninety [8].

***In vitro* cytotoxicity**

To assess the effect of solubilization of paclitaxel in SSMM on cytotoxicity against breast cancer cell line, as well as to evaluate the influence of active VIP-targeting in this regard, we evaluated the *in vitro* cytotoxicity of the formulations.

The cytotoxic profiles of paclitaxel in various formulations tested against breast adenocarcinoma cell line MCF-7, which is VIP-R positive [24,30], are depicted on Fig. 2. Paclitaxel solubilized in SSMM showed similar cytotoxicity compared to a solution of paclitaxel in DMSO with ED50 values of 7.80±0.53nM and 8.17±1.08nM, respectively. Remarkably, a two fold increase in ED50 (3.76±0.26 nM) was observed in case of P-SSMM-VIP. This increase in cytotoxicity could be attributed, in part, to the accelerated rate of intracellular drug accumulation by VIP-R internalization [23]. Blank carriers, SSMM and SSMM-VIP showed no toxicity to the cells at the concentration range corresponding to the drug-containing test solutions (Fig. 2).

Nanocarriers alter biodistribution of paclitaxel

The ultimate goal of targeted delivery is to achieve increased accumulation of drug at the cancer site and diminished exposure to systemic tissues, to reduce drug toxicity. Therefore, the biodistribution data for the drug administered in a nanocarrier in comparison with conventional formulation would provide an understanding of possible outcome on therapeutic application of targeted delivery system. Thus, in these studies biodistribution of P- SSMM-VIP, P-SSMM, and commercial PTX in rats bearing MNU-induced breast cancer was compared. The formulations were administered at a single clinical paclitaxel dose of 5mg/kg. The drug accumulation in various organs was evaluated up to 24 hours post injection.

Rapid distribution of P-SSMM and P-SSMM-VIP to the mammary tumor within first 15 min was significantly ($p < 0.05$) higher ($\sim 2\times$) than for PTX (Fig. 3. a). In contrast, distribution of P-SSMM-VIP and P-SSMM to the bone marrow was significantly lower followed by rapid clearance in comparison with PTX (Fig. 3. b). The later could be attributed to the notorious propensity of molecular paclitaxel to accumulate in bone marrow causing myelosuppression [36]. In contrast, SSMM encapsulation minimized, to some extent, drug contact with marrow and contributed to rapid clearance along the concentration gradient back to the circulation through inherently “leaky” vascular fenestration (85–100 nm) of marrow [37].

Moreover, P-SSMM-VIP accumulated significantly more at the tumor site (AUC_{0-t} 87.8 ± 9.5 $\mu\text{g}\cdot\text{h/g}$; $p < 0.05$) compared to both P-SSMM (45.8 ± 2.3 $\mu\text{g}\cdot\text{h/g}$) and PTX (26.7 ± 3.2 $\mu\text{g}\cdot\text{h/g}$) as shown on Fig. 4. This confirms our hypothesis that active VIP-targeting results in retention of the micelles at the tumor causing an increase in drug concentration with time in VIP-R positive tumor mass. Furthermore, binding to VIP-R promote cellular accumulation of the carrier with its entrapped load via internalization, as was previously shown by us [23], assisting in retention of the drug within the tissue. In contrast, non-actively targeted micelles have less potential to hold up in the tissue and can escape back into the circulation.

Lower cumulative exposure to micellar paclitaxel in comparison with commercial PXT product was observed for other tissues, including heart, kidneys, lung, and spleen (Fig. 4). No statistically significant formulation-dependent differences were observed for the liver exposure (Fig. 4). This finding may be rationalized by the fact that paclitaxel metabolism and elimination takes place in the liver [38] and translocation of the drug from other organs to the liver with time may contribute to this matter. In addition, formulation dependant differences in paclitaxel distribution on the cellular level (hepatocytes vs. Kupffer cells) may exist, contributing to evident similarity of drug deposition in liver. Variances in mechanisms of body elimination for the particulates in comparison with molecular drug may, at least partially, play a role in observed biodistribution differences.

Based on several other reports on biodistribution studies for various nanoparticulate drug systems [39–41], we conclude that biodistribution strongly depends on the carrier properties, such as size, composition, steric stabilization, in addition to the features of the drug itself, such as hydrophobicity and the mechanisms of its elimination. Nevertheless, in most cases drug delivery in a carrier system usually results in a favorable tumor accumulation.

***In vivo* efficacy of P-SSMM-VIP against MNU-induced breast cancer in rats**

MNU-induced rat mammary cancer is a well-established animal model, widely used in the study of carcinogenesis and efficacy evaluation of chemotherapeutic and chemopreventive agents [12,42,43]. It is an orthotopically developed model in which the carcinogen induces and promotes tumor formation in the mammary gland and mimics the multistage process of human mammary carcinogenesis. It differs from a xenograft model, derived from a single-cell clone that lacks the development of cellular architecture of the natural tumor environment. Furthermore, we have shown in MNU-induced breast cancer rat model that the VIP-Rs are overexpressed approximately 5 times more in the breast cancer tissue than in surrounding normal breast [19]. Hence, the MNU-induced cancer animal model, with many resemblances of the human disease, such as realistic anatomic location and targeting environment, was chosen for this study.

Tumor response profiles to each of the treatments are illustrated in Fig. 5. Both drug-free vehicle controls, SSMM and SSMM-VIP, did not show any anticancer action *in vivo*. Tumor burden in both cases was increased about 100-fold similar to untreated animals at the end of the observation period (data not shown). Treatment with P-SSMM-VIP at a low paclitaxel dose of 1mg/kg resulted in a significant ($80 \pm 3.6\%$) reduction of the tumor size,

compared to both P-SSMM ($40\pm3.6\%$) and PTX ($26\pm4.5\%$) at the end of the observation period (day 30) as presented in Fig. 5. a. Treatment with the clinical paclitaxel dose of 5mg/kg resulted in a dramatic increase in efficacy with near complete cancer eradication for P-SSMM-VIP ($95\pm0.3\%$) and P-SSMM ($93\pm2.0\%$) compared to PTX which resulted in $45\pm4.5\%$ tumor regression (Fig. 5. b). In addition, the rate of tumor regression was faster for P-SSMM and P-SSMM-VIP compared to PTX. However, at 5mg/kg paclitaxel no significant differences were observed between P-SSMM and P-SSMM-VIP. This may be due to the fact that at the dose of 5mg/kg paclitaxel the passive targeting was able to achieve the maximal response to the treatment and active targeting could not show any further improvement. However, it is likely that actively-targeted P-SSMM-VIP can be superior to P-SSMM at a dose lower than 5mg/kg paclitaxel and/or with a reduced dosing frequency may be sufficient to obtain complete cancer eradication. It is worth noting expression of VIP-R in metastatic sites at the same or higher level than in primary patient neoplasms, including breast cancer [21,22]. Therefore, actively VIP targeted carriers are capable of delivery of their cargo directly to the primary as well as metastatic tumor sites.

Safety evaluation of P-SSMM-VIP

VIP is an endogenous peptide and its receptors are widely distributed in the body. Therefore, exogenously administered VIP may cause side effects such as hypotension due to its well-known vasodilatory action [44]. Unlike other receptors for ligands presently tested in clinical trials, such as folate and transferrin [45,46], VIP-Rs are not expressed on microvascular endothelial cells [25]. Therefore, VIP needs to extravasate in order to interact with its receptors expressed on subluminal smooth muscle cells and elicit side effects. A distinct advantage of targeting nanocarriers with VIP is a restriction of the extravasation of the entire construct, due to its size, only at the site of action and as consequent total elimination of VIP-caused hypotension.

In this study we evaluated the safety of VIP when used as a targeting ligand, conjugated to the surface of nanocarrier SSMM, encapsulating cytotoxic drug paclitaxel. Systolic blood pressure following single administration of either P-SSMM-VIP or aqueous VIP (equivalent to peptide dose $4.5\ \mu\text{mol/kg}$) to MNU-induced tumor bearing rats was determined by a non-invasive tail cuff method. We observed that administration of aqueous VIP evoked a rapid decrease in systemic arterial pressure (Fig. 6). By contrast, P-SSMM-VIP had no significant effects on systemic arterial pressure. These data suggest that P-SSMM-VIP did not extravasate from normal vasculature to interact with VIP-Rs. As mentioned above, the vasculature in breast tumors is leaky due to the discontinuous endothelial lining of the cancer neovasculature. Thus, this presents a unique and novel opportunity where VIP-conjugated SSMM preferentially extravasates only at tumor site.

As additional safety measure, if necessary, all D-VIP (inactive enantiomer) rather than L-VIP (native peptide) could be used as an active targeting, but metabolically inert, moiety [47].

Abraxane[®] is a relatively new paclitaxel nanomedicine that is used for breast cancer treatment [41,48]. Vehicle toxicity of Taxol[®] is eliminated with this product, since paclitaxel is bound to albumin nanoparticles (130nm), instead of being solubilized in Cremophor EL and ethanol. Being a nanoparticulate system, Abraxane[®] may also utilize, at least in part, passive targeting mechanism to improve efficacy and reduce toxicity of the drug. However, Abraxane[®] lacks active targeting mechanism and steric stabilization property to avoid RES uptake. We believe our proposed paclitaxel formulation, P-SSMM-VIP that uses safe biocompatible excipients as the vehicle with steric stabilization and active targeting should be superior to currently available paclitaxel formulations with respect to the drug safety and efficacy.

Conclusions

In conclusion, these studies demonstrated for the first time the feasibility of *in vivo* active targeting using human vasoactive intestinal peptide surface conjugated to nanomicelles. We have successfully developed a nanomedicine for a model drug paclitaxel with surface grafted VIP, using biocompatible phospholipids. P-SSMM-VIP demonstrated improved *in vitro* cytotoxicity compared to non-targeted system. Furthermore, the *in vivo* studies demonstrated that paclitaxel delivered in the targeted carrier accumulated significantly more at the mammary tumors due to its size, long circulation and interaction with target receptors. In addition, targeted delivery resulted in reduced accumulation of paclitaxel in healthy tissues especially those associated with systemic toxicities such as bone marrow. Moreover, the increase in tumor accumulation due to targeting resulted in significantly enhanced drug activity in treating rats with MNU-induced breast cancer. Using the clinical dose of paclitaxel (5mg/kg), the complete tumor eradication was achieved by P-SSMM-VIP, whereas the commercial cremophore-based formulation of paclitaxel, at the same dose regimen showed only 45% tumor regression. Therefore, we propose further development of SSMM-VIP as a targeted delivery platform for paclitaxel and other anticancer drugs against cancers that overexpress VIP-R.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Integrated heat change per injection for the titration of PE-PEG₃₄₀₀-VIP and SSMM. Saturation of SSMM by PE-PEG₃₄₀₀-VIP was achieved between injection 22 and 24, corresponding to the molar ratio of ~17:1 total lipid to the peptide conjugate

Fig. 2.

Cytotoxic activity of paclitaxel solubilized in SSMM-VIP, SSMM, and DMSO against human breast adenocarcinoma MCF-7 cell line. Drug free vehicles, SSMM-VIP and SSMM, were also tested at concentrations equivalent to the test solutions. Data represent the mean $\pm$ SD of triplicates (n=3/formulation)

Fig. 3. Paclitaxel exposures over 24 hours in rats bearing MNU-induced breast cancer following intravenous administration of 5 mg/kg paclitaxel in P-SSMM-VIP, P-SSMM, and commercial product Paclitaxel for injections (PXT): **a)** Breast cancer; **b)** Bone marrow. Data represent the mean $\pm$ SEM; n=4 rats/formulation/time point

Fig. 4.

Comparative paclitaxel tissue distribution (AUC_{0-24h}) in rats bearing MNU-induced breast cancer following intravenous administration of 5mg/kg paclitaxel formulated in SSMM-VIP, SSMM, and commercial product Paclitaxel for injections (PXT). Data represent the mean $\pm$ SEM; n=4 rats/formulation/time point; * p<0.05 compared to PXT; # p<0.05 compared to PXT; ^ p<0.05 compared to P-SSMM

Fig. 5.

In vivo efficacy of paclitaxel against MNU-induced breast cancer in rats; rate of tumor regression on treatment with micellar formulations of paclitaxel, P-SSMM-VIP and P-SSMM, compared to commercial product Paclitaxel for injections (PXT): **a)** Paclitaxel dose 1 mg/kg. **b)** Paclitaxel dose 5 mg/kg. Data represent the mean $\pm$ SEM; n=6 rats/formulation; $\uparrow$ symbolize dosing. *-indicate statistical significance ($p < 0.05$) between specified groups at the end of the study

Fig. 6.
Effect of P-SSMM-VIP and aqueous VIP on systemic arterial pressure of breast cancer bearing rats on intravenous administration at a single injection at the peptide dose corresponded to $4.5\mu\text{mol/kg}$. Data represent the mean $\pm$ SEM; $n=3$ rats/formulation; * $p<0.05$ compared to P-SSMM-VIP

Table 1

Characteristics of optimized P-SSMM-VIP and P-SSMM formulations

Formulation	Average diameter (nm)	Phospholipid content (mM)	Paclitaxel concentration ($\mu\text{g/ml}$)	VIP content (mM)
P-SSMM	14.2 $\pm$ 0.26	14.8 $\pm$ 0.09	999.0 $\pm$ 2.10	
P-SSMM-VIP	14.5 $\pm$ 1.19	15.8 $\pm$ 0.07	987.1 $\pm$ 22.03	0.854 $\pm$ 0.067

values are mean $\pm$ SD from three separate formulation preparations