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<td>The purpose of this research is to explore the ability of tyrosine-derived nanospheres to improve the anti-tumor activity while simultaneously targeting the drug-nanoparticle complex to diseased cells, thereby minimizing unwanted effects on healthy cells. This report describes the optimization of nanospheres binding efficiency of three anti-cancer agents: camptothecin, paclitaxel and vitamin D3. Tyrosine-derived nanospheres efficiently encapsulate vitamin D3 and paclitaxel under all investigated conditions. However, our nanospheres bind only limited amounts of camptothecin, regardless of presence of Vitamin D3, and/or optimization of experimental conditions and fabrication technique. The optimum formulation for highest binding and stability of camptothecin, vitamin D3 is DTO-SA/5K nanospheres. Preliminary In Vitro and In Vivo studies suggest that (a) tyrosine-derived nanospheres provide highly effective delivery of hydrophobic paclitaxel to human tumor cells in vitro; (b) tyrosine-derived nanospheres exhibit no toxicity as compared to CrEL; (c) Tyrosine-derived nanospheres containing paclitaxel exhibit anti-tumor activity in a breast cancer xenograft model that is similar to that of an equivalent dose and schedule of clinically used formulation of Cremophor-paclitaxel. In conclusion, we are confident that tyrosine-derived nanospheres offer the potential for effective parenteral delivery of a wide array of hydrophobic drugs without the cytotoxicity problems commonly exhibited by surfactant-based drug delivery systems.</td>
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To whom it might concern:

We have made substantial progress in the preparation and optimization of the proposed chemotherapeutic and chemoprotective formulations, however the In Vivo evaluations have not been completed. Our subcontractors at UMDNJ-RWJMS were significantly delayed in obtaining the necessary animal protocol approval from their institution and have recently withdrawn their agreement to conduct the remaining studies. We have established a new collaboration with Prof. Tamara Minko at the College of Pharmacy of Rutgers University, and applied for a one-year no-cost extension of funding that was accepted only on August 26, 2008. Prof. Minko is ready to execute our work plan as designed in the original proposal and previously approved by ACURO veterinarians. All appropriate documentation is being prepared and will be sent to the USAMRMC Animal Care and Use Review Office to obtain the approval for the new AICUC protocol under Prof. Minko’s supervision as soon as we receive approval by Rutgers.

Thus, the report below is a summary of the preliminary data obtained for the optimization of drug-loaded nanosphere formulation but its ultimate evaluation of anti-tumor efficacy has not been accomplished yet.

Introduction

It is widely recognized that nanotechnology will have a profound effect on the delivery of pharmaceuticals with poor bioavailability. Our goal is to develop a tunable polymeric architecture that will provide the basis for a technology platform for drug delivery that is: a) biocompatible and biodegradable; b) enables injectable complexes with a wide array of drugs; and c) retains high therapeutic efficacy. Our specific objectives in this research program are:

- Optimize the process for nanosphere formation and complexation with a specific class of anti-tumor drugs
- Demonstrate in vivo the biological efficacy and safety of the nanosphere delivery system for treating a range of human cancers.

Background

Self-assembling nanospheres offer a promising route to the delivery of pharmaceuticals that have poor bioavailability by improving the drugs’ stability, circulation times in the body, and permeability through cell membranes, while reducing their toxicities. Many drugs, including anti-tumor agents, anti-depressants and statins, are lipophilic and therefore require a solubilization process to enable their parenteral delivery. Of the many alternative approaches proposed to overcome the obstacle of poor bioavailability of the drug, perhaps the most promising is the use of amphiphilic block copolymers that self-assemble into supramolecular nanoparticles. These nanoparticles can be designed to provide stable dispersions of lipophilic drugs with low cytotoxicity, thus making them attractive alternatives to less mechanically stable liposomes. In addition, these nanoparticles are often superior to more cytotoxic surfactant dispersant systems such as the CremophorEL that has been associated with some of the serious clinical side effects of Taxol®. The amphiphilic block copolymers typically form a core-shell architecture. The hydrophobic core serves as the reservoir for the incorporation of lipophilic drugs and diagnostic agents and the hydrophilic shell enables stable dispersion in an aqueous environment and frequently also offers protection from protein adsorption and subsequent biological attack.  Amphiphilic block copolymers with poly(ethylene glycol) as the hydrophilic block and polyester, poly(amine acid), or polyether as the hydrophobic block have been explored for applications in drug delivery. Particle size has been shown to be a critical design parameter, as particles with diameters less than 200 nm and having poly(ethylene glycol) (PEG) shells avoid entrapment by the reticuloendothelial system.
(RES) and accumulate preferentially in tumors that typically exhibit an enhanced permeability and retention effect (EPR). The biodistribution and uptake by the tumor of the nanoparticles is further dictated by charge density, conformation, hydrophobicity, and immunogenicity. The drug loading efficiency of the nanoparticles is also governed by a number of critical parameters, particularly the chemical and structural affinities of the loaded drug to the nanoparticle core.

We have previously reported on the design and synthesis of unique ABA-type amphiphilic triblock copolymers that self-assemble into nanospheres at low critical aggregation concentration. The A-blocks of these copolymers are composed of PEG and the B-blocks are composed of polyarylate oligomers of desaminotyrosyl-tyrosine alkyl esters (DTR) and non-toxic diacids (Scheme 1). A major breakthrough was achieved in 2006, when Rutgers scientists led by Prof. Joachim Kohn and TyRx Pharma, Inc., announced the FDA’s clearance of a new medical device for hernia repair that incorporates a biodegradable technology developed from novel tyrosine-based polyarylates. In January 2008, FDA approved an additional product, AIGISRX™ Cardiac Rhythm Medical Device (CRMD) Anti-Bacterial Envelope, which is also based on a biodegradable technology developed from tyrosine-derived polymers (Rutgers and TyRx Pharma, Inc.). The hydrophobic core of the triblock copolymer platform proposed to use in this research is an oligomeric version of the above-discussed polyarylates. Tyrosine-derived triblock copolymers self-assemble into spherical structures with hydrodynamic diameters between 50 nm and 100 nm, thus providing particle size and surface chemical properties superior to conventional drug delivery designs. In addition to their biocompatibility, biodegradability and lack of cellular toxicity, these nanospheres strongly bind and retain anti-tumor cytotoxicity of the hydrophobic chemotherapeutic agent, paclitaxel. It is our belief that this novel technology can potentially address the key military and civilian requirements for effective breast cancer chemotherapy: nontoxic administration, increased bioavailability, prolonged circulation and targeting cancer cells, leading to substantially greater drug efficacy and lower toxicity. Further exploration of the proposed multidisciplinary research, while potentially high risk, may result in the introduction of innovative, high impact treatments for breast cancer.

Rationale

Camptothecin (CPT) and its derivatives, such as 9-aminocamptothecin and 9-nitrocamptothecin, are inhibitors of topoisomerase I and have been investigated for their chemotherapeutic activity and inhibition of human breast carcinoma cells. The integrity of the lactone ring system of camptothecins is a key determinant for the chemotherapeutic efficacy. The hydrolytic instability of camptothecins and their hydrophobic nature have complicated clinical development of these compounds. Paclitaxel (Taxol®) is used widely for the treatment of breast, ovarian, non-small cell lung carcinoma, prostate, and other types of solid tumor cancers. Paclitaxel (PTX) is only sparingly soluble in water, and its intravenous administration depends on the use of Cremophor® EL (polyethoxylated castor oil) to obtain a sufficiently concentrated solution. The use of Cremophor increases patient toxicity and can lead to clinically important adverse effects, including acute hypersensitivity reactions and peripheral neuropathy.

It is postulated that our nanosphere delivery of these classes of drugs will be far superior to other available methods: effective parenteral delivery of a wide array of hydrophobic drugs without the cytotoxicity problems commonly exhibited by surfactant-based drug delivery systems. In addition, our formulation might open new avenues for adjuvant therapies such as simultaneous administration of several hydrophobic anticancer drugs with different mechanisms of activity. Thus, complexation of vitamin D3, another hydrophobic chemotherapeutic and chemopreventive agent, with our nanospheres in the presence of camptothecin or paclitaxel may provide a novel pathway in breast cancer treatment.
Objectives

It is been proposed to investigate multifunctional nanospheres that may be capable of overcoming the physicochemical and biological barriers to breast cancer drug delivery. Our goal is to parenterally deliver multiple therapeutic agents at high local concentrations and with physiologically appropriate timing directly to cancer cells, thereby interrupting the growth and metastasis of the tumor. Our initial focus will be on the delivery of camptothecin with triblock copolymer-derived nanospheres that will increase the solubility of the drug and provide protection to the lactone ring, resulting in increased bioavailability to breast cancer cells. In addition, we will formulate and evaluate the potential of our nanospheres to bind and deliver paclitaxel. Further, we will evaluate the relative efficacy and potential synergies of nanospheres containing camptothecin or paclitaxel alone, vitamin D3 alone, mixtures of these formulations delivered simultaneously, and a single nanosphere complex containing both vitamin D3 and paclitaxel or camptothecin.

Body

Special Note on Nomenclature

The abbreviation, DTR-XA/5K, is used to designate the various copolymer compositions in the family of ABA triblocks copolymers. The PEG A-blocks are abbreviated as 5K, indicating the molecular weight and units of the PEG components (i.e., 5K = PEG5000). The oligo B-blocks are distinguished by both their alkyl pendent chain “R” linked to the DTR (desaminotyrosyl-tyrosine alkyl ester) unit and/or the diacid “XA” to form the DTR-ester (DTR-XA). The three pendent chains “R” used are (B) butyl, (O) n-octyl, (D) n-dodecyl or (Bn) benzyl and the diacid “XA” is (SA) suberic acid (Scheme 1). Therefore, DTO-SA/5K stands for the triblock copolymer PEG_{5K}-b-oligo(desaminotyrosyl-tyrosine octyl suberate)-b-PEG_{5K}. Additional abbreviations used in this report are: VD3 for vitamin D3, CPT for camptothecin, and PTX for paclitaxel.

1. Copolymer Syntheses and Nanosphere Formulation

The first objective of this study involved determination of triblock copolymer structure-activity relations (SAR’s) for optimum binding of camptothecin, paclitaxel and vitamin D3 and the evaluation of process improvements so as to achieve the highest possible stable nanosphere-drug complex concentrations in aqueous solutions. Systematic synthetic variations were made in the copolymer structures (Scheme 1) to expand the range of nanosphere hydrophobicities. The synthesis of desaminotyrosyl-tyrosine esters, DTR,(30) and triblock copolymers has been previously described.(22, 23, 31).

![Scheme 1. Structure of PEG-b-oligo(DTR-XA)-b-PEG triblocks copolymers.](image)

We expected to identify an optimum ratio of triblock hydrophobicity/hydrophilicity, determined by the physical and chemical properties of the copolymer blocks, that provides for effective delivery of each selected drug. To this end the following triblock copolymer compositions were synthesized and characterized.
Table 1. Molecular weight properties of the PEG₅K-b-oligo(DTR-XA)-b-PEG₅K triblock copolymers and their corresponding nanospheres hydrodynamic diameters.

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<th>Copolymer/nanospheres composition</th>
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<th>Mw</th>
<th>Mw/Mn</th>
<th>DPᵃ</th>
<th>Nanospheres hydrodynamic diameter, nmᵇ</th>
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<td>DTB-SA/5K</td>
<td>20000</td>
<td>27000</td>
<td>1.35</td>
<td>18</td>
<td>69 ± 1.5</td>
</tr>
<tr>
<td>DTO-SA/5K</td>
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<td>29000</td>
<td>1.36</td>
<td>18</td>
<td>55 ± 1.3</td>
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<tr>
<td>DTD-SA/5K</td>
<td>24000</td>
<td>32000</td>
<td>1.33</td>
<td>21</td>
<td>72 ± 1.6</td>
</tr>
<tr>
<td>DTBz-SA/5K</td>
<td>22500</td>
<td>29600</td>
<td>1.31</td>
<td>21</td>
<td>76 ± 1.7</td>
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ᵃ DP, degree of polymerization, was determined by the following equation: \( \frac{(M_n^{DTR-XA/5K} - 2x(M_n^{mPEG}))}{MW_{DTR-XA}} \)

ᵇ Cumulant fit. The SD value was for the nanosphere mean hydrodynamic diameter obtained for the three measurements of a single batch.

The chemical structure of the tyrosine-based triblock copolymers with varying pendent R chains is illustrated in Scheme 1. With a copolymer synthesis reaction time of one hour, the copolymers are obtained with narrow molecular weight distributions centered on 29 kDa (Table 1). Based on the copolymers investigated so far, it can be concluded that the copolymer molecular weights are not strongly affected by the pendent ester in the DTR monomers. The triblock copolymers were induced to self-assemble in dilute aqueous solution using a conventional injection method.(31) The resulting turbid dispersion was purified and sequentially filtered through 0.45, 0.22 and 0.1 micrometer size syringe filters. The final filtrate was used for all subsequent characterizations. The triblock copolymer nanospheres have hydrodynamic diameters that go through a minimum size as the pendent ester chain lengths increase from ethyl (C₄) to octyl (C₈) to dodecyl (C₁₂) (Table 1). This apparent minimum is reminiscent of the Ferguson effect observed in surfactant systems.(32) Given that the B-block chain lengths, as reflected by their degree of polymerization (DP) are very similar (DP ~ 19), it can be suggested that the DTO-containing nanospheres will have the most densely packed hydrophobic cores. The degree of polymerization was also measured by ¹H NMR and very similar values were obtained (data not shown). The observed variations in self-organization behavior as a function of the DTR-XA core-forming blocks are consistent with their thermal properties.(33, 34) Poly(DTB-XA)’s are semi-amorphous materials characterized by a glass transition and they can be readily plasticized by water. In contrast, poly(DTD-XA)’s possess long range structural order with highly layered mesogenic properties, while poly(DTO-XA)’s have less ordered structures typical in non-mesogenic macromolecules. An increase in the length of the core-forming block is expected to cause an increase in the core size of the nanospheres which, in turn, may result in an increased drug loading capacity per nanosphere.(35) In conclusion, all of the copolymer formulations and their resultant nanospheres investigated so far appear to be suitable for use in drug delivery based on their structural composition, polymer molecular weight distribution and nanosphere size.

2. Nanospheres Drugs Compatibility and Binding Efficiency

First, we evaluated nanospheres encapsulation efficiency with all proposed drugs. There are two ways to analyze encapsulation efficiency: binding and loading. The binding is generally determined as a yield of encapsulation process (the ratio of the mass of drug in final formulation to the mass of drug in feed) and loading is the mass of drug that is retained per mass of the nanospheres. In general, the binding efficiency is higher than the loading since loading is dependent on both binding affinity and the mass of the nanospheres per volume of formulation. We do not expect a 100% binding efficiency since there is always some drug-water solubility
and drug loss is expected due to the multi-step preparation and purification procedures (Scheme 2).(23) It should be noted that the binding and loading efficiencies reported below were measured following meticulous purification procedure, which includes filtration through 0.22 μm filters, ultracentrifugation and additional filtration through 0.22 μm filters for sterilization purposes. The initial filtration step strongly affects the drug binding efficiency because all nanosphere-drug particles and particles alone that are larger than 220 nm will be removed. It was found that this filtration step reduced nanosphere yield as well as drug content in the nanospheres by 25% for paclitaxel-containing nanospheres and 35% in camptothecin-bounded nanospheres (data not shown).

The data presented in Figure 1 clearly shows the strong correlation between drug’s hydrophobicity and binding efficiency: the amount of drug entrapped in the purified DTO-SA/5K nanospheres increases as a function of the drug’s hydrophobicity, as reflected in the drug’s oil:water partition coefficient, Log D.

![Figure 1](image)

**Figure 1.** Effect of drug hydrophobicity on the binding efficiency of DTO-SA/5K nanospheres. Data expressed as ± SD of three independent measurements. (■) Drug binding efficiency (% wt/wt); (■) drug loading efficiency (% wt/wt). These efficiencies were detected by extraction method and HPLC analysis.

\[
\text{Binding Efficiency} (\%) = \frac{\text{mass of drug in the nanospheres}}{\text{mass of drug in the feed}} 
\]

\[
\text{Loading Efficiency} (\%) = \frac{\text{mass of drug in the nanospheres}}{\text{mass of nanospheres}} 
\]

The binding efficiency of the most hydrophobic drug vitamin D3 (Log D 9.72, molar solubility at pH 7 of 3.2x10^-8 mol/L) by the DTO-SA/5K nanospheres is 72%, which is indicative of an extremely high compatibility and solubility of this drug with our nanospheres. With sparingly soluble paclitaxel (molar solubility at pH 7 of 2.9x10^-7 mol/L) the aqueous nanosphere formulation bound only 43% of the initial input of the drug; however this amount is still at least 5000-fold higher than the solubility of PTX obtainable in water. With the most hydrophilic of these drugs, camptothecin (molar solubility at pH 7 of 6.43x10^-3 mol/L), we could achieve only 5% binding efficiency. Despite its relatively high log D value and higher molar solubility at pH 7, CPT is also poorly soluble in most organic solvents and has some tendency to self-aggregate. Hence, it is not surprising that it has low solubility in the hydrophobic DTO-SA block and, as reported below, the majority is lost during the purification process.

Next, we evaluated the effect of nanospheres composition on the binding of CPT. To this end, we chose nanosphere formulations that contain short and/or long alkyl pendent chains (Butyl (C4), Octyl (C8) and DTD (C12)), benzyl ring pendent (DTBz), and nanospheres composed of mixtures of these copolymers (Scheme 1). The rationale for choosing these formulations is based on different packing densities of the resultant nanospheres. With shorter R groups, there can be more flexible packing while longer pendent chains are expected to cause an increase in the core size of the nanospheres which, in turn, may result in an increased drug loading capacity per nanosphere. (19,33) Based on the results presented in Table 1, we suggest that DTO-containing nanospheres produce the most densely packed hydrophobic
cores. The introduction of the benzyl group might affect the rigidity and therefore self-assembly organization of the nanospheres. The presence of \( \pi-\pi \) interaction between the aromatic group and/or double bond of drug molecules (Figure 5) and the phenyl group of DTBz pendent chain could increase the affinity of the drug to the nanosphere core and thus, increase binding efficiency and stability of nanospheres-drug complexes. CPT-binding efficiency of these nanospheres was measured for a constant quantity of the polymer and VD3 or CPT. HPLC methods were developed and validated for quantitative determination of CPT and VD3 in all copolymer systems.

Figure 2. Camptothecin (A) and vitamin D3 (B) binding efficiency as a function of triblock hydrophobicity. In this figure nanosphere compositions are abbreviated as their respective DTR components: DTO stands for 100% DTO-SA/5K, DT(B+O+D+Bz) stands for the copolymer composed of 25% wt/wt of each DTO-SA/5K, DTB-SA/5K, DTD-SA/5K, and DTBz-SA/5K triblock copolymers. DTO-DT stands for 50% wt/wt of each DTO-SA/5K and DTB-SA/5K triblock copolymers. DTO-DTBz stands for 72% wt/wt of each DTO-SA/5K and DTB-SA/5K triblock copolymers.

Figure 2 represents binding efficiency of VD3 and CPT by DTR-XA/5K nanospheres. In all copolymer compositions, VD3 binding is at least 10 times higher than binding of CPT by respective nanosphere formulations (note the difference in the scale of Y-axes of each graph). Both CPT and VD3 binding is strongly affected by the nanospheres composition. The highest binding efficiency was measured in formulations composed of DTO-SA/5K: 5% wt/wt of CPT and VD3, respectively. In the case of nanospheres composed of short (C4) or long (C12) DTR-SA/5K, different binding profiles were obtained for each drug. CPT binding efficiency in DTB and DTBz-containing nanospheres was significantly lower and less reproducible (Fig. 2-A, ± StDev). In the case of VD3, the same nanospheres compositions had a smaller effect on binding efficiency. For both of the drugs, the lowest binding efficiency was measured in DTBz-SA/5K nanospheres. To better understand these results we have to refer to the fabrication and purification processes of drug-loaded nanospheres illustrated in Scheme 2.

Scheme 2. Process steps for formation and purification of drug-loaded nanosphere formulation.

According to our standard operating procedure, a drug's binding is measured after isolation of purified drug-nanosphere complexes by ultracentrifugation. Ultracentrifugation produces gel-like pellets containing purified drug-loaded nanospheres that generally are fully re-suspended in
phosphate saline buffer (PBS) after several hours of gentle shaking. Following careful
evaluation, in the case of DTB and especially DTBz-containing nanospheres, the majority of
the pellet remained in the gel form. In addition, in some cases DTBz-containing pellets had yellow
precipitates that were indicative of the presence of un-bound CPT. The amount of yellow
precipitates varied in all experiments and this could explain lower reproducibility of CPT binding
by DTBz-containing nanospheres. Since CPT binding was measured (Figure 2-A) from only the
re-suspended fraction, lower binding of CPT by DTBz-containing nanospheres can be attributed
to the retention of the drug in the non-fully recovered formulation (gels). Thus, one might
suggest that the presence of \(\pi-\pi\) interactions between the phenyl groups of DTBz pendent
chains themselves and aromatic groups of CPT causes: (a) pellets aggregation and general
loss of formulation yield, and (b) increase in encapsulation and stability of CPT within the
nanospheres of DTBz-composition. To explore these suggestions and perhaps reduce the effect
of pellets aggregation, we tested other nanospheres composition in which DTBz content varied
from 50 to 25% wt/wt. In DTO-DTBz, DTB-DTBz, and DTD-DTBz-containing nanospheres the
amount of non-re-suspended pellets reduced significantly but CPT binding was still lower than in
DTO-SA/5K nanospheres. When phenyl group content was reduced to 25 % wt/wt, DT(B+O+D+Bz)-SA/5K nanospheres retained a similar amount of CPT to the 100% wt/wt DTO-
SA/5K nanospheres. Therefore, at this point we cannot conclude if the presence of a benzyl
group increases CPT encapsulation or DTBz-containing pellets provide better conditions for
CPT’s self-aggregation and precipitation.

Similarly, but less significantly, the effect of the decrease in drug binding was observed in
VD3-loaded DTBz-containing nanospheres (Fig. 2-B). Since theoretically \(\pi-\pi\) interactions
between the phenyl groups of DTBz and the double bond of VD3 would be much weaker than
\(\pi-\pi\) interactions between the phenyl groups of DTBz and CPT, in this case reduction
of measured drug binding is attributed to only non-fully re-suspended formulation. Several
attempts were made to optimize the purification of drug-loaded DTBz-containing nanospheres
(see section below), but in all, no full re-suspension of the pellet was observed.

In summary, given that the highest and most reproducible binding efficiency of both VD3
and CPT were obtained with DTO-SA/5K nanospheres, we have continued our research
program using only this triblock formulation. In addition, as previously reported (23) the highest
binding and loading efficiency of paclitaxel was also observed in DTO-SA/5K.

3. Optimization of Camptothecin Binding Efficiency by Tyrosine-Derived
Nanospheres (DTO-SA/5K)

**Effect of Vitamin D3 presence and mass of the drug in feed**

We began with exploration of the relative efficacy and potential synergies of nanospheres
containing CPT and VD3. We hypothesized that the presence of highly hydrophobic VD3 will
increase the solubility of CPT within the nanosphere core and provide protection to the
camptothecin’s lactone ring, resulting in its increased bioavailability to breast cancer cells.

Figure 3 summarizes the effect of CPT input (mass in feed) and the presence of VD3 on
the camptothecin binding efficiency by DTO-SA/5K nanospheres. CPT binding was evaluated
for the constant quantity of the polymer with varying ratios of CPT to VD3. CPT’s binding
efficiency goes through a maximum as a function of the initial CPT to VD3 ratio: increases with
increasing CPT:VD3 feed ratios between 1:0.16 to 1:1:14 wt/wt and then decreases at higher
feed ratios (Figure 3, green bars).
We believe that the fall off in binding is due to competitive equilibria between the drug and nanosphere core and between the drug self-nucleation and precipitation. This explanation is supported by visual observations of yellow precipitates of camptothecin at a feed ratio of 1:1.4 = VD3:CPT wt/wt. Further, we evaluated the effect of VD3 presence on CPT binding efficiency. As seen in Figure 3 (highlighted in red square boxes) regardless of VD3 presence, exactly the same binding efficiency of CPT was measured, suggesting that there is no interaction between VD3 and CPT during nanosphere formation and drug encapsulation. The effects of VD3 concentration and the nature (hydrophobicity, confirmation and structural flexibility) of the co-drug on the retention of CPT by DTO-SA/5K nanospheres are illustrated in Figure 4.

CPT binding is not affected by the increased presence of VD3 (Figure 4-A), suggesting that these drugs do not interact with each other during encapsulation and most likely have different binding sites within the nanospheres. Further, to confirm this hypothesis we measured CPT binding in the presence of paclitaxel (PTX). As shown in Figure 4-B, CPT binding in the presence of either VD3 or PTX is similar to that of CPT alone. This outcome led to the consideration that not only hydrophobicity and solubility but also physical factors such as rigidity, conformation/configuration, and compatibility between the hydrophobic inner core-forming polymer block and the drug contributes to a stable incorporation of each particular drug. Figure 5 depicts chemical structures of all drugs investigated here.
From these structures it can be clearly seen that each molecule has different degrees of free rotation around single bonds and number of H-acceptors/donors that contribute to binding with the nanospheres and/or the co-drug. When rotation is possible, the molecule can have an infinite number of conformations, which increases the number of docking possibilities within the nanosphere core. CPT is the most rigid molecule among these with only 1 rotation bond, and this property could contribute to its limited incorporation into the nanospheres and/or interaction with both PTX and VD3. On the other hand, 14 and 5 rotation bonds are counted for PTX and VD3, respectively, and thus better packing of these drugs within the nanospheres is observed. We believe that other parameters such number of H-acceptors/donors, π-π interactions, molar volume and molecular weight play an important role in governing efficient drug encapsulation. To better understand the interaction and binding affinity of drugs with our nanospheres, our group began developing a computational method that combines Molecular Dynamics (MD) simulations and docking studies. We hope that this approach will provide additional insights into understanding of poor camptothecin encapsulation by our nanospheres in the current experimental conditions, as well as assist in prediction of suitable drug candidates for delivery by our nanospheres.

**Effect of fabrication and purification processes on camptothecin binding**

Camptotecin is a weak acid and therefore the lactone ring highly susceptible to ring opening by hydrolysis, forming carboxylate. The integrity of the lactone ring system of camptothecins is a key determinant for the chemotherapeutic efficacy. The closed form (Fig. 5) is more hydrophobic and favored in acidic conditions. The rationale behind the experiment, illustrated in Figure 6, was to optimize experimental conditions that will drive the drug to stay in a more hydrophobic form and to have greater solubility in the hydrophobic core of the nanospheres than in aqueous solutions.
CPT-loaded nanospheres were fabricated in the presence or absence of vitamin D3 in three different media: water, phosphate buffer saline (PBS) at pH 4-5 (cancer cells microenvironment), and PBS at pH 7.4. Our results demonstrate (Figure 6) that in a lower pH where the lactone form is prominent and theoretically the drug is more hydrophobic, less CPT was retained by the DTO-SA/5K nanospheres. Presence of vitamin D3 did not affect CPT binding in all tested conditions. Similar results were obtained when CPT-nanospheres were made in water (DI, pH 6 to 6.5). We do not have a confirmed explanation of these observations, but similar results were obtained when CPT in vitro release was measured as a function of pH of dialysis outer solution. Faster release kinetics were measured at pH 5.5 (Figure 9).

As previously reported, self-assembly of the polymers into drug-loaded nanospheres is induced by the drop-wise addition of the triblock copolymer and drug in DMF solution into aqueous solution under mild agitation. Purified drug-loaded nanospheres are isolated by the ultracentrifugation.(23,24) As a part of the process improvement during fabrication and purification (Scheme 2), we have investigated the effects of (a) polymer-camptothecin pre-mixing, and (b) effect of the centrifugation time on camptothecin binding efficiency. The intention in the pre-mixing technique experiment was to achieve a balance of intermolecular forces between the solvent and solutes and thus the maximum of their interaction. In this experiment CPT, VD3 and DTO-SA/5K triblock copolymer were mixed for 15 min (standard procedure) or 18 hours (overnight). Alternatively, polymer and drug/s were dissolved in DMF and lyophilized to create a homogeneous polymer-drug solid. As shown in Figure 7, neither prolonged mixing nor lyophilization and presence of VD3 significantly improved CPT’s binding efficiency.

Figure 7. Camptothecin binding efficiency as a function of CPT-polymer pre-mixing. 
(■) CPT alone; (■) in the presence of VD3.

Prior to the addition to PBS:
15 min: polymer+CPT+VD3 in DMF for 15 min
18 hrs: polymer+CPT+VD3 in DMF for 18 hrs
Lyophilized: polymer+CPT+VD3 in DMF for 15 min followed by lyophilization overnight; then re-suspended in DMF for 15 min

In view of the fact that CPT’s water solubility is relatively high for the hydrophobic drug and its hydrolytic instability, we have examined the effect of ultracentrifugation time on its binding efficiency. In general, the ultracentrifugation is done for 3 hours to ensure complete pelleting of the drug-loaded nanospheres. As shown in Figure 8, regardless of the VD3 concentration most of the CPT is actually found in supernatant (calculated as a ratio to the drug in feed) and even more CPT is lost if the centrifugation time is shorter.
Figure 8. Camptothecin binding efficiency as a function centrifugation time. (■) CPT in the presence of 1:1 wt/wt ratio of VD3; (▲) CPT in the presence of 1:3 wt/wt ratio of VD3; and (▲) CPT in the presence of 1:6 wt/wt ratio of VD3

The insufficient pelleting of the nanosphere-drug complexes and some of the formulation still being present in the supernatant could explain about 80% loss of CPT after 1 hour of ultracentrifugation. This assumption was confirmed by HPLC and $^1$H NMR analyses of the supernatant (data not shown). No significant difference in CPT binding was measured after 2 and 3 hours, and in both cases about 60% of initially added drug was found in supernatant. We have previously considered replacing ultracentrifugation by either dialysis or ultrafiltration. However, both of these techniques require prolonged exposure of CPT-loaded nanospheres to large quantities of aqueous washing solutions, which will increase the likelihood of CPT disassociation from the nanosphere formulation. This belief and results obtained in the ultracentrifugation effect experiment are supported by an in vitro released study performed using CPT-loaded and PTX-loaded nanospheres (Figure 9). (24)

Figure 9. Release profiles of CPT- and PTX-loaded DTO-SA/5K nanospheres in different pH media at 37 °C. (23,24)

Dialysis equilibrium profiles of CPT and PTX from DTO-SA/5K nanospheres in phosphate buffered saline are indicative of initial burst release of the drugs followed by a slow release phase (Figure 9). The initial burst release occurs within the first few hours of incubation and may be attributable to drug molecules associated with the interface of the nanosphere hydrophobic core and PEG corona while the slow release process depends on diffusion of drug from the nanosphere core region. The burst release of CPT amounts to 70% of the total bound drug in 5 hours (pH 7.4), compared to 25% of the total bound PTX released in the same amount of time. The faster release of CPT compared to PTX can be attributed to the lower initial loading of CPT in the nanospheres and the higher aqueous solubility of CPT. We have previously reported on the high, extended stability of our nanospheres under physiological conditions for several months and also their very low critical aggregation concentrations. (23,24) Nanosphere
dissociation and degradation is therefore not a significant factor in the observed drug release profiles. The relatively fast release kinetics of both drugs can however be attributed to the low Tg (21 °C) of the hydrophobic copolymer core.(23) The low Tg is indicative of a mobile polymeric matrix that enables rapid diffusion of small drug molecules.(8)

In addition, this experiment supports the data represented in Figure 6, where CPT-loaded nanospheres were made in PBS pH 4-5 and pH 7.4. The release kinetics of both PTX and CPT are strongly dependent upon the pH of the outer dialysis solutions; the lower the pH, the faster the release since most likely the solubility of the drug in this pH is higher (Figure 9). After 72 hours 100% of CPT is released at pH 5.5 compared to just 84% at pH 7.4 and only 60% at pH 10. For PCL, the release rates are substantially less than for CPT at all times. At 72 hours, the release of PCL is 72% at pH 5.5 and only 20% at pH 10. The measured hydrodynamic diameters of PCL-containing nanospheres recovered from the dialysis cassettes after 72 hours of dialysis at pH 5.5, 7.4 and 10 are 53 ± 1.2, 54 ± 1.3 and 43 ± 0.9 nm, respectively. Thus, the higher release rate at pH 5.5 than at pH 10 was not due to the destruction of the nanospheres. The pH dependence of the drug release profiles is expected to have an important therapeutic advantage in that the drugs will be bound to the nanospheres at pH 7.4, which is typical of the blood stream, but drug release will be enhanced at pH 5.5, which is typical of the intracellular environment.

In addition to the process improvements mentioned above, we have also evaluated other parameters that might increase CPT binding and stability. Among these are: (a) different re-suspension times of pelleted drug-loaded nanospheres, (b) ratio of organic phase containing the polymer and drug/s to the aqueous phase, and (c) reverse procedure of nanospheres preparation: slow addition of the aqueous phase to the organic containing the polymer and drug/s. Different re-suspension times from 4 to 48 hours (prolonged for DTBz-containing nanospheres) and smaller ratio between organic and aqueous phases did not show any effect. Increased volume ratio of aqueous to organic phase resulted in significant reduction of CPT binding. Using the reverse procedure, we had hoped to decrease CPT’s chance to preferentially dissolve in aqueous phase. Unfortunately, none of these attempts resulted in improved CPT binding by our nanospheres.

In summary, what causes such rapid and massive CPT loss is complete incompatibility with our nanospheres or fabrication and purification conditions that promote CPT disassociation. Figure 10 summarizes quantification of CPT during all steps of preparation and purification of CPT-loaded nanospheres.

![Figure 10. Quantification of CPT presence in each step of preparation and purification of CPT-loaded nanospheres.](image)

As mentioned earlier, the first filtration step is responsible for at least 35% loss of CPT’s initial input. That includes CPT entrapped in nanospheres that are larger than 200 nm, CPT dissolved in fabrication medium and the unbound drug that precipitates out. This lowers our
input into the centrifugation step where about 60% of CPT is washed away and in some cases minor CPT precipitation is also found in pellets. After the second filtration (sterilization), we are left with about 5% wt/wt bound CPT that is rapidly released from nanosphere formulation under physiological conditions.

To conclude, at this point we are not convinced that camptothecin and its derivates are suitable candidates for the encapsulation and delivery by our nanospheres. On the other hand, paclitaxel and vitamin D3 showed much greater binding to our nanospheres and extended stability within the nanospheres at all complex formations and purification steps (Figure 1). We have previously reported on optimization of paclitaxel-loaded nanospheres and these studies revealed that the optimum formulation for PTX binding and in vitro delivery is DTO-SA/5K.(24) Hence, our efforts for (i) optimization of the process for (PTX+VD3)-loaded nanosphere formation and (ii) in vivo evaluation of the biological efficacy and safety of the nanosphere delivery system for treating a range of human cancers, will concentrate on using paclitaxel and/or Vitamin D3 in DTO-SA/5K nanospheres.

4. In Vitro and In Vivo Biological Efficacy and Safety of the PTX-Nanosphere Formulation (PRELIMINARY RESULTS)

Below we report on preliminary data obtained in in vitro and in vivo evaluation of paclitaxel-loaded DTO-SA/5K performed in collaboration with former subcontractors at UMDNJ-RWJMS. Please note that these are incomplete studies and that further investigation and optimization to complete them will be performed in the research period of Sept. 2008 - Sept. 2009 in collaboration with Prof. Minko at the College of Pharmacy of Rutgers University.

In Vitro Cytotoxicity of nanospheres alone and PTX-loaded nanospheres

In the investigated copolymer compositions (Figure 11-A) and DTO-SA/5K nanosphere concentration range (Figure 11-B), no significant decrease of the cell metabolic activity of KB cervical carcinoma cells was detected, confirming that these nanospheres do not induce any short-term cytotoxicity. Cell viability was analyzed by MTS colorimetric assay after 3 days.

![Figure 11: Metabolic activity of KB cervical carcinoma cells exposed to tyrosine-derived nanospheres prepared in PBS.](image)

Further, effectiveness and non-toxicity of nanosphere formulation was compared to the Cremophor® EL (CrEL) vehicle (Figure 12) in MDA-MB-435 breast cancer cells exposed to PTX delivered in these formulations (Figure 12). Viability of cells was expressed as metabolic activity of cells remaining in the wells after treatment (MTS assay, 490 nm).
This experiment clearly demonstrates that tyrosine-derived nanospheres exhibit no toxicity as compared to CrEL (green bars). Further data analysis revealed that our nanospheres provide substantially enhanced PTX delivery as compared to CrEL. By the decoupling of the effect of vehicle cytotoxicity (subtraction of formulation toxicity from the toxicity of the vehicle – orange bars) the efficiency of delivered PTX is similar in both formulations containing 30 µg of PTX and significantly higher in 150 µg PTX dosage. Cytotoxicity of nanospheres loaded with VD3 and (VD3+PTX) to MDA-MB-435 breast cancer cells is currently under investigation.

**In Vivo toxicity of PTX-loaded nanospheres (Preliminary Data)**

Preliminary assessment of tyrosine-derived nanospheres toxicity was evaluated in NCR nu/nu mice. The mice were treated (q2dx5, tail vein, i.v.) with 5, 50, 100, and 200 mg/kg doses of the nanospheres alone and/or with nanospheres-PTX (15 mg of drug/kg). The change in total body weight (toxicity) was measured and mice were observed for other physical stress. This study revealed no significant weight loss (<15%), no change in vital behavior, no skin irritation at the injection spot as well as no skin irritation and sensitization in any of the treated groups, confirming the non-toxic nature of nanospheres as a vehicle and nanospheres-PTX formulation at current drug dose. Maximum tolerated dose (MTD) for the PTX-nanospheres formulation in NCR nu/nu mice is planned to be evaluated in the next round of experiments.

**In Vivo anti-tumor efficacy of PTX-loaded nanospheres (Preliminary Data)**

Anti-tumor efficacy of PTX-loaded nanospheres was assessed in mice bearing subcutaneous MDA-MB-435 breast cancer xenografts (Figure 14).
Nanospheres-PTX (N=8) were administered via tail vein injections using a dose of 15 mg/kg on a q2dx5 schedule. As a control, CrEL-PTX (N=7) and saline (N=7) were administered to a separate group of xenografted mice using an identical dose and schedule. Tumor volume was measured based on length and width. Using the T-C method, the growth delay was calculated to be 244% for PTX and 181% for Nano-PTX. These results suggest that tyrosine-derived nanospheres containing paclitaxel exhibit anti-tumor activity in a breast cancer xenograft model that is similar to that of an equivalent dose and schedule of clinically used formulation of Cremophor-paclitaxel. Next, we will investigate the relative efficacy and potential synergy of nanospheres containing VD3 alone and mixture of vitamin D3 and paclitaxel.

**In Vivo biodistribution of PTX-loaded nanospheres (Preliminary Data)**

*In Vivo* Biodistribution of PTX delivered nanospheres or CrEL was assessed in mice bearing subcutaneous MDA-MB-435 breast cancer xenografts (Figure 15).

This initial test revealed that in general, accumulation of PTX is comparable in all tested tissues regardless of administration vehicle: rapid and significant uptake (0.5-1 hrs) and very low concentrations in plasma at 24 hours. The main difference in PTX distribution (24 hours) was observed in the lung tissue: 43% of PTX delivered via nanospheres remained in lungs, while no PTX administrated via CrEL was detected. To show further advantages of tyrosine-derived nanospheres to CrEL, pharmacokinetic studies and more detailed evaluation of nanospheres EPR effect will be further investigated.
Key Research Accomplishments and Preliminary Conclusions

(1) Copolymer syntheses and nanosphere formulation
(2) Nanospheres Drugs Compatibility and Binding Efficiency:
   a. Tyrosine-derived nanospheres efficiently encapsulate vitamin D3 and paclitaxel under all investigated conditions
   b. However, our nanospheres encapsulate only a limited amount of camptothecin regardless of Vitamin D3, and/or optimization of CPT-loaded nanospheres fabrication and purification techniques
   c. The optimum nanosphere formulation for camptothecin, vitamin D3 and paclitaxel delivery is DTO-SA/5K
(3) Preliminary in vitro and in vivo studies using paclitaxel-loaded nanospheres suggest that:
   a. tyrosine-derived nanospheres provide highly effective delivery of hydrophobic paclitaxel to human tumor cells in vitro
   b. tyrosine-derived nanospheres exhibit no toxicity as compared to clinically used CremophorEL formulation;
   c. tyrosine-derived nanospheres containing paclitaxel exhibit anti-tumor activity in a breast cancer xenograft model that is similar to that of an equivalent dose and schedule of Cremophor-paclitaxel. Thus, tyrosine-derived nanospheres offer the potential for effective parenteral delivery of a wide array of hydrophobic drugs without the cytotoxicity problems commonly exhibited by surfactant-based drug delivery systems

Further investigation and optimization of tyrosine-derived nanospheres as a novel pathway in breast cancer treatment are ongoing and will be reported in the completion of the research program.

Reportable Outcomes

This research has been reported as a poster presentation at the DOD Breast Cancer Research Program – Era of Hope Meeting (June 2008, Baltimore; poster session number and order: 49-11). No other reportable outcomes have yet resulted from the research described in this report.

Conclusion

As stated in the beginning of this report, due to the delay in in vivo evaluation of our drug-nanospheres formulation and current pending approval of animal protocols by both Rutgers and USAMRMC Animal Care and Use Review Office, no significant progress was achieved in this part of the research. Even though we are confident that this work may result in the introduction of innovative treatments for breast cancer, at this point we do not have enough confirmation to comment or summarize the implications of the completed research.
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