Algorithm for Designing Nanoscale Supramolecular Therapeutics with Increased Anticancer Efficacy


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ABSTRACT: In the chemical world, evolution is mirrored in the origin of nanoscale supramolecular structures from molecular subunits. The complexity of function acquired in a supramolecular system over a molecular subunit can be harnessed in the treatment of cancer. However, the design of supramolecular nanostructures is hindered by a limited atomistic level understanding of interactions between building blocks. Here, we report the development of a computational algorithm, which we term Volvox after the first multicellular organism, that sequentially integrates quantum mechanical energy-state- and force-field-based models with large-scale all-atomistic explicit water molecular dynamics simulations to design stable nanoscale lipidic supramolecular structures. In one example, we demonstrate that Volvox enables the design of a nanoscale taxane supramolecular therapeutic. In another example, we demonstrate that Volvox can be extended to optimizing the ratio of excipients to form a stable nanoscale supramolecular therapeutic. The nanoscale taxane supramolecular therapeutic exerts greater antitumor efficacy than a clinically used taxane in vivo. Volvox can emerge as a powerful tool in the design of nanoscale supramolecular therapeutics for effective treatment of cancer.

KEYWORDS: cancer, supramolecular nanoparticles, molecular dynamics simulations, taxanes, drug delivery

Supporting Information

N ature has inspired the design of many engineered materials and devices.1,2 For example, the nanotopography of gecko feet inspired the development of surgical adhesives.3 Similarly, a cephalopod’s ability to display vivid colors by reversibly activating chromatophores through muscular contractions recently inspired the development of an electro-mechanochemically responsive elastomer system that can exhibit a wide variety of fluorescent patterns under the control of electric fields.4 In the evolution of organisms, an extensively studied system is the Volvocine family, which includes the unicellular Chlamydomonas and the first multicellular organism, Volvox. Simplistically, Volvox comprises 2000 somatic cells (and a few germ cells) that resemble Chlamydomonas cells routinely arranged in a self-assembling extracellular matrix.5 The evolution from unicellularity to multicellularity confers complexity of function. In the chemical world, such an evolution is mirrored in the origin of supramolecular structures from molecular subunits.6 We rationalized that the complexity of function acquired in a supramolecular system over a molecular subunit could be potentially harnessed in the treatment of cancer.

Mortality due to cancer is expected to increase from 7.6 million in 2008 to 12 million deaths in 2030, making it one of the major causes of mortality.7 Even emerging approaches such as immunotherapy produce durable response in only a fraction

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of the cancer patients and need to be combined with small-molecule chemotherapy. An approach that can preferentially target the small-molecule therapeutics to the tumor and minimize exposure to systemic tissues can therefore emerge as a paradigm shift in the treatment of cancer. We rationalized that this goal can be achieved using supramolecular therapeutics, where molecular subunits self-assemble through supramolecular “weak” interactions to form large complex nanoscale structures. This evolution in dimensional complexity confers functional advantages over the molecular subunits in that they home into tumors through an enhanced permeability and retention (EPR) effect arising from leaky tumor vasculature with minimal systemic organ exposure. However, the current approaches of engineering nanoscale supramolecular therapeutics are stochastic with limited insights into the mechanisms of self-assembly, which pose a significant barrier to expanding the repertoire of supramolecular therapeutics.

Here, we report the development of a computational algorithm, which we term Volvox, for the rational design of nanoscale supramolecular therapeutics. Starting with a quantum mechanical energy-state- and force-field-optimized molecular structure of a template subunit, for example, a known drug, we use large-scale all-atomic explicit water molecular dynamics simulations to model the interaction of high concentrations of the subunits with colipids to form a stable nanoassembly. Based on the inputs from these simulations, we sequentially and rationally design subunits from the template and use their quantum mechanical energy-state- and force-field-optimized molecular structures to repeat the above simulations, cyclically iterating the process until a molecular subunit is identified that confers optimal stability to the supramolecular nanostructure. Interestingly, the algorithm could additionally be used to analyze the synthesis of the current generation of nanoparticles that relies on drug encapsulation. We experimentally demonstrate that the final predicted structure indeed leads to a stable supramolecular nanostructure. In one example, we demonstrate that Volvox enables the design of a nanoscale taxane supramolecular therapeutic. In another example, we demonstrate that Volvox can also facilitate the optimization of a PEGylated colipid to form a stable nanoscale supramolecular nanotherapeutic. We demonstrate that the nanoscale taxane supramolecular therapeutic significantly improves the antitumor outcome in two aggressive in vivo 4T1 breast and K-RasLSL, G12D/Ptenfl/fl ovarian cancer models as compared with a clinically used taxane. Such nanoscale supramolecular therapeutics, inspired by the evolution of multicellularity from self-assembly of unicellular building blocks, can emerge as a paradigm in the treatment of cancer.

RESULTS AND DISCUSSION

Simulating Large-Scale Molecular Subunit Excipient Interactions. Volvox was set up as three connected iterative recursive modules. In the first module, the goal was to develop the force field for the molecular subunits. The second module involved the all-atomic molecular dynamics (MD) simulation of the interactions of these quantum mechanical (QM) energy-optimized structures packed with colipids. The third module used analysis to quantify stability of the system (Figure 1a and Supporting Information Figure S1). Analogous to Volvox, where the individual cells are arrayed in an extracellular matrix for multicellularity, the molecular subunits needed to be arranged in the excipients-based matrix to form a stable supramolecular structure. We therefore needed to construct a large simulation cell to pack a high concentration of structurally large molecular subunits (for a detailed algorithm, see Supporting Information) (Figure S2a). Following an all-atomic simulation, the trajectory was analyzed using three different parameters that we developed as a measure of stability: (i) ripple formation, (ii) lipid tail ordering, and (iii) the probability of water molecules entering the bilayer. We set

![Volvox Algorithm](image-url)
Figure 2. All-atomistic simulation predicts a stable supramolecular assembly with STX2 but not with PTX. Volvox generated trajectories were analyzed for stability using several parameters. (a) Schematic representation for tilt angle calculation. Tilt angle is the angle between the vector joining the center of mass of phospholipid tails and the z-axis (axis perpendicular to bilayer plane). Value of tilt angle is positive or negative depending on direction of the ripple. Its value tends to 0° when no ripples form. (b) Distribution of tilt angle averaged over the last 5 ns of the MD trajectory. The broader the distribution, the larger the tilt angle and, hence, the higher the extent of ripple formation. Filler distribution for PTX (red line) than STX2 (blue line) represents larger ripple formation for PTX compared to STX2. Further, peak height of distribution at 0° is higher for STX2 than for PTX. Thus, PTX is away from no ripple state more than STX2. (c) Schematic representation of angle (θ) between the vector defined on the C–C bond on the SOPC tail with the z-axis (axis perpendicular to bilayer plane). Deuterium order parameter (S_{CD}) is calculated using θ. S_{CD} is calculated on each carbon atom of a phospholipid tail. The higher the S_{CD} or the greater the lipid tail ordering. (d) Deuterium order parameter on each methylene group on the saturated tail of SOPC is depicted. Lipid tail ordering for the PTX-containing SOPC bilayer is the least. Tail ordering of STX2 containing the SOPC bilayer is lower than pure SOPC but higher than PTX containing a bilayer. (e) Representation of unit volume inside the hydrophobic core of a lipid bilayer, where the number of water molecules per unit volume is computed. Penetration of water molecules into the hydrophobic core of the bilayer is indicative of its instability. The hydrophobic region of the bilayer is divided into grids, and the number of water molecules falling in these grids is counted. The count is divided by volume of the grid to obtain the number of water molecules per unit volume. (f) Probability of finding water molecules per unit volume was calculated by normalizing the count per unit volume with number of frames in the last 5 ns of the MD trajectory. More water molecules penetrate the PTX-containing bilayer compared to the STX2-containing bilayer. It indicates that the PTX-containing bilayer is less stable than the STX2-containing bilayer. (g) Cluster size of PTX/STX2 molecules in the lipid bilayer. It represents aggregation of PTX/STX2 molecules in the lipid bilayer. Higher peaks at larger cluster size for PTX compared to those of STX2 indicate more aggregation of PTX molecules in the PTX-containing bilayer.

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bounds, using (1) trajectory obtained with a pure lipid bilayer (upper bound) and (2) trajectory obtained using a bilayer spiked with the starting template molecule that destabilizes the lipid bilayer (lower bound). Starting from a known bioactive molecular template and tweaking the molecular structure iteratively after every run based on inputs from the trajectory analysis with the goal of achieving the upper bound, the recursive loop could progressively lead to the configuration of a final molecular subunit that facilitated the formation of a stable supramolecular therapeutic. All-atomistic simulations have been previously used to investigate the fluidity and packing of preassembled lipid bilayers and to study the permeability and distribution of different solutes and drugs through lipid bilayers as a surrogate for the plasma membrane. Recently, atomic simulations have also been used to understand the interactions of nanomaterials with the cell membrane. However, the use of all-atomistic simulations to analyze the effect of high concentrations of large molecular subunits on the stability of a supramolecular structure and to use this information to in silico design subunits that form stable supramolecular structures is yet unknown. Indeed, it is only recently that a coarse-grained model was used to simulate the effect of distributions of a low concentration of drug molecules inside a liposomal carrier. Furthermore, previous reports only describe the insertion of a single large molecule in the bilayer by creating a cavity. In contrast, in our study, we needed to pack high concentrations of large molecular weight drug subunits in a lipid-based system. Additionally, previous studies have achieved packing by either randomly placing small molecules into the bilayer and eliminating the overlaps between the molecules and lipids by energy minimization or randomly replacing lipid molecules to minimize the overlaps right at outset. These approaches were, however, incompatible with molecular subunits that have significantly larger volume as in the case of the current study. As a result, we initially placed the molecular subunits in a symmetric position and ran an initial energy-minimizing (at NPT) pre-equilibration step, which randomized the arrangement of the molecular subunits (Supporting Information Figure S2a). This randomized conformation was the starting structure
for the all-atomistic simulation, thus closely mimicking the randomization observed in the physical system.

**Designing a Volvox-Inspired Taxane Supramolecular Subunit.** We set a high bar to test the applicability of Volvox in the development of a stable nanoscale supramolecular therapeutic. We therefore selected paclitaxel (PTX) as the starting template to engineer a taxane-based subunit that facilitates the assembly of a nanoscale supramolecular structure. Paclitaxel is one of the most widely used anticancer drugs but is notorious for conferring instability to lipid bilayers. The hydrophobic, asymmetric, nonflexible structure of PTX (Figure 1b) means that entrapping in lipid-based nanoparticles has remained a holy grail even after two decades of research.

As the first step toward developing a taxane-based nanoscale supramolecular therapeutic, we used Volvox to simulate the dynamic interactions of PTX with colipids. Paclitaxel structures obtained after QM optimization and short MD simulation in vacuum are shown in Figure 1b and Supporting Information Figure S2b. Simulations were performed at increasing concentrations (5, 20, and 30 mol %) of PTX incorporated in a bilayer system made of 1-octadecanoyl-2-(9Z-octadece- noyl)-sn-glycero-3-phosphocholine (SOPC), where the force-field parameters for SOPC were adapted from an atomistic CHARMM-based force field proposed for phosphatidylcholine lipids and using a TIP3P water model. The composition of the models constructed is shown in Supporting Information Table S1. As shown in movie 2, the unbiased atomistic simulations revealed that 20 mol % of PTX formed large ripples in the bilayer structure (Figure 1c). Such periodic ripple formations have been associated with pretransition between fluid and gel phases. At 5 mol %, PTX formed a stable bilayer (Supporting Information Figure S2c and movie 1). Highly mobile fluidic domains, mainly composed of PTX molecules and mostly oriented near the lipid headgroup and water interface, were also observed (Figure 1c and Supporting Information Figure S2d). The formation of such large fluidic domains has been reported to increase the bilayer instability, hindering the formation of a stable supramolecular structure.

Based on this in silico trajectory analysis, we rationalized that endowing the taxane with a hydrophobic tail that inserts into the bilayer and reduces lipid mobility, while orienting the active taxane moiety toward the bilayer and reduces lipid mobility, while orienting the active taxane moiety could further stabilize the supramolecular assembly. Paclitaxel was chosen based on the criteria that it should not cause any structural changes in the taxane, keep the orientation of the taxane near headgroup–water interface, and provide optimal insertion within the bilayer. We termed this molecule as Suprataxel-2 (STX2) (Figure 1b and Supporting Information Figure S2g). Indeed, simulations revealed the formation of a stable bilayer even at 20 mol % of STX2 molecules in contrast to the PTX simulations (Figure 1c and movie 4).

As a measure of the degree of stability of the supramolecular bilayer, we quantified ripple formation by calculating the angle between the vector joining the center of mass of lipid tails and the phosphorus (P) atom on the headgroup of the SOPC molecule with the z-axis (bilayer normal) (Figure 2a). These angles were calculated and averaged along the x-axis (perpendicular to the bilayer axis) by dividing the simulation cell into 20 equal slabs. Compared to PTX, STX2 exhibited reduced ripple formation (Figure 2b). To quantify the change in ordering of lipid tails, we calculated the deuteron order parameter (−S°C,D) for the SOPC lipid saturated tail from the last 5 ns of the trajectory (Figure 2c). As shown in Figure 2d, STX2 was found to increase the ordering of the aliphatic tail groups by ~30% compared to PTX.

The formation of ripples and the decrease in lipid tail order can result in perforations in the bilayer, which can facilitate the penetration of water molecules. To compare the permeability of the PTX and STX bilayer systems, we calculated the probability of finding water molecules per unit volume of the hydrophobic region of the bilayer (Figure 2e). We observed that higher concentrations of PTX increased the probability, while the presence of STX2 hindered the permeation of water into the supramolecular structure (Figure 2f). Thus, the computationally optimized STX2-based supramolecular bilayer shows less perforation, consistent with a stable supramolecular assembly. We previously observed that PTX has a greater tendency to aggregate in the lipid bilayer, potentially arising from partial phase separation of lipids and PTX. The size of clusters formed by PTX or STX2 was calculated by searching for neighbors within a cutoff of 2 nm (distance between center of masses) and was represented as the distribution of cluster size from the last 5 ns of the trajectory. As shown in Figure 2g, PTX molecules aggregated in larger size clusters compared to STX2. Studying the tilt angle and number of PTX molecules present in the same slab plotted as a function of slab number on the x-axis revealed that the count of PTX molecules were more in the particular slabs where the ripples appear (Supporting Information Figure S2d). This suggested that the aggregation of molecules can trigger ripple formation and instability, which can translate to a rapid release of the drug. On the contrary, the presence of the flexible hydrophobic tail in STX2 decreased this aggregation by pinning the taxane moiety in the interface region. A comparison of lipid tail ordering and probability of finding water in the bilayers containing increasing concentrations of PTX or STX2 revealed that the most optimal structure (i.e., loading vs stability) is achieved with 20 mol % of STX2. A 30 mol % of STX2 resulted in a less stable supramolecular assembly than 20 mol % (Supporting Information Figure S2h). As shown in Figure S2j, STX1 performed poorly compared with STX2 on all these parameters.
and was closer to PTX in performance. These results indicate that Volvox can emerge as a powerful tool in the design of subunits for supramolecular assembly.

Physicochemical Studies on a Taxane Supramolecule.
To experimentally test the in silico predictions, we chemically synthesized STX2 subunits (Supporting Information Figure S3) and tested its ability to form stable nanoscale supramolecular structures compared to PTX. Consistent with the in silico model, we observed the formation of stable supramolecular nanostructures using 5 mol % of PTX. However, at 20 mol % of PTX, the supramolecular nanostructures did not form unless 30 mol % of a PEGylated colipid, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-aminopolyethylene glycol)-2000 (DSPE-PEG2000), was added. Even then PTX precipitated out of this system within 24 h, consistent with an unstable system (Figure 3a and Supporting Information Figure S4a–c).

Indeed, high-resolution cryo-transmission electron microscopy (cryo-TEM) revealed the disruption of the bilayer with PTX as

Figure 3. STX2 but not PTX forms stable supramolecular structure. (a) PTX precipitates out of the lipidic nanoparticles within 24 h, as shown in the image. (b) Representative cryo-TEM image of a PTX-loaded nanoparticle fabricated with colipids (PC and DSPE-PEG at 50 and 30 mol %, respectively) shows disruption of the lipidic structure in the presence of PTX (20 mol %) as predicted from all-atomistic simulations. (c) In contrast, STX2 forms stable formulations with different colipids during storage conditions at 40 °C after 24 h. (d) High-resolution cryo-TEM image of STX2 supramolecules (scale bar = 100 nm). (e) Size distribution of STX2 supramolecules as measured by DLS. The hydrodynamic radius was found to be 180 ± 4 nm. The DSC graphs of (f) pure lipidic nanoparticles and (g) STX2 supramolecules. Tm is phase transition temperature. (h,i) Height and deflection images and their corresponding indentation histograms fitted with Gaussian distribution curves generated using AFM for (h) PTX-loaded lipidic nanoparticles and (i) STX2 supramolecules (insets show plots of force vs z-piezomotion). (j) Change in size of the PTX-loaded lipidic nanoparticles and STX2 supramolecules in plasma and PBS measured using DLS as a quantification of stability over time. (k) Release of PTX from PTX-loaded nanoparticles or from STX2 supramolecules in PBS (pH 7.4) or when incubated with 4T1 cell lysate.
predicted by Volvox (Figure 3b). In contrast, STX2 facilitated the assembly of stable supramolecular structures even at 20 mol % (Figure 3c−e). Furthermore, STX2 formed stable supramolecular structures with a range of lipids with varying degrees of saturation and length, such as SOPC, egg-PC, DOPC, and soy-PC (Figure 3c and Supporting Information Figure S4b). Additionally, we observed that the efficiency to pack STX2 increased when we added increasing molar concentrations of DSPE-PEG2000, resulting in a loading efficiency of 88 ± 4% at 20 mol % of STX2 (Figure S4d). It has been reported that...
optimal molecular weight PEG–lipid conjugates undergo steric exclusion from the surface of the lipid bilayer and can contribute to the thermodynamic stability of the lipid bilayer. Cryo-TEM analysis revealed that STX2 forms spherical nanostructures of ~100 nm (Figure 3d), while dynamic light scattering (DLS) revealed a hydrodynamic radius of 180 ± 10 nm with a narrow polydispersity index of 0.188 (Figure 3e and Supporting Information Figure S4b,d). Differential scanning calorimetry (DSC) revealed that the STX2-based supramolecular bilayer exhibited a similar Tm as the pure lipid-only bilayer (Figure 3f,g). To further validate the stability data, we used atomic force microscopy (AFM) in contact mode imaging followed by force mapping. Nanoindentation is an effective technique to measure the stiffness of the lipid bilayer system, where higher and lower indentation values suggest lower and higher stiffness values, respectively, and thereby structural stability of the nanostructures. As shown in Figure 3h,i, in contrast to the STX2 supramolecular nanostructures, the PTX nanostructures exhibited a variation in the height features indicative of precipitation or aggregation. Force curves were plotted and further analyzed to get the indentation (nanometer) values. The mean (±standard deviation) for PTX nanostructures was found to be 127.00 ± 2.00 nm in contrast to 13.00 ± 0.01 nm of STX2-based supramolecular nanostructures, which was similar to 4.00 ± 0.01 nm for lipid-only bilayers (Supporting Information Figure S4e). Indeed, the STX2-based supramolecular nanostructure showed a very narrow Gaussian distribution comparable to a lipid-only bilayer, suggesting that despite high drug concentrations, STX2 maintains the structural stiffness of the lipid bilayer. In contrast, the PTX colipid nanostructures exhibited a broad distribution, indicative of a very fragile lipid bilayer system (Figure 3h,i). Indeed, monitoring the change in size over a defined time period at 4 °C in phosphate-buffered saline (PBS) and serum (Figure 3j), as an indirect measure of physical stability, further validated that the STX2-based supramolecular structures were stable unlike PTX-based nanostructures. Furthermore, consistent with the Volvox-based simulations, where PTX-induced ripple formation was predicted to facilitate rapid drug release, the PTX-based nanostructures indeed exhibited a burst release. In contrast, the STX2-based supramolecular nanostructures enabled a sustained release of drug in the presence of cell lysate, due to acidic and enzymatic conditions, with slower release in PBS (pH 7.4) (Figure 3k).

**Volvox Predicts Optimization of Excipients.** We next evaluated whether the Volvox-based structural insights can be extended to optimizing excipients for increasing the stability of the supramolecular nanostructures. We selected cabazitaxel (CBZ), a recently approved taxane that di...
Figure 5. Nanoscale STX2-based supramolecular therapeutic exerts greater efficacy than paclitaxel in vivo. (a) Representative images of animals with K-rasLSL/Ptenfl/fl ovarian cancer pre- and post-treatment with vehicle, PTX-loaded lipidic nanoparticles or STX2 supramolecules. Animals were given three cycles of treatment at a paclitaxel dose equivalent of 3 mg/kg. Tumor images were obtained using an IVIS Lumina II imaging system. (b) Quantification of bioluminescence. Mice received 150 mg/kg of α-luciferin firefly potassium salt via intraperitoneal injection prior to imaging. (c) Table shows the mortality of mice treated with increasing doses of PTX or STX2 supramolecules as a measure of maximal tolerated dose. *Animal was sacrificed as a result of distress. (d) Effect of PTX and STX2-based supramolecular therapeutics on tumor growth in 4T1 animal model. 4T1 breast-tumor-bearing animals were treated with 30 mg/kg of PTX or STX2-based supramolecular therapeutics (dose equivalent to 50 mg/kg paclitaxel) once the implanted tumors reached 100 mm³ in volume (considered as day 0). The days of dosing are labeled with arrows. (e) Plasma pharmacokinetic profile of STX2-based supramolecules in Balb/c mice. The graph shows plasma concentration of drug at different time post-injection. (f) In vivo biodistribution profile of NIR-dye-labeled STX2-based supramolecules in 4T1 tumor-bearing mice at different time points post-injection. (g) Quantitative analysis of relative organ accumulation in the 4T1 tumor model system. Error bars represent mean ± SEM analyzed by Maestro (CRI) small-animal in vivo imaging system. (h) Representative immunofluorescence images show cross sections of tumors from animals treated with vehicle, PTX-loaded lipidic nanoparticles, or STX2-based supramolecules. Cryosections were labeled for von Willebrand factor, which delineates the vasculature. The sections were counterstained with DAPI to label the nuclei. Images are shown in pseudocolor. Graph shows quantification of the inhibition of tumor angiogenesis following treatment. Data shown are mean ± SEM from n ≥ 3. *P < 0.05, **P < 0.01, P < 0.001 (ANOVA followed by Newman Keul's post-hoc test).

aggressive syngeneic mouse models that have been extensively used in cancer biology.49,50 The advantage of murine syngeneic and transgenic tumor models over xenografts lies in the intact immune component that is absent in the latter. Recent reports indicate that the immune component could play an important role in antitumor efficacy of cytotoxic agents.51,52 As transgenic models capture the normal progression of tumor growth, we first used a transgenic K-RasLSL/Ptenfl/fl ovarian cancer. The animals were treated with a three cycles of STX2-based supramolecular therapeutic or PTX at a dose equivalent to 3 mg/kg of PTX. As shown in Figure 5a,b, bioluminescence quantification revealed that treatment with the STX2-based supramolecular therapeutic exhibited significantly greater antitumor efficacy than PTX. We performed a maximum tolerated dose (MTD) study in mice. As shown in Figure 5c,d, MTD was reached at 30 mg/kg of PTX, while only one animal in the STX2-based supramolecular therapeutic-treated groups had to be sacrificed at a dose level of 80 mg/kg, indicating that the supramolecular therapeutics possess an improved safety profile as compared with the parent taxane molecule. We next tested the antitumor efficacy in a 4T1 breast cancer model, where tumor-bearing animals were injected with a dose equivalent to MTD dose (30 mg/kg) of PTX or STX2-based supramolecular therapeutics at the maximum dose (50 mg/kg paclitaxel equivalent) that can be administered i.v. As shown in Figure 5d, the STX2-based supramolecular therapeutic exerted an antitumor outcome significantly superior to that of the high dose of PTX.

It has been previously reported that the preferential accumulation of nanoparticles in the tumor arises from an enhanced circulation half-life. Indeed, pharmacokinetic analysis of the STX2-based supramolecular therapeutic revealed a T1/2 of 10.95 ± 0.99 h (Figure 5e and Supporting Information Table S2), which is significantly higher than 1.5 h recently reported for paclitaxel.53 Next, to test the biodistribution of the STX2-based supramolecular therapeutic, we spiked the supramolecular structures with a near-infrared dye and used imaging to visualize the distribution in vivo at different time points post-i.v. administration. As shown in Figure 5f,g, the supramolecules were found to progressively accumulate in the tumor with time and reached a plateau by 24 h. Quantifications of the distribution at an organ level indicated a preferential accumulation in the tumor and negligible concentrations in lungs, heart, or the kidneys. The accumulation in spleen, a reticuloendothelial organ, was significantly lower than in the tumor, while the levels in the liver were consistent with a
Targeting. An advantage of the modularity, that is, that factors of the former.

PTX, which mechanistically could explain the increased efficacy of STX2-based supramolecular therapeutic as compared to PTX, which mechanistically could explain the increased efficacy of the former.

Modular Design Facilitates Integration of Tumor Targeting. An advantage of the modularity, that is, that different excipients and the molecular subunits could be brought together for supramolecular assembly, is the possibility of integrating active tumor-targeting capability to the supramolecular therapeutic. The need to target the drugs specifically to the tumor is the holy grail in cancer chemotherapy. For example, antibody–drug conjugates (ADCs) have evolved to target conjugated cytotoxic payloads to the tumor. Recent studies have revealed that iRGD peptides can bind to the neuropilin receptors in tumors and promote penetration of drugs and nanoparticles to tumors. We rationalized that conjugating iRGD peptides to the terminal end of the PEG chain of DSPE-PEGs (Figure 6a) could facilitate the targeting of the supramolecular therapeutics to the tumor. The iRGD-endowed STX2-based supramolecular therapeutic had a hydrodynamic radii of 216.00 ± 6.45 nm and a ζ-potential of −42.70 ± 4.56 mV (Supporting Information Figure S8e). We tested the in vivo efficacy of iRGD-endowed STX2-based supramolecular therapeutic using the 4T1 tumor model. As shown in Figure 6b,c, three cycles of PTX (3 mg/kg) had no effect on the growth of 4T1 breast cancer. We selected a submaximal dose of STX2-based supramolecular therapeutic to dissect the effects of targeting. Interestingly, we observed a dramatic increase in tumor growth inhibition with the iRGD-STX2-based supramolecular therapeutics. Previous studies have noted no intrinsic antitumor activity of iRGD, suggesting that the increased efficacy stems from targeted delivery to the tumor. We did not observe any change in the body weight, a measure of systemic toxicity at these dose levels, which further indicated that the increased antitumor efficacy was specific (Figure 6d). Taken together with the results from the previous efficacy study, where a higher dose exhibited a greater efficacy, these results indicate that increasing the delivery of the active agent to the tumor enhances the antitumor outcome. To understand the mechanisms underlying the increased efficacy of STX2-based supramolecular therapeutics, we measured the degree of apoptosis using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). As shown in Figure 6e,f, there was a significant correlation between the tumor growth inhibition and the proportion of apoptotic cells in the tumor. We did not observe any change in the body weight, an indication of bioluminescence was achieved by using the Living Image software 3.1. Mice received 150 mg/kg of D-[1-14C]luciferin sodium salt via intraperitoneal injection prior to imaging. (i,j) Effect of different treatments on (i) blood cell counts and (j) degree of apoptosis quantified as the ratio of TUNEL+ve cells to total cells in cross sections of different organs as a measure of systemic toxicity. Data shown are mean ± SEM from n ≥ 3. *P < 0.05, **P < 0.001. Statistical analysis was performed with ANOVA.
respective treatment groups. Treatment with iRGD-STX2-based supramolecular therapeutics significantly increased the number of apoptotic cells compared with other groups.

We next tested the efficacy of the targeted supramolecules in the transgenic K-Ras<sup>LSL+/+</sup>/Pten<sup>fl/fl</sup> ovarian cancer model. Bioluminescence quantification revealed that treatment with the iRGD-STX2-based supramolecular therapeutic (3 mg/kg PTX equivalent) significantly inhibited the tumor progression as compared with PTX (3 mg/kg) (Figure 6g,h). Furthermore, systemic toxicities of the different treatments were assessed by monitoring body weight, behavioral changes, blood cell counts, and quantification of apoptosis in the RES system and the kidney. As shown in Figure 6i, no statistically significant difference was evident in the blood cell counts between any of the groups. Furthermore, while we did observe a bioluminescence signal from the liver and spleen, labeling the organs for TUNEL to quantify apoptosis revealed that the STX supramolecule treatment resulted in significantly reduced cell death in these tissues as compared with PTX treatment (Figure 6j).

CONCLUSIONS

Supramolecular nanochemistry is emerging as a powerful technology platform for use in cancer diagnosis and therapy. The ability of the building blocks to assemble via weak interactions results in high loading efficiency and controlled drug release, while the increase in dimension confers the advantage of preferentially accumulating in the tumor by leveraging the tumor vascular pathophysiology. In evolutionary terms, this mimics the early evolution from unicellular to multicellular structures, which confers functional complexities to the evolved species. However, an in silico platform for automating the rational design of a nanoscale supramolecular therapeutic did not exist prior to this study.

Traditionally, the experimental approaches to deliver drugs preferentially to the tumor have relied on nanoparticles of different types, which are typically engineered using a drug of choice and stochastically choosing excipients from a standard repertoire. In most cases, these existing systems allow high loading efficiency at only low mol % of drug, which translates into an overall low total drug concentration/injectable volume. This becomes a challenge during clinical translation, where volume of injection is a limiting factor. As seen with paclitaxel and cabazitaxel, the nanoparticles become unstable at high mol % of drug, as validated by both experimental and computational observations. Volvox could delineate the implications of minor structural variations between the active drugs, for example, between cabazitaxel and paclitaxel, on the stability of the assembled supramolecular nanostructure. Indeed, the use of Volvox to facilitate the formation of a stable supramolecular nanostructure at a high mol % of drug by facilitating the design of an optimal molecular subunit could address the above challenges. While cholesterol is known to increase the Young’s modulus and bending modulus of bilayers, both theoretical and experimental observations with STX1 and STX2 indicated that the cholesterol by itself as the tether, as in STX1, was not sufficient to form a stable supramolecular nanostructure. The introduction of an optimal linker in STX2 following Volvox-based iterations had a critical implication on the stability of the supramolecular nanostructure. Both simulation and experimental results showed that the same linker failed to confer stability in the case of SCBZ. These observations indicate that minor changes in the structure of supramolecular subunits result in distinct behavior in the nanoscale and can impact the stability of a supramolecular assembly. This indicates that the currently used “one size fits all” strategy is unlikely to work, and the true design of next-generation nanomedicines and supramolecular therapeutics needs an atomistic understanding of intermolecular interactions. Such atomistic resolutions can be challenging to obtain from experimental studies due to thermal fluctuations of soft matter.

Our results indicate that Volvox can potentially be used to optimize the excipients in a supramolecular structure. For example, the insertion of DSPE-PEG at an optimal molar percentage into the bilayer could contribute to the stability of the SCBZ-based supramolecular nanostructure, although not by increasing lipid tail ordering but via the reduction of ripple formation and inhibiting the entry of water molecules in the lipid bilayer, which could arise due to the steric hindrance as well as the known effect of PEG2000 in dehydrating the lipid bilayer. This does highlight that the three parameters that we use as a measure of stability could have distinct weightages on the outcome, which needs to be elucidated in future studies. It is well-established that PEGylation can mask foreign bodies from clearance by the reticuloendothelial system and result in increased circulation time in vivo. The circulation half-life of 10.95 h observed in the current study with the nanoscale taxane-based supramolecular therapeutic is consistent with these previous observations and significantly greater than 1.5 h reported earlier for paclitaxel. Even Abraxane, the only approved albumin-bound paclitaxel nanoparticle, is reported to exhibit a half-life ranging from 3.8 ± 0.7 to 6.2 h, indicating that the albumin–drug assembly dissociates in circulation. This dissociation could underlie a nonspecific deposition in systemic organs in a preclinical model reported with Abraxane. An increased circulation time has been correlated with preferential accumulation in the tumor. Indeed, biodistribution studies revealed that the supramolecular taxane preferentially accumulates in the tumor as compared with highly vascularized structures such as lungs or heart, which is consistent with the increased stability and circulation time. The localization into the tumor was further validated by NIR tracking studies. It is likely that the enhanced antitumor efficacy is a result of the increased tumoral accumulation. The sustained release of the molecular subunit as observed in the release kinetics study could create a condition within the tumor that mimics a metronomic dosing schedule (i.e., sustained low dose) that contributes toward the observed antiangiogenic effect. While IC<sub>50</sub> values of both the parent taxane and the supramolecular therapeutic were in the nanomolar range, the increased therapeutic index together with an antiangiogenic effect seen with the supramolecular system offers the possibility of enhancing the efficacy of therapy in vivo using the supramolecular taxane.

Interestingly, recent studies have highlighted the effect of stiffness, elasticity, or size of nanostructures on endocytosis of the drug into the cell. The shape of nanostructures can also be optimized to increase anticancer efficacy. Furthermore, Ohura et al. recently demonstrated that it is possible to modulate the biological properties of the nanoparticles by dynamically altering the surface properties and shape. Indeed, in the current study, we observed that using a tumor-targeting iRGD peptide with the taxane supramolecule completely abrogated tumor growth. These observations suggest that the efficacy of supramolecular therapeutics can be further enhanced by...
optimizing geometrical and biomechanical properties for maximizing tumor accumulation. It may be possible to computationally predict and synthesize supramolecular nanostructures with different shapes and elasticity by altering the compositions. For example, a calculation of the additive packing parameter for the building blocks used in the current study generated a score of ~0.75, which is consistent with bilayers we observed here. Increasing concentrations of polyethylene glycol can push a lipid bilayer toward a bicellar or micellar structure. A further validation of Volvox using a larger number of animal sets will be required to evaluate the performance of different designs in tumor-targeting applications. Such experiments would allow us to extend the concept of using our algorithm to identify different particle designs, shapes, and types for optimal tumor-targeting applications.

Several components of Volvox-based designer nanoscale supramolecular therapeutics can facilitate future therapy in humans. First, the granularity in understanding the interactions of drug and excipients at an atomistic level means that it can enable a rational design of a supramolecular therapeutic with optimal pharmacodynamics and pharmacokinetic properties. This can result in increased efficacy and reduced adverse effects. Second, while we demonstrate the application of this in silico design platform on the taxane class of molecules in this study, Volvox can potentially expand the repertoire of drug templates and excipients that can be harnessed to engineer supramolecular therapeutics. Third, the modularity in the assembly of a supramolecular therapeutic means that multifunctionality can be dialed into the design, consistent with the complexities emerging through evolution. For example, the modular design of a supramolecule, where the targeting moiety and the active agent are separate but align via supramolecular interactions, offers the opportunity to tailor the stoichiometry between the drug and the targeting antibody or peptide, which can be an advantage over current technologies such as ADCs. Similarly, two molecular subunits can assemble into a single supramolecular structure, thus enabling combination therapies. The Volvox platform technology-enabled design of supramolecular therapeutics can thus emerge as a powerful tool in the search for an optimal cancer therapy.

METHODS

Materials. All chemical reagents were of analytical grade and used as supplied without further purification unless indicated. All reactions were performed under inert conditions unless otherwise indicated. Dichloromethane (DCM), anhydrous DCM, methanol, cholesterol, dimethylamino pyridine (DMAP), succinic anhydride, sodium sulfate, pyridine, 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide (EDC), L-α-phosphatidylcholine, and Sephadex G-25 were purchased from Sigma-Aldrich. Paclitaxel and cabazitaxel were purchased from LC Laboratories. Soy L-α-phosphatidylcholine (SOY-PC), egg-PC, SOPC, DOPC, 1,2-distearoyl-sn-glyero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2000], and the mini-hand-held Extruder kit (including 0.2 μm Whatman Nucleopore Track-Etch Membrane, Whatman filter supports and 1.0 mL Hamiltonian syringes) were purchased from Avanti Polar Lipids. Analytical thin-layer chromatography (TLC) was performed using precoated silica gel aluminum sheets 60 F 254 bought from EMD Laboratories. Spots on the TLC were visualized under UV light and/or by treatment with alkaline permanganate solution followed by heating. MTS reagent was supplied by Promega. Column chromatography was conducted using silica gel (230-400 mesh) from Qualigens. 1H and 13C NMR spectra were recorded on a Bruker DPX 400 MHz spectrometer. Chemical shifts are reported in δ (ppm) units using residual H signals from deuterated solvents as references. Spectra were analyzed with Mest-Re-C Lite (Mestrelab Research) and/or XWinPlot (Bruker Biospin). Electrospray ionization mass spectra were recorded on a Micromass Q-Tof 2 (Waters), and data were analyzed with MassLynx 4.0 (Waters) software. Cell lines were obtained ATCC and used within 6 months of resuscitation of frozen stock. All the simulations were performed on an in-house server containing 32 CPU cores (Intel Xeon CPU E5-2690 at 2.90 GHz). In addition to the CPU cores, the server contains one NVIDIA Tesla K20c graphical processing unit (GPU). All the simulations were performed with CPU–GPU balancing as implemented in Gromacs. Simulations were performed with a GPU-enabled Gromacs code.

Computational Simulations. As the first step in this module, we performed geometry optimization of the subunits using quantum mechanical methods to obtain the lowest energy conformation of the molecular subunit. Following the QM optimization step, partial charges on each atom of the drug molecules were computed using Gaussian 09 with the B3LYP exchange correlation functional and 6311G basis set, and electrostatic potentials were fitted with CHELPG scheme. Next, we developed the force-field parameters of the molecular subunit. Bond and angle potential parameters were taken from CHARMM force field. Most of the dihedral potentials were taken from CHARMM force field, and those that were not present in the CHARMM force field were calculated and parametrized. In terms of nonbonded potentials, Lennard-Jones parameters were adapted from CHARMM force field, while for coulomb potential, partial charges on each atom obtained from QM calculations were used. Following the force-field development step, the subunit was energy optimized using the developed force field using the steepest descent algorithm. We next performed a short MD simulation of the molecular subunit in vacuum using the developed force field, and the output structure obtained was matched with the structure obtained after QM optimization of the drug molecule. If the structures obtained did not match, we looped back to the force-field development circle and different parameters were tweaked, and this iteration continued until the structures obtained after QM optimization and MD in vacuum matched, at which stage it was advanced to the second module. All the MD simulations were performed using the Gromacs 4.6.1 package. The partial charges of individual atoms in PTX, STX1, STX2, CBS, and SCBZ were computed using Gaussian 09 with the B3LYP exchange correlation functional and 6311G basis set, and electrostatic potentials were fitted with CHELPG scheme. Quantum chemically geometry-optimized structures of PTX, STX1, STX2, CBZ, and SCBZ were considered as the starting structure for MD simulations. All other bonded and nonbonded parameters were taken from CHARMM force field. All covalent bonds in SOPC and PTX or STXs were constrained with the LINCS algorithm. SETTLE method was used to constrain the covalent bonds in water. A time step of 2 fs was used for all of the simulations. Isothermal and isobaric ensemble (NPT) and periodic boundary conditions were used. Temperature was kept constant at 300 K for all the systems using a Nose-Hoover thermostat with a coupling constant of 0.5 ps. Pressure was kept constant at 1 atm using a Parrinello–Rahman barostat with a coupling constant of 10 ps. Semi-isotropic pressure coupling was used. Neighbor searching was done with the Verlet algorithm as implemented in Gromacs 4.6.1. For long-range electrostatic interactions, we used a particle mesh Ewald scheme with a real space cutoff of 1.4 nm. For van der Waals interactions, a 1.5 nm cutoff was applied with a switching function at 1.4 nm. PTX or STX molecules were inserted inside the lipid bilayer structure and equilibrated until 10 ns for all systems. Models were constructed such that at the high drug concentration (20%), the system contained 256 molecules of SOPC and 64 molecules of geometry-optimized structure of PTX or CBZ, while the low (5%) concentration setting contained 128 molecules of SOPC and seven molecules of molecular subunit CBZ. After this, trajectories were saved for further analysis. For more details, see the Supporting Information.

STX2/CBZ Supramolecule Synthesis and Characterization. Cholesterol-4-[(2-aminoethyl)amino]-4-oxobutanoic acid was conjugated to PTX or CBZ using EDC and DMAP coupling reaction to form a STX2 or SCBZ, respectively. The products were characterized by 1H NMR spectroscopy and mass spectrometry. Next, 6.5 mg (50
mol %) of t-ε-phosphatidylcholine, 4.7 mg (20 mol %) of STX2 or SCBZ, and 14.1 mg (30 mol %) of DSPE-PEG were dissolved in 1.0 mL of DCM. Solvent was evaporated into a thin and uniform lipid–drug film using a rotary evaporator. The lipid–drug film was then hydrated with 1.0 mL of H2O for 2 h at 70 °C. The hydrated nanoparticles looked light yellow to white with little viscous texture. It was considered at 70 °C using a 400 nm membrane with a 100 μL sample volume to obtain sub-200 nm particles. The nanoparticles were further passed to the Sephadex G-25 column to remove free STX2 or SCBZ. STX2 or SCBZ incorporated in the nanoparticles was determined using high-performance liquid chromatography (HPLC). A standard curve of STX2 or SCBZ conjugate was generated at 280 nm using acetonitrile as the solvent system. Different dilutions of nanoparticles were injected into the column of the HPLC system. Then the area under the curve at 280 nm was determined. The incorporation efficiency was determined as the percentage of drug recovered from the nanoparticle fractions compared to the initial loading amount. For synthesis of iRGD-STX2 supramolecules, DSPE-PEG-maleimide was conjugated to the iRGD peptide using thiol–maleimide coupling reaction. The iRGD-STX2-SNP was synthesized using a lipid–film hydration method with the same molar concentrations of the drug and the lipids as above.

The mean particle size of the nanoparticles was measured by DLS using a Zetasizer Nano ZS90 (Malvern, UK). Ten microliters of a nanoparticle solution was diluted to 1 mL using DI water, and three sets of 10 measurements each were performed at 90° scattering angle to obtain the average particle size. The ζ-potential was measured using a Zetasizer ZS90 with the nanoparticles diluted in water for measurement according to the manufacturer’s manual. The physical stability of nanoparticles was evaluated by measuring changes in mean particle size and ζ-potential during storage conditions at 4 °C.

PTX-NP Synthesis. t-ε-Phosphatidylcholine (13.8 mg, 85 mol %), PTX (1 mg, 5 mol %), and DSPE-PEG (5.9 mg, 10 mol %) were dissolved in 1.0 mL of DCM. Solvent was evaporated into a thin and uniform lipid–drug film using a rotary evaporator. The lipid–drug film was then hydrated with 1.0 mL of H2O for 2 h at 70 °C. The hydrated nanoparticles looked light white with slight viscous texture. They were passed through a Sephadex G-25 column and extruded at 70 °C to obtain sub-200 nm particles.

Differential Scanning Calorimetry. The DSC studies were carried out in MicroCal VP-DSC microcalorimeter (MicroCal Incorporated) differential scanning calorimeter equipped to perform ascending and descending temperature change operations. The data were analyzed using MicroCal Origin 7 software. The lipid concentration for each sample used was 20 mg/mL. Scans were recorded in a range of 20–110 °C at a scan rate of 30 °C/h. The final scan was obtained by subtracting a baseline scan made with a reference sample.

Additive Packing Parameter. Additive packing parameter (P_add) for a multicomponent system is defined as the sum of the product of individual packing parameter of each component multiplied by its mole fraction, that is, 

$$P_{add} = P_{add} = \sum_{i} x_i P_{j}$$

Here, P_i and x_i are the packing parameter and mole fraction of the i-th component, respectively, and v_i, a_i, and l_i are volume of the hydrophobic part, area of the headgroup, and length of hydrophobic part of the i-th component, respectively. P_DSPE-PEG is adapted for the brush regime. As the PTX/CBZ unit in the STX2/SCBZ molecule positions itself below the hydrophilic headgroup in the lipid bilayer, to compute P_STX2/SCBZ the volume of PTX/CBZ is added to the volume of cholesterol to obtain P_STX2/SCBZ. The linker group aligns itself near the hydrophilic headgroups contributing to the δ_STX2/SCBZ - δ_STX2/SCBZ is same as the length of cholesterol because cholesterol remains buried in the hydrophobic core. P_add for a system containing 20 mol % of STX2/SCBZ, 30 mol % of DSPE-PEG, and 50 mol % of SOPC to be calculated as

$$P_{add} = 0.98 \times 0.2 + 0.487 \times 0.3 + 0.82 \times 0.5 = 0.75,$$

for STX2

AFM Studies. The supramolecular nanoparticles were adsorbed to a poly-l-lysine-treated glass disc. In each experiment, 500 μL of supramolecular solution was placed on a surface-treated glass surface followed by the addition of 4 mL of PBS solution. The Petri dish was gently shaken and rotated such that it allowed formation of a homogeneous supramolecular solution and completely covered the glass pieces. The amount of PBS was also precisely sufficient to form a meniscus on the lower side of the cantilever holder (while it was in contact with the liquid). Care was taken to avoid the air bubbles. Asylum MFP-3D (placed in an acoustic and environmental isolation hood) was used to perform the AFM experiments. Silicon nitride cantilevers (Veeco, USA) with a blunt, unsharpened V-shaped tip with a spring constant of 0.02 N/m was used. The cantilever was calibrated in air using the built-in thermal calibration method, which uses the Sader formula for spring constant measurement. Contact mode imaging followed by force measurements in the form of 32 × 32 grids were performed throughout. A constant relative positive trigger of 50 nm was applied in order to achieve an applied force of 1 nN, and a tip velocity of 2 μm/s was maintained. Data were analyzed with the help of a code using Igor Pro 5.2 (WaveMetrics, USA).

Co-Transmission Electron Microscopy. The sample was preserved in vitrified ice supported by holey carbon films on 400 mesh copper grids. The sample was prepared by applying 3 μL of sample suspension to a cleaned grid, blotting away with filter paper, and immediately proceeding with vitrification in liquid ethane. Grids were stored under liquid nitrogen until transferred to the electron microscope for imaging. Electron microscopy was performed using an FEI Tecnai Cryo-Bio 200 kV FEG TEM, operating at 120 kV, equipped with two Gatan Sirius CCD cameras one 2K*2K and one 4K*4K pixel. Vitreous ice grids were transferred into the electron microscope using a cryostage that maintains the grids at a temperature below −170 °C. Images of the grid were acquired at multiple scales to assess the overall distribution of the specimen. After potentially target areas for imaging at lower magnification were identified, high-magnification images were acquired at nominal magnification of 50 000× (0.21 nm/pixel) and 20 000× (0.50 nm/pixel). Images were acquired at a nominal underfocus of −5 μm (20 000×) and −4 μm (50 000×) at electron doses of 10−15 e−/Å².

Release Kinetics Studies. Drug-loaded nanoparticles (1 mg drug/mL, 5 mL) were suspended in PBS buffer (pH 7.4) and 4T1 cell lysate and sealed in a dialysis tube (MWCO = 3500 Da, Spectrum Lab). The dialysis tube was suspended in 1 L of PBS at pH 7.4 with gentle stirring to simulate the infinite sink tank condition. A 100 μL portion of the aliquot was collected from the incubation medium at predetermined time intervals and replaced by an equal volume of PBS buffer, and the released drug was quantified by HPLC and plotted as cumulative drug release.

In Vitro Cell Viability Assays. 4T1 and MDA MB 231 breast cancer cells were cultured in RPMI, while H460 lung and PC3 prostate cancer cells were cultured in DMEM, supplemented with 10% FBS and 1% antibiotic–antimycotic 100× solution. Cells (4 × 10⁴) were seeded into 96-well flat-bottomed plates and incubated with free drug or drug-loaded nanoparticles (normalized to equivalent amounts of free drug) for desired time periods. Cell viability was quantified using the CellTiter 96 Aqueous One Solution assay (Promega).

Capsaicin Activation Studies. For capsaicin activation studies, 4T1 breast cancer cells (5 × 10⁴) were seeded into 6-well plates and incubated with free drug or equivalent amount of drug-loaded nanoparticles in appropriate concentrations. After 24 h of incubation in 5% CO₂ atmosphere at 37 °C, cells were washed twice with ice cold PBS and protein was collected. The protein lysates were electrophoresed transferred to membranes, which were incubated with antibodies against cleaved caspase-3 (1:1000 dilution) and β-actin (1:2000 dilution) (all antibodies from Cell Signaling Technology), and probed with horseradish peroxidase conjugated secondary antibody. Expression was normalized to β-actin levels. Detection was done using
imaging, mice received 150 mg/kg of D-luciferin using Living Image software 3.1 (Caliper Life Sciences). Prior to three treatments, and 1 day after taken a day prior to initial treatment (day 0, baseline image), after luciferin administration for an exposure time of 30 s. Images were captured using the formula L × B^2/2. All animal procedures were approved by the Harvard Institutional Use and Care of Animals Committee.

**Maximum Tolerated Dose Studies.** MTD studies for free PTX and STX2 supramolecules were carried out in healthy Balb/c female mice (4−6 weeks, 18–20 g weight). Animals (n = 5 in each group) were randomized into the following groups: vehicle, free PTX (10 mg/kg), STX2 supramolecules (20 mg/kg), and iRGD-STX2 supramolecules (3 mg/kg), and all drugs were treated with iRGD-STX2 supramolecules to test for the effect of active targeting on efficacy. Animals were dosed every fourth day. The tumors were measured regularly, and tumor volume (Vt) was calculated using the formula L × B^2/2. All animal procedures were approved by the Harvard Institutional Use and Care of Animals Committee.

**Murine 4T1 Breast Cancer Model.** 4T1 breast cancer cells (1 × 10^6) were implanted subcutaneously in the flanks of 4 week old Balb/c mice. The drug therapy was started on day 10. Animals were randomized into the following treatment groups: (i) vehicle, (ii) free PTX (10 mg/kg), and (iii) STX2 supramolecules (at dose equivalent to 50 mg/kg of the PTX). To compare the effect of higher drug doses on the anticancer efficacy, we included two groups: free PTX (10 mg/kg) and STX2 supramolecules (at dose equivalent of 10 mg/kg of PTX). In a separate experiment, we included an additional group treated with iRGD-STX2 supramolecules to test for the effect of active targeting on efficacy. Animals were dosed every fourth day. The tumors were measured regularly, and tumor volume (Vt) was calculated using the formula L × B^2/2. All animal procedures were approved by the Harvard Institutional Use and Care of Animals Committee.

**Ovarian Tumors.** To evaluate tumor-induced symptoms and their body weight loss of more than 20% of the control and causes neither death due to severe toxicity nor any significant general changes in the mice within 1 week after the drug administration.

**Murine Ovarian Cancer Model: Efficacy Study of STX2 Supramolecules in Murine Ovarian Cancer Model.** Ovarian adenocarcinomas were induced in genetically engineered K-ras<sup>G12D</sup>/p53<sup>−/−</sup> mice via intrabursal delivery of adenovirus-carrying Cre recombinase. Tumor cells were also engineered to express luciferase once activated by Adeno-Cre to make tumor imaging possible before and after drug treatment. Once mice developed medium to large tumors, they were placed into different treatment groups (vehicle, free PTX 3 mg/kg, STX2 supramolecules 3 mg/kg, and iRGD-STX2 supramolecules 3 mg/kg), and all drugs were administered via tail vein injection. Treatment was administered five times over a 20 day period with a 4 day period between treatments. Tumor imaging in vivo was performed using an IVIS Lumina II imaging system. Quantification of bioluminescence was achieved by using Living Image software 3.1 (Caliper Life Sciences). Prior to imaging, mice received 150 mg/kg of β-luciferin firefly pitomy salt via intraperitoneal injection. Five minutes post-luciferin injection, mice were anesthetized in a 2.5% isoflurane flow of isoflurane and the body temperature was maintained steady using a 37 °C temperature stage. A bioluminescent signal was collected 15 min after administration for an exposure time of 30 s. Images were acquired for further analysis using Maestro software. Once a spectrum for the signal was determined, the drug was injected via the tail vein of healthy female Balb/c mice (4−6 weeks old, weight ~20 g). Twenty microliters of blood was withdrawn from the tail vein at 0.167, 1, 4, 12, 24, and 48 h in heparin tubes. The samples were centrifuged at the rate of 8000 rpm for 10 min at 4 °C, and the supernatant was stored at ~20 °C until further analysis. The drug was extracted from the plasma samples using dichloromethane; the organic layer was separated and evaporated. The extracted residue was dissolved in acetonitrile and injected into the HPLC system (Waters Inc.). The standard curve was obtained using different concentrations of STX. The data set was fit to a noncompartment model to obtain different pharmacokinetic parameters.

**Tumor Histocytochemistry.** For studying apoptosis, formalin-fixed tumor sections were stained with a standard TMR red fluorescent terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling kit following the manufacturer’s protocol (in situ cell detection kit, TMR-Red, Roche). As a measure of angiogenesis, cold methanol fixed tumor cryo-sections were labeled with an antibody against vWF (Dako). All imaging was done using a Nikon Ti epifluorescence microscope.

**Statistics.** The statistical analysis was done using a two-tailed Student’s t test or one-way ANOVA followed by Newman Keuls post-hoc test, with P < 0.05 as the threshold for significance.

### ASSOCIATED CONTENT

1. Supporting Information

   The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.6b00241.

   Supporting results, figures, and tables (PDF)
   - Movie 1 (AVI)
   - Movie 2 (AVI)
   - Movie 3 (AVI)
   - Movie 4 (AVI)
   - Movie 5 (AVI)
   - Movie 6 (AVI)
   - Movie 7 (AVI)

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**Notes**

The authors declare the following competing financial interest(s): SS holds equity in Invictus Oncology.
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