ABSTRACT: A major limitation of immune checkpoint inhibitors is that only a small subset of patients achieve durable clinical responses. This necessitates the development of combinatorial regimens with immunotherapy. However, some combinations, such as MEK- or PI3K-inhibitors with a PD1-PDL1 checkpoint inhibitor, are pharmacologically challenging to implement. We rationalized that such combinations can be enabled using nanoscale supramolecular targeted therapeutics, which spatially home into tumors and exert temporally sustained inhibition of the target. Here we describe two case studies where nanoscale MEK- and PI3K-targeting supramolecular therapeutics were engineered using a quantum mechanical all-atomistic simulation-based approach. The combinations of nanoscale MEK- and PI3K-targeting supramolecular therapeutics with checkpoint PDL1 and PD1 inhibitors exert enhanced antitumor outcome in melanoma and breast cancers in vivo, respectively. Additionally, the temporal sequence of administration impacts the outcome. The combination of supramolecular therapeutics and immunotherapy could emerge as a paradigm shift in the treatment of cancer.

KEYWORDS: cancer, supramolecular therapeutics, immune checkpoint inhibitors, targeted therapy

Among the most promising approaches in the treatment of cancer is the blockade of immune check points.\textsuperscript{1,2} Cancer progression occurs as a result of the ability of cancer cells to overcome immunosurveillance.\textsuperscript{3,4} Molecules such as the programmed cell death ligand 1 (PDL1), expressed on cancer cells, can ligate with the PD1 receptor that is expressed on cell surface of T cells and cause T cell anergy.\textsuperscript{5} A number of antibodies that disrupt the PD1-PDL1 immune checkpoint are currently in the clinics.\textsuperscript{6} However, the percentage of patients who show durable response to immunotherapy is small.\textsuperscript{7} This stems from the strong immunosuppressive environment within the tumor, and also potential intrinsic, acquired or drug-induced resistance mechanisms that can limit the activity of an immunotherapy agent.\textsuperscript{8} As a result, it is increasingly being realized that a combination of an immunotherapy agent with a second therapeutic agent may be the optimal strategy for a maximal antitumor outcome.\textsuperscript{9−11}

A large body of data is evolving on the combinations of immune checkpoint inhibitors with chemotherapy, radiotherapy, or targeted therapies.\textsuperscript{9−16} The emerging paradigm is however to move from empirical combinations to one of rational design, based on the compatibility of mechanisms that can act synergistically.\textsuperscript{16} For example, there is currently intensive investigation into PD1-PDL1 inhibition in combination with either BRAF inhibitors alone or with BRAF- and MEK-inhibitors. This is based on the observations that vemurafenib, a BRAF inhibitor, increases the responsiveness of T cells against melanoma by enhancing the expression of T

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Figure 1. Computer-aided design of a MEK-inhibiting supramolecular therapeutic. (a) Selumetinib forms highly unstable lipid nanoparticles and precipitates out within 4 h of synthesis. (b) MD-simulation optimized structure of selumetinib. (c) Snapshot of all-atomic simulation of selumetinib interacting with SOPC lipid bilayer at 500 ns shows aggregates of selumetinib at the interface of hydrophilic lipid head and water interface. Lipid hydrophilic heads are shown in orange and blue spheres, and lipid tails are shown in gray color. Selumetinib is shown in red color. (d) MD simulation optimized structure of supratinib (molecular subunit of supramolecular nanostructure STB). (e) Snapshot of all-atomic simulation of supratinib interacting with SOPC lipid bilayer at 500 ns shows stable supramolecular structure. Lipid hydrophilic heads are shown in orange and blue spheres, and lipid tails are shown in gray color. Supratinib is shown in red color. (f) Schematic representation for tilt angle calculation. Tilt angle is the angle between vector joining center of mass of phospholipid tails and z-axis (axis perpendicular to bilayer plane). Value of tilt angle is positive or negative depending on direction of the ripple; its value is close to 0° when no ripples form. (g) Distribution of tilt angle averaged over last 5 ns of MD trajectory. Broader the distribution larger is the tilt angle, hence, higher is the extent of bilayer instability. (h) Schematic representation of angle (θ) between vector defined on C–C bond on SOPC tail with 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 phospholipid tail. The higher the S_{CD}, the higher is the lipid tail ordering. (i) Deuterium order parameter on each methylene group on saturated tail of SOPC is depicted. Lipid tail ordering for selumetinib-containing SOPC bilayer is the least. Lipid tail ordering of supratinib-containing SOPC bilayer is similar to pure SOPC bilayer. (j) Probability of finding water molecules per unit volume was calculated normalizing the count per unit volume with number of frames in last 5 ns of MD trajectory. (k) High-resolution cryo-TEM image of STB supramolecular therapeutic showing size of ~100 nm and spherical morphology (scale bar =100 nm).

cell antigens. Similarly, the PI3K-AKT pathway, which is highly dysregulated in breast cancer, has also been implicated in conferring resistance to T cell-mediated killing through increased expression of anti-apoptotic proteins. Both PI3K and MAPK pathways have been reported to regulate the expression of PDL1, although the effect is variable across different cancers. Together, these observations indicate that targeted therapeutics that inhibit PI3K or MEK could be rationally combined with immunotherapies that modulate T cell functions. However, concerns exist about the potential cumulative toxicities with these combinations. Furthermore, both PI3K and MAPK pathways are implicated in the activation of T cells. PDL1-PD1 ligation downregulates these pathways in the T cells, leading to T cell exhaustion. Thus, a systemic shutdown of these pathways could potentially limit the effect of a PD1-PDL1 inhibition. We rationalized that the preferential targeting of the inhibitors of these aberrant pathways to the cancer cells could emerge as an alternative approach to enable a combination regimen with T cell activating treatments. Nanoscale technologies allow the preferential delivery of therapeutic payloads to tumors by evading the systemic immune system. While nanoparticles have recently been used as vaccines or as mimics of professional antigen-presenting cells to induce an immune response against the cancer cells, combination regimens of immunotherapy with nanoparticles that target specific signaling pathways in cancer are yet to be studied. We have previously demonstrated that a polymeric nanoparticle that targeted MAPK could preferentially accumulate in the tumor and exert an enhanced antitumor effect. In another study, we demonstrated that nanoparticles can exert a sustained inhibition of PI3K signaling in the tumor in vivo. Taken together, these pharmacodynamics properties and the preferential biodistribution of MEK- and PI3K-targeting nanoparticles to the tumors could potentially be harnessed for combining with PD1-PDL1 checkpoint inhibitors. As described by Jean-Marie Lehn, supramolecular assembly defines the assembly of complex structures from molecular subunit via weak interactions, such as coordination bonds, van der Waal’s forces, hydrophobic–hydrophilic bonds, etc. In a recent study, we have demonstrated that a quantum mechanical (QM) all-atomic simulation-based algorithm could be used to engineer molecular building blocks that are pharmacologically active and can self-assemble into nanoscale structures via...
supramolecular assembly. These nanoscale supramolecular structures overcome the challenges of stability and drug loading associated with traditional lipid-based nanoparticles.32 Here we describe the use of this computational algorithm to design inhibitors of MEK and PI3K, which act as molecular subunits in the assembly of supramolecular nanostructures. We demonstrate that the nanoscale supramolecular therapeutics preferentially accumulate in the tumor, and the combination of the MEK-inhibiting supramolecular nanoparticle with a PDL1 inhibitor and the combination of the PI3K-inhibiting supramolecule with a PD1 blockade can result in an enhanced antitumor outcome in in vivo models of melanoma and breast cancer, respectively. The combination of nanoscale supramolecular targeted therapeutics and immunotherapy can emerge as a paradigm shift in the treatment of cancer.

RESULTS AND DISCUSSION

Engineering a MEK-Targeting Nanoscale Supramolecular Therapeutic. Classical approaches toward engineering a nanoparticle, such as a liposome, involves the entrapment of the drug in lipid or polymeric matrices. However, attempts to entrap selumetinib, a clinical stage MEK inhibitor, in a liposome (at 20 mol % of drug) failed with the drug precipitating out within 4 h (Figure 1a). Indeed, the stability of nanoparticles at drug concentrations required for clinical efficacy has been a major challenge in the clinical translation of nanomedicines. We rationalized that this hurdle could be overcome by designing a molecular subunit that facilitates the assembly into a nanoscale structure through supramolecular interactions while retaining its biological MEK-inhibiting activity.

As the first step, we used a QM all-atomistic simulation-based platform technology32 to understand the mechanism underlying the failure of selumetinib to stay in the lipid-based nanoparticles. As shown in Figure 1b, we first obtained energy-minimized structures of selumetinib using QM optimization and a molecular dynamics (MD) simulation in vacuum. In parallel, we designed an analogue, which we term supratinib, by

Figure 2. Characterization of MEK-inhibiting supramolecular therapeutics. (a) Graph shows distribution of hydrodynamic diameter of MEK-inhibiting supramolecular therapeutics (STB) as measured using DLS. (b) Graph shows the changes in size and ζ potential of STB over time as a measure of physical stability of STB during storage condition at 4 °C. Absence of change reflects structural integrity. (c) Graph shows release kinetics profiles of the drug from STBs in PBS (pH 7.4) or cell lysate. Data shown are mean ± SEM (at least triplicates at each time point). The drug is released in a sustained manner in PBS, whereas a rapid release is observed in cell lysate, consistent with the faster degradation of supramolecular therapeutics under enzymatic conditions. (d) Graphs show effect of increasing concentrations of STB or selumetinib on viability of A549 lung cancer cells and B16-F10 melanoma cells. (e) Table shows the IC50 values of selumetinib and STBs in A549 and B16-F10 cells. (f) Western blot shows expression of phospho and total ERK 1/2 in B16-F10 cells at different time points post-treatment with either 5 μM of selumetinib or STB. Graph shows internalization of selumetinib and STBs at 3 and 18 h in (g) A549 lung cancer cells and (h) B16-F10 melanoma cells. Statistical analysis was performed with student t test. Data show mean ± SEM; n.s., not significant; **p < 0.01; ***p < 0.001.
Figure 3: Combination of MEK-inhibiting supramolecular therapeutic and PDL1-immune checkpoint inhibitor increases antitumor efficacy in a B16-F10 melanoma model. (a) Graph shows the effect of MEK-inhibiting supramolecular therapeutic (STB) treatments on tumor volume in B16-F10 tumor bearing mice as compared to selumetinib- and vehicle-treated groups ($n = 4$). Each animal was injected intravenously with four doses of vehicle (as control group), 5 mg/kg of selumetinib, or STB (equivalent to selumetinib dose). (b) Western blot showing expressions of phospho and total ERK 1/2, and β-actin for normalization, in tumors after different treatments. Graph shows quantification of expressions of pERK/Total ERK in different treatment groups after normalization with β-actin levels. Statistical analysis was performed with one-way ANOVA test. Data shows mean ± SEM; n.s., not significant; ***$p < 0.001$. (c) Representative images show biodistribution of NIR-dye labeled STBs in tumor-bearing mice at different time points post-i.v. administration. The tumor position is shown dotted circle. (d) Graph shows changes in tumor volume after four doses of selumetinib (5 mg/kg), 2.5 mg/kg αPD-L1, selumetinib (5 mg/kg) + αPD-L1 (2.5 mg/kg), or STB (5 mg/kg) + αPD-L1 (2.5 mg/kg) on each alternate days. First day of treatment was considered as day 0. Tumor volumes were measured on every alternate day for 10 days. Statistical analysis was performed with one way ANOVA test. Data shown are mean ± SEM.
derivatizing selumetinib with a cholesterol-based tether through an optimal linker. This was based on previous findings that cholesterol can affect a bilayer by decreasing its free volume, increasing lipid tail order and packing, and reducing the lipid mobility. Indeed, in a previous study with taxanes, we have observed that such molecular subunits can facilitate the assembly into a stable supramolecular nanostructure. The energy-minimized structure of supratinin using QM optimization and a short MD simulation in vacuum is shown in Figure 1c. We next simulated the interaction of a high (20 mol %) concentration of selumetinib or supratinin with a model colipid, 1-octadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine (SOPC). 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. Models were constructed such that the system contained 256 molecules of SOPC and 64 molecules of selumetinib or supratinin, i.e., to mimic 20 mol % of the drug in the lipid bilayer. We have previously observed that three distinct parameters: (1) ripple formation in the bilayer, (2) decreased lipid tail ordering, and (3) the probability of water molecules entering the bilayer are good predictors of instability. The unbiased atomistic simulations did not reveal any significant ripple formations for either selumetinib- or supratinin-containing bilayers (Figure 1c, and e, Supplementary Movies 1 and 2), quantified as the angle between vector joining the center of mass of lipid tails and the phosphorus (P) atom on the headgroup of SOPC molecule with z-axis (bilayer normal), where the greater the angle, the higher is the degree of instability (Figure 1f and g). However, selumetinib did decrease the ordering of the aliphatic tail groups that was quantified by calculating the deuterium order parameter (S_CD) for the SOPC lipid saturated tail from last 5 ns of the trajectory (Figure 1h and i), which could explain the instability seen in experimental observations. In contrast, simulating the interaction of a high (20 mol %) concentration of supratinin with SOPC revealed a stable supramolecular assembly, characterized by absence of ripple formation (Figure 1e-g), a lipid tail ordering similar to the SOPC-only bilayer (Figure 1h and i), and no permeation of water into the supramolecular structures (Figure 1j).

Given that supratinin-based supramolecular nanostructure (STB) satisfied all three parameters of stability in silico, we next synthesized (Supplementary Figure 1) and tested its ability to form a stable supramolecular nanostructure with colipid excipients using a thin-film hydration technique. Indeed, as shown in Figure 1k, cryo-transmission electron microscopy (cryo-TEM) revealed that supratinin was able to form stable supramolecular nanostructures (STB) with an average mean diameter of 108.6 ± 27 nm and loading efficiency of 87 ± 5%.

Characterization of the MEK-Inhibiting Supramolecular Nanotherapeutic (STB). We next physicochemically characterized the MEK-inhibiting supramolecular therapeutic (STB). Dynamic laser light scattering revealed that the hydrodynamic diameter of STB was 191 ± 38 nm. The difference in dimension compared with the findings from electron microscopy is consistent with PEGylation of the structures (Figure 2a). The ζ potential was measured to be 18.4 ± 6.48 mV. Both the radius and the ζ potential remained within a constant range over a sustained period of time at 4 °C or when incubated with serum, suggesting that the structures were stable and that the surface PEGylation prevents the opsonization by serum proteins (Figure 2b, Supplementary Figure 2a and b). Incubating STB with cancer cell lysate, to mimic the tumor environment, resulted in a sustained and complete release within a 7 day period (Figure 2c). Similarly, a faster release was observed when incubated at an acidic pH (Supplementary Figure 2c). In contrast, limited release of the drug was observed at physiological pH 7.4 (Figure 2c) or in lysate from a normal splenocyte (Supplementary Figure 2d).

We next tested the efficacy of STB in melanoma B16/F10 and lung carcinoma A549 cell lines in vitro. As shown in Figure 2d and e, STB was more potent than selumetinib in reducing cell viability of both cell lines, as evident from the lower IC50 values. At the same concentration range, neither selumetinib nor STB had any significant inhibitory effect on the viability of T cells (Supplementary Figure 3a). Furthermore, as shown in Supplementary Figure 3b, treatment with STB resulted in an increase in PDL1 expression of B16/F10 melanoma cells. We did not observe a similar increase in PD1 expression on T cells in response to STB (Supplementary Figure 3c). Additionally, Western blotting revealed that treatment of melanoma cells with selumetinib resulted in an early inhibition of ERK phosphorylation. However, this inhibition was not long lasting. In contrast, we observed a sustained inhibition of ERK phosphorylation following STB treatment (Figure 2f). A similar sustained inhibition of phosphorylation of ERK was also observed when A549 cells were incubated with STB (Supplementary Figure 3b). Furthermore, the quantification of intracellular drug concentration revealed that while at an early time point (3 h post-treatment) both selumetinib and STB attained similar levels, the latter resulted in a greater drug build up at a later time point (Figure 2g and h), which could explain the sustained inhibition of ERK phosphorylation and the increased efficacy.

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Combination with a PDL1 Antibody in Vivo. Based on the in vitro observations, we next tested the efficacy of STB in a syngeneic murine melanoma model. A large subset of melanoma presents with constitutive activation of the MAPK pathway, underpinning the use of MEK inhibitors in the treatment of melanoma. Consistent with our in vitro findings, we observed an increase in the antitumor efficacy with STB compared with selumetinib (Figure 3a). It should be noted that here we used submaximal doses so that we could dissect whether the combination chemotherapy results in enhanced efficacy. Western blotting of in vivo tumor analytes revealed a significantly greater inhibition of MAPK signaling (i.e., reduced phosphorylated ERK levels) with STB treatment as compared with vehicle- or selumetinib-treated control groups, consistent with the observed efficacy (Figure 3b).

Tumors that overexpress PDL1 are reported to respond well to PDL1 immune checkpoint inhibitors. Based on our prior observation that STB induces PDL1 expression in melanoma, we rationalized that a combination of STB and a PDL1-immune checkpoint inhibitor could result in an increased antitumor outcome. However, previous studies have reported that T cell activation is maintained through ERK signaling, and MEK inhibitors can adversely affect T cell proliferation, cytokine production, and antigen-specific expansion, suggesting that for a MEK inhibitor to be rationally combined with an immune checkpoint inhibitor, it should ideally be preferentially delivered to the tumor. Although our in vitro studies showed that STB has no effect on T cell proliferation at therapeutic concentrations, we first tested if the supramolecular nanostructures can preferentially target the tumor. Tumor-bearing mice

Figure 4. Computer simulations predicts stable PI3K-inhibiting supramolecular nanostructures. (a) MD simulation optimized structure of PI103. (b) Snapshot of all-atomistic simulation of PI103 interacting with SOPC lipid bilayer at 500 ns shows aromatic stacking-mediated aggregates of PI103 coming out of the bilayer. Lipid hydrophobic heads are shown in orange and blue spheres, and lipid tails are shown in gray color. PI103 is shown in red color. (c) MD simulation optimized structure of SPI molecular subunit. Bottom panel shows snapshot of all-atomistic simulation of SPI lipid bilayer at 500 ns, which shows a stable supramolecular structure. (d) Graph depicts the cluster size distribution of PI103 and SPI molecular subunit. PI103 shows more occurrence of larger cluster sizes as compared to SPI molecular subunit. (e) Graph shows the estimation of number of pairs of PI103 and SPI molecular subunits stacking in lipid bilayer. (f) Deuterium order parameter on each methylene group on saturated tail of SOPC is depicted. Lipid tail ordering for PI103-containing SOPC bilayer is least. Lipid tail ordering of SPI-containing SOPC bilayer is higher than pure SOPC bilayer, which shows that SPI molecular subunit stabilizes the supramolecular structure. (g) Probability of finding water molecules per unit volume was calculated normalizing the count per unit volume with number of frames in last 5 ns of MD trajectory. (h) Distribution of tilt angle averaged over last 5 ns of MD trajectory. (i) High-resolution cryo-TEM of supramolecular structure (scale bar = 100 nm).
were injected with a NIR dye-tagged supramolecular nanostructure and imaged at different time points. At 48 h post-administration, the animals were sacrificed, and the biodistribution of the supramolecular nanostructure was noted. As shown in Figure 3c, the supramolecular nanostructures were found to progressively accumulate in the tumor, reaching saturation by 24 h. Additionally, a negligible quantity of the nanoparticles distributed to normal tissues like lungs, heart and kidneys, and the amount/g of tissue was still lower in the major reticuloendothelial system organs such as liver and spleen as compared with the levels in the tumor (Figure 3d), consistent with previous findings.28 Furthermore, an analysis of the liver and kidney of animals treated with STB or selumetinib exhibited similar levels of cleaved caspase 3, a marker of apoptosis, compared with vehicle-treated control animals (Supplementary Figure 4a). Taken together, these results indicated that STB could emerge as an attractive approach to preferentially inhibit MEK in the tumor.

We next studied whether the combination of a PDL1 inhibitor and the STB exerted a greater antitumor efficacy compared with the immune checkpoint inhibition alone. Melanoma-bearing animals were injected with three cycles of PDL1 antibodies (2.5 mg/kg) alone or in combination with MEK-inhibiting supramolecular nanoparticles (dose equivalent to 5 mg/kg of selumetinib). As a control, we also included a group where the tumor-bearing animals were treated with a combination of selumetinib and PDL1 antibodies. As shown in Figure 3e, we observed that while PDL1 inhibition did inhibit tumor growth, a significantly enhanced antitumor effect was evident when it was combined with STB. In contrast, we did not see a similar increase in efficacy when the PDL1 inhibitor was combined with selumetinib. We did not observe any change in body weights following the treatments (Figure 3f).

To mechanistically dissect the enhanced efficacy seen with the combination, we performed Western blotting of tumor analytes, which revealed that the treatment with STB resulted in a decrease in the phosphorylated ERK levels as compared with PDL1 inhibition alone (Figure 3g). Immunolabeling the tumor sections for phospho-ERK confirmed that the phosphorylated ERK levels were significantly lower in the tumors treated with the combination of STB and PDL1 inhibitor as opposed to the latter alone (Figure 3h). Additionally, labeling the tumor sections for CD45, a marker for lymphocytes, or using H&E staining (Supplementary Figure 4b) revealed that the checkpoint inhibition increased the number of tumor infiltrating lymphocytes (TILs). The level was significantly enhanced when the immune checkpoint inhibitor was combined with STB as compared to the combination of PDL1 antibody and selumetinib (Figure 3h and i). Furthermore, an analysis of the tumor sections for apoptosis using TUNEL revealed that the treatment with STB induced significantly greater cell death as compared with selumetinib (Figure 3h and j). The combination of STB and the immune checkpoint inhibition resulted in greater tumor apoptosis as compared with PDL1 inhibition alone or in combination with selumetinib. Taken together, the mechanistic observations were consistent with the tumor growth inhibition.

We next validated these results by analyzing the tumor immune cells using fluorescence activated cell sorting (FACS). As shown in Figure 3k and l, the treatment with STB as well as the PDL1 inhibitor increased the intratumoral ratio of CD8 cells to CD4 cells as compared with selumetinib or vehicle treatments. The combination of STB with the PDL1 inhibitor further increased CD8+ T cells in the tumor compared with the combination of the immune checkpoint inhibitor with selumetinib. Additionally, quantifying CD44CD62L (activated) fraction of the TILs revealed that treatment with the combination of the PDL1 inhibitor and STB resulted in a greater percentage of activated TILs as compared with the combination of PDL1 inhibitor and selumetinib (Figure 3l). None of the treatments altered the levels of Tregs in the tumor (Figure 3m and Supplementary Figure 4c). Taken together, these findings indicate that a MEK-inhibiting supramolecular therapeutic is not associated with T cell inhibitory effects and can be rationally combined with immune check point inhibitors for enhanced antitumor efficacy. Additionally, the enhanced efficacy relies on the enhanced recruitment of activated TILs.

**Engineeering a PI3K-Targeting Supramolecular Therapeutic.** While the above results suggested that a nanoscale supramolecular therapeutic could preferentially accumulate in tumors and enable a combinatorial approach with immune checkpoint inhibitors, we rationalized that the findings needed to be validated in a different setting using a distinct target and tumor model. The PTEN/PI3K pathway is a major oncogenic driver implicated in 30−40% of primary breast tumors.41 However, PI3K is also the dominant downstream signaling pathway that is needed for the activation and proliferation of T cells.42 The ligation of PD1 by PDL1 leads to inhibition of PI3K signaling, resulting in T cell exhaustion,43 indicating that a PI3K inhibitor should ideally be preferentially targeted to the tumor to be combined rationally with an immune checkpoint inhibitor, as systemically they can be counteractive.

PI103 (Figure 4a) is a well-established inhibitor of class IA PI3Ks as well as mTOR.44 However, like selumetinib, it precipitates out of a lipid-based nanoparticle at concentrations required for clinical translation.45 To understand the incompatibility of PI103 with formulation in a classical liposome, we ran an all-atomatic simulation of PI103 interacting with lipids in a bilayer, which revealed that the PI103 molecules precipitate out into the water layer after stacking with each other to form clusters (Figure 4b and Supplementary Movie 3). We rationalized that the approach we used for selumetinib, i.e., bioconjugation with cholesterol to form a molecular subunit, could be applied to PI103 to prevent stacking and facilitate a stable supramolecular nanoscale assembly. The molecular subunit, indeed formed a stable supramolecular structure, which we termed as SPI (Figure 4c and Supplementary Movie 4). To compute stacking, a vector was defined among the atoms shown on PI103 molecule. The angle between this vector on different molecules was calculated within a distance of 0.5 nm. Stacking was considered if the angle was >125° or <75°. The count of such stacked pairs was plotted for each frame in the last 20 ns of the trajectory. The number of molecules stacked was found to be higher for PI103 as compared to the SPI system (Figure 4d and e). Additionally, we observed that 20 mol % PI103 system reduced the lipid tail ordering, while it was significantly elevated in the 20 mol % SPI system compared with a pure SOPC (Figure 4f). This was again reflected in the calculation of number of water molecules per unit volume in the lipid bilayer, i.e., SPI showed the least perforation among all the systems (Figure 4g). It should be noted that neither system exhibited any ripple formation (Figure 4h). Consistent with the theoretical model, SPI resulted in the formation of extremely stable nanoparticles of ~150 nm diameter (Figure 4i) with a loading efficiency of 65 ± 10%, which were stable even in the presence of serum proteins.
Figure 5. Combination of PI3K-inhibiting supramolecule with PD1 antibody enhances antitumor efficacy in 4T1 breast cancer model. (a) Growth curves show effect of PI3K-inhibiting supramolecule (SPI) treatment on tumor volume in 4T1 tumor bearing mice as compared to PI103- and vehicle-treated groups. Each animals were injected intravenously with three doses of either vehicle (for control group), 2.5 mg/kg of PI103, or SPI (∼2.5 mg/kg of PI103). SPI shows statistically significant tumor growth inhibition as compared to PI103. Data shown are mean ± SEM (n = 5); ***p < 0.001. (b) Western blot showing expressions of phospho and total Akt and mTOR in tumors after different treatments. Graph shows quantification of expressions of pAkt/Total Akt in different treatment groups after normalization with β-actin levels. Statistical analysis was performed with one-way ANOVA test. Data show mean ± SEM; *p < 0.05; **p < 0.01. (c) Graph shows changes in tumor volume after three doses of αPD-1 (5 mg/kg), PI103 (2.5 mg/kg) + αPD-1 (5 mg/kg), or SPI (2.5 mg/kg) + αPD-1 (5 mg/kg) on alternate days. First day of treatment was considered as day 0. Tumor volumes were measured on every alternate day for 17 days. End point for each animal was tumor size >4000 mm³ or tumor ulceration or necrosis or animal death. Statistical analysis was performed with one way
ANOVA test. Data show mean ± SEM; **p < 0.01; ***p < 0.001. SPI in combination with immune checkpoint inhibitor antibody PD1 shows the greatest tumor growth inhibition as compared to other treatment groups. (d) Graph shows effect of treatments on overall body weight as a measure of any systemic toxicity. (e) Representative FACS data from 4T1 tumors after different treatments (day 17). The left panel shows percentage of CD4+ vs CD8+ cells in the isolated lymphocyte population. The right panel shows the percentage of activated CD8+ cells (CD44HI/CD62LLO). (f) Representative FACS data from spleen of 4T1 tumor bearing mice after different treatments (day 17). The left panel shows percentage of CD4+ vs CD8+ cells in the isolated lymphocyte population from spleen. The right panel shows the percentage of activated CD8+ cells (CD44HI/CD62LLO) in the spleen. (g) Graph shows the number of CD8+ cells in 4T1 tumors (normalized to tumor mass) after different treatments as quantified using FACS. (h) Graph shows the number of activated CD8+ cells in 4T1 tumors (normalized to tumor mass) after different treatments as quantified using FACS. Data represents mean ± SEM (n = 3); *p < 0.05 (ANOVA with Newman Keuls post-hoc test). (i) Graph shows the ratio of activated CD8+ T cells to naïve T cells in spleen in 4T1-tumor bearing mice after different treatments. Data represent mean ± SEM (n = 3); *p < 0.05 (ANOVA with Newman Keuls post-hoc test).

Figure 6. Efficacy of PI3K-inhibiting supramolecule and PD1 antibody combination is enhanced by temporally sequencing the administration. (a) Schematic shows the sequence of dosing schedules of PI3K-inhibiting supramolecule (SPI) and PD1 antibody combination. SPI was administered on days 0, 2, and 4 for both the schedules. PD-1 (immediate) represents the dosing schedule 1 where the PD-1 antibody was injected on every alternate day from day 1, and PD-1 (after) represents the dosing schedule 2 where PD-1 antibody is injected on days 6, 8, and 10, i.e., after the SPI-treatment cycle. (b) Graph shows tumor volumes of 4T1 tumor bearing mice after treatments with different dosing schedules (day 16). The efficacy of SPI with PD-1 antibody combination is improved with dosing schedule 2 where PD-1 antibody is administered after SPI treatment as compared to schedule 1. (c) Representative epifluorescent images of tumor sections from animals after different treatments (day 16), labeled for apoptosis using TUNEL (red) and counterstained with DAPI (blue). (d) Graph shows the quantification of apoptosis from the labeled tumor sections as a percentage of TUNEL+ve cells as a function of total nuclei. Statistical analysis was performed with ANOVA with Newman Keuls post-hoc test. Data shown are mean ± SEM (n = 3–5); *p < 0.05. (e) Kaplan Meir curves showing effect of treatments on survival (n = 5). (f) Representative FACS data from 4T1 tumors after treatments with different dosing schedules (day 16). The FACS data show the percentage of activated CD8+ cells (CD44HI/CD62LLO). (g) Graph shows the number of activated CD8+ cells in 4T1 tumors (normalized to tumor mass) after different treatments (n = 6). Statistical analysis was performed with student t test. Data shown are mean ± SEM; *p < 0.05.
(Supplementary Figure 5a and b) and exhibited limited drug release in lysate from normal splenocytes (Supplementary Figure 5c).

**Combination of PI3K-Inhibiting Supramolecule and PD1 Inhibitor in Vivo.** We first tested the effect of PI103 and SPI on T cell viability in vitro. As shown in Supplementary Figure 5d, PI103 was more toxic to T cells than SPI. In a separate study, we treated naïve T cells with PI103 or SPI (at concentration equivalent 500 nM of PI103) for 36 h and then activated the cells using CD3/CD28 Dynabeads. Interestingly, both PI103 and SPI did affect the activation of T cells in this static system, which mimics a systemic exposure (Supplementary Figure 5e and f). Interestingly, neither selumetinib nor STB exhibited a similar effect (Supplementary Figure 3a), which highlights the differences in the role of the PI3K and MAPK signaling in T cell activation. This finding further validated that it may be necessary to preferentially target the PI3K inhibitor to the tumor for combining it with an immune checkpoint inhibitor. Indeed, in a separate study, we demonstrated that the PI3K-inhibiting supramolecular therapeutic (SPI) can preferentially accumulate in the tumor and enables a sustained inhibition of PI3K and downstream targets.29 Consistent with these findings, we observed that SPI exerted a greater antitumor effect than PI103 (Figure 5a). Furthermore, a Western blot analysis of the tumors revealed that the treatment with SPI results in a significantly greater inhibition of the PI3K signaling pathway, i.e., reduced phosphorylation of Akt and downstream mTOR, as compared with PI103- or vehicle-treated tumors (Figure 5b).

PI3K inhibitors have been reported to decrease the expression of PD-L1 in breast cancer,35 and hence a PD1L inhibitor is unlikely to be effective in such a setting. For this reason, we used a PD1-blocking antibody as the immune checkpoint inhibitor in this study for combining with the PI3K inhibitors. In our previous study, we have shown that a higher concentration of SPI can ablate the tumor.29 As a result, for the current study, we used a submaximal dose of the SPI to be able to dissect the effect of combining it with the PD1 inhibitor. Treatment with the anti-PD1 antibody inhibited breast tumor growth as compared with the vehicle-treated group. Interestingly, the combination of PI103 with PD1 inhibitor decreased the antitumor efficacy of the latter. In contrast, animals treated with the combination of SPI and PD1 inhibitor exhibited greater antitumor outcome as compared to the PD1 inhibitor-treated animals (Figure 5c). We did not observe any changes in the body weight as a result of drug treatment in any of the groups (Figure 5d).

We next studied the effect of different treatments on the intratumoral recruitment and activation of T cells. As shown in Figure 5e and g, the different treatments did not significantly affect the total number of CD8+ T cells recruited to the tumor. However, an analysis of the activation status of the intratumoral CD8+ T cells by monitoring for CD44CD62LLo expression revealed that the groups treated with the PD1 inhibitor as well as SPI exhibited a larger fraction of activated T cells compared with vehicle-treated controls (Figure 5e and h). The combination of the PD1 inhibitor with the PI3K-inhibiting supramolecular therapeutic did not further increase the fraction of activated CD44CD62LLo CD8+ T cells. The fraction of activated T cells in the vehicle- and the PI3K inhibitor (PI103)-treated groups were found to be similar. We additionally monitored the splenocytes for CD8 positive T cells counts as well as the effect of treatment on the activation of CD8+ T cells. As shown in Figure 5f and i, increased ratio of activated CD8+ T cells to naïve cells was observed following treatment with PD1 inhibitor and the PI3K-inhibiting supramolecular therapeutic as compared with PI103 treatment.

**Sequencing Drug Administration Affects Outcome.** Recent reports have highlighted the importance of sequencing the administration of cancer therapies on outcomes.30,36,47 We therefore used two distinct schedules to treat tumor-bearing animals with a combination of PD1 antibody and SPI. In one schedule, animals were injected with the immune checkpoint inhibitor on alternate days of administration of SPI (“immediate” schedule). In the second schedule, the PD1 inhibitor was administered after the cycle of SPI treatment was complete (“after” schedule) (Figure 6a). Interestingly, as shown in Figure 6b, the antitumor efficacy was significantly greater with the “after” schedule. These findings were validated by quantifying the degree of cell death within the tumors. As shown in Figure 6c and d, the “after” schedule resulted in maximal levels of intratumoral apoptosis as compared to the “immediate” schedule. Excitingly, the “after” schedule resulted in a significant increase in life span as compared to vehicle or the regimen where both drugs were administered simultaneously. It should be noted that a combination of PI103 and PD1 did not increase life span as compared with vehicle-treated animals (Figure 6e). Interestingly, we observed an increase in the mean level of activated intratumoral CD8+ T cells with the after schedule as compared with the immediate schedule (Figure 6f and g).

**CONCLUSIONS**

Combinations of immunotherapy with targeted therapeutics, such as MEK or PI3K inhibitors, are currently the holy grails in the management of cancer. Here we demonstrate that the ability of nanoscale supramolecular therapeutics to preferentially home into tumors can be leveraged to rationally combine targeted therapeutics with immunotherapy. We show that the combinations of a nanoscale MEK-targeting supramolecular nanotherapeutics with a PD1L antibody, and a PI3K-inhibiting supramolecular nanotherapeutic with a PD1 inhibitor, can result in increased antitumor efficacy in immunocompetent melanoma and breast carcinoma models, respectively. Such combinations can shift the paradigm in the treatment of cancer.

While nanotechnology enables the synthesis of nanoparticles or liposomes, which have had limited success with clinical translation due to the instability of the formed nanostructures. Here we used a distinct approach, where the active agents were first redesigned based on a QM all-atomistic simulation to facilitate self-assembly into stable nanoscale supramolecular structures. Computational simulations, such as those based on coarse grain models, have recently been used to understand the interactions between the drug and excipients that can assemble into a nanostructure.48 However, all-atomistic simulations can enable resolutions at the nanoscale. Indeed, our results revealed significant insights, for example, both selumetinib and PI103 induced instability in the bilayer but through distinct mechanisms. It should be noted that such atomistic resolutions can be challenging to obtain from experimental studies due to thermal fluctuations of soft matter. The correlation between the simulation and the experimental
observations suggests that computer-aided design can indeed emerge as a powerful tool for the fabrication of stable nanostructures.

Sterically stabilized nanoparticles have been reported to preferentially home into tumors by leveraging the leaky pathophysiology of the tumor blood vessels and result in greater intratumoral concentrations of the active drug as compared to when the drug is administered in free form.\textsuperscript{26,27} Our observation from the biodistribution experiment in the current study, where the supramolecular nanotherapeutics preferentially accumulated in the tumors, is consistent with these previous reports.\textsuperscript{49} Furthermore, we observed that unlike packing the drugs in a liposome, which result in instability, the redesign of the molecular subunit to facilitate supramolecular assembly increases the stability of the nanostructure at high mol %.

In a recent study, we have demonstrated that this stability is not a simple function of conjugating a drug to cholesterol and that the linker can also influence the supramolecular assembly.\textsuperscript{12} This ability of the subunit to facilitate the supramolecular assembly is a key distinction from fabricating a classical drug-loaded liposome. Interestingly, the cholesterol derivatization does not mean that the drug partitions into the lipid raft-rich cell membrane. Confocal imaging of fluorophore-tagged cholesterol-based supramolecular nanostructures revealed that the structures are internalized into the cytoplasm (Supplementary Figure 6).

Furthermore, we observed a sustained release of the active agent in the tumor lysate and an accelerated release at an acidic pH. Interestingly, the release rate was slower in physiological pH and lysates of normal cells. Indeed, previous studies have reported differences in esterases and pH between normal and cancerous environments that could contribute to this difference.\textsuperscript{30} Taken together, these attributes can lead to a higher and sustained concentration of the active drug in the cancer cells as compared to free drug. Indeed, this is validated by pharmacodynamics observations, where the nanoscale supramolecular therapeutics exerted a prolonged and greater inhibition of the target pathway as well as greater antitumor efficacy compared to selumetinib or P1I03 treatment. Additionally, in a separate study, we have shown that the nanoscale supramolecular therapeutics are less toxic systemically.\textsuperscript{31} In the current study, we did observe that the P1I03 was more toxic against splenocytes than SPI. Taken together, these observations support the hypothesis that nanoscale supramolecular therapeutics could emerge as a better choice than current inhibitors of PI3K or MAPK for combining with immune checkpoint inhibitors.

Interestingly, while both PI3K- and MEK-inhibiting supramolecular therapeutics resulted in increased antitumor efficacy when combined with the immune checkpoint inhibitors, our results indicate that the underlying mechanisms driving such an outcome are distinct. The combination of PDL1 inhibition with MEK-inhibiting supramolecular therapeutic resulted in an increase in the total activated TILs and increased intratumoral cytotoxicity as compared with the animals treated with either the PDL1 antibody or the MEK-inhibiting supramolecular therapeutic alone. In a recent study, the triple therapy combination of trametinib, a MEK inhibitor, with a BRAF inhibitor and adoptive cell transfer was indeed shown to increase T cell infiltration and improved \textit{in vivo} efficacy.\textsuperscript{32} The rationale for combining a MEK inhibitor was to reduce the toxicity associated with BRAF inhibitors attributed to the paradoxical activation of MAPK by the BRAF inhibitors in BRAF wild-type cells.\textsuperscript{32} The current findings suggest that the ability of the supramolecular nanotherapeutic to preferentially home into the tumor can offer an alternative and simpler approach toward combination immunotherapy. Additionally, MEK inhibitors have also been reported to upregulate melanocyte differentiation antigens and MHC class I expression, which could underlie the increased TILs seen with STB treatment.\textsuperscript{22} In contrast, treatment with the PI3K-inhibiting supramolecular nanotherapeutic and the PD1 inhibitor or the combination did not increase total CD8+ T cell infiltration, but both significantly increased the fraction of activated CD44\textsuperscript{+}CD62L\textsuperscript{+} CD8+ TILs. We did not observe any further increase in TILs when the PD1 inhibitor was combined with the PI3K-inhibiting supramolecular therapeutic, indicating that we had reached a maximal ceiling in the level of activated T cells. This suggests that the increased efficacy observed in the case of simultaneous combination treatment with the PD1 inhibitor and the PI3K-inhibiting supramolecular therapeutic is likely due to a direct anticancer effect as a result of PI3K-inhibition, and not via the augmentation of the activity of the immune checkpoint inhibitor. However, we did observe an increase in the efficacy as well as mean activated TILs when the sequence of administration of the combination was temporally staggered. Indeed, previous reports have suggested that PI3K-AKT inhibitors can diminish the expression of antiapoptotic proteins such as cIAP1 and 2, BCL-2, and BCL-XL, which can then sensitize the tumor cells to immunemediated destruction.\textsuperscript{16} Indeed, SPI induced greater apoptosis compared with the parent drug. Taken together with the observation that PI3K-inhibiting supramolecule enables drug release in a sustained manner, it is logical that sufficient drug buildup induces maximal apoptosis of cancer cells, which can then amplify the outcome to immunotherapies. Thus, temporality or the sequence of administration is likely to emerge as a key parameter in the design of combination regimens with immunotherapies.\textsuperscript{16} Interestingly, P1I03 resulted in a decrease in the efficacy of the PD1-inhibitor, consistent with the dominant role of PI3K signaling in the activation of T cells.

Several aspects of this study can facilitate future therapy in humans. First, the ability of the nanoscale supramolecular therapeutics to preferentially home into tumors indicates that many of the adverse effects that have emerged in recent clinical trials with combination of targeted therapy with immunotherapy could potentially be minimized. Second, the increased intratumoral buildup of the active agent can result in a sustained pharmacodynamic effect, which can increase the antitumor efficacy. Third, the versatility of the computer-aided design of the supramolecular nanotherapeutics means that a repertoire of such agents can be designed for additional immunomodulatory targets. Finally, the nanoscale supramolecular therapeutics can indeed emerge as a natural choice for enabling combination therapy with immune checkpoint inhibitors that are pharmacologically incompatible, thus enabling a paradigm shift toward a comprehensive “integrative” approach in the treatment of cancer.

**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. Anhydrous dichloromethane (DCM), methanol, cholesteryl hemi-succinate, dimethylamino pyridine (DMAP), succin anhydride, 1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide (EDC), 1-α-phos-
phatidylcholine, and sephadex G-25 were purchased from Sigma-Aldrich. PI103 and selumetinib were purchased from LC Laboratories. PD-1 and PD-L1 antibodies were purchased from BioXCell. 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[(aminopolyethylene glycol)2000] and the mini hand-held extruder kit (including 0.2 and 0.4 μm polycarbonate membrane, Whatman filter supports, and 1.0 mL Hamiltonian syringes) were bought from Avanti Polar Lipids Inc. 31H NMR spectra were recorded on Bruker DRX 400 MHz spectrometer. Electrospray ionization mass spectra were recorded on a Micromass Q ToF 2 (Waters), and data were analyzed with MassLynx 4.0 (Waters) software. A549, B16-F10, and 4T1 cell lines were obtained from ATCC and used within 6 months of resuscitation of frozen stock. MTS reagent was supplied by Promega. EasySep Mouse CD90.2 positive selection kit was purchased form Stemcell Technologies. FACS antibodies for CD4, CD8, CD44, and CD62L and isotypes were purchased from Biolegend, and intracellular Foxp3 staining kit was purchased from eBioscience. Pierce BCA protein assay kit and Vybrant Lipid Raft labeling kit and protease and phosphatase inhibitors were purchased from Thermo Fisher Scientific.

**All-Atomistic Computational Simulations.** As the first step, we performed geometry optimization of the molecular subunits using QM 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 B3LYP exchange–correlation functional and 6-31G 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 parameterized. In terms of nonbonded potentials, Lennard-Jones (lJ) 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 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. This iteration continued until the structures obtained after QM optimization and MD in vacuum matched. All the MD simulations were performed using GROMACS-4.6.1 package. QM geometry-optimized structures of selumetinib, STB, PI103, and SPI were considered as the starting structure for MD simulations. All the other bonded and nonbonded parameters were taken from CHARMM force field. All covalent bonds in SOPC and the drugs were fixed.

The reaction mixture was stirred at rt for 12 h under argon. Upon completion of reaction as monitored by TLC, the reaction mixture was diluted with 10 mL DCM and washed with dilute HCl and water. The organic layers were separated, combined, and dried over anhydrous sodium sulfate. The solvent was evaporated under vacuum, and the crude product was purified by using column chromatography, eluting with a 10% chloroform/methanol chloride gradient, to give the supratinin as a light-yellow solid (54% yield). 2H NMR (CDCl3, 400 MHz): δ 9.75 (s, 1H), 8.03 (m, 1H), 7.9 (s, 1H), 7.54 (d, 1H), 7.1 (m, 4H), 5.36 (s, 1H), 4.67 (m, 1H), 4.3 (m, 1H), 4.12 (m, 1H), 3.95 (m, 2H), 3.85 (s, 1H), 2.65 (m, 3H), 2.31 (m, 2H), 2.06 (m, 2H), 1.86 (m, 3H), 1.72 (s, 3H), 1.29 (m, 33H), 0.74 (m, 3H). HRMS calculated for C30H40BrCIFNO + H+: 925.37. Found: 925.39. Cholesterol hemisuccinate was similarly conjugated to PI103 using EDC and DMAP coupling reaction to form molecular subunit of SPI.  

**Synthesis and Characterization of Nanoscale Supramolecular Therapeutics (STB and SPI).** For supramolecule synthesis, 6.5 mg (50 mol %) of 1-octadecylphatidylcholine, 3.1 mg supratinin, or 2.7 mg molecular subunit of SPI (20 mol %) and 14.1 mg (30 mol %) of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[(aminopolyethylene glycol)2000] (DSPE-PEG) were dissolved in 1 mL 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 H2O for 2 h at 70 °C. It was extruded at 70 °C using 400 nm and then 200 nm membrane with 100 μL sample volume to obtain sub-200 nm particles. The nanoscale supramolecular therapeutics were further passed to Sephadex G-25 column to remove free molecular subunits. Drugs incorporated in the STB or SPI were determined using UV–vis spectroscopy. The incorporation efficiency was determined as the percentage of drug recovered from the nanoscale supramolecular therapeutic fractions compared to the initial loading amount. The mean particle size of the nanoscale supramolecular therapeutics was measured by dynamic light scattering (DLS) method using Zetasizer Nano ZS90 (Malvern, U.K.). The ζ potential was measured using a Zetasizer ZS90 with the nanoscale supramolecular therapeutics diluted in water for measurement according to the manufacturer’s manual. The physical stability of nanoscale supramolecular therapeutics was evaluated by measuring changes in mean particle size and ζ potential during storage condition at 4 °C. For serum stability study, STB and SPI (100 μL) were incubated with 100 μL of human serum plasma. The particle size and ζ potential were measured as a function of time using Malvern Zetasizer.

**Cryo-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 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 keV equipped with 2 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 identifying potentially suitable target areas for imaging at lower magnification, high-magnification images were acquired at nominal magnification of 52,000× (0.21 nm/pixel) and 21,000× (0.50 nm/pixel). Images were acquired at a nominal defocus of ∼5 μm (21,000×) and ∼4 μm (52,000×) at electron doses of 10−15 e/Å².

**Release Kinetics Studies.** Nanoscale supramolecular therapeutics (1 mg drug/mL, 5 μL) were suspended in PBS buffer (pH 7.4) or acidic conditions (pH 5.5) or AS49 cell lysate and sealed in a dialysis tube (MWCO = 3500 Da. Spectrum Lab). The dialysis tube was suspended in 1L PBS pH 7.4 with gentle stirring to simulate the infant sink tank condition. A 100 μL portion of the aliquot was collected from the incubation medium at predetermined time intervals and replaced by equal volume of PBS buffer, and the released drug was quantified by UV–vis spectrophotometer and plotted as cumulative drug release.
Release Kinetics from Splenocytes Lysate. Spleen was isolated from Balb/C (female, 4–6 weeks old) mice, and lysate was prepared using NP40 lysis buffer in the presence of Halt protease and phosphatase inhibitor. STB and SPI (equivalent to 3 mg/mL of drug, 5 mL) were suspended in splenocytes lysate and sealed in a dialysis tube (MWCO = 3500 Da, Spectrum Lab). The dialysis tube was suspended in 1 L PBS 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 equal volume of PBS buffer, and the released drug was quantified by UV–vis spectrophotometer and plotted as cumulative drug release.

In Vitro Uptake of FITC-Cholesterol Supramolecular Nanoparticles. B16-F10 melanoma cells were labeled and cross-linked using Vybrant 5SS lipid raft labeling kit according to manufacturer’s protocols. The labeled cells were then seeded on 8 chamber tissue culture-treated slides at 50,000 cells/well density and were allowed to attain 70% subconfluence. The cells were treated with diiodotyrosine (DT) for desired time periods. Cell viability was quantified using CellTiter Aqueous One solution and were incubated at 37 °C. After treatment, the cells were washed with ice-cold PBS to remove supramolecular nanoparticles that have not been internalized. The cells were later fixed using 4% paraformaldehyde and were mounted using ProLong Gold Antifade reagent and were imaged using Vybrant 555 lipid raft labeling kit according to manufacturer’s protocol. The isolated CD90.2 positive T cells were cultured in Immunocult expansion medium and treated with free or equivalent amount of supramolecular therapeutics (0.5 μm). After 36 h of incubation, the cells were activated for 12 h using CD3/CD28 Dynabeads according to the manufacturer’s protocol. The cells were collected from both sets of experiments and quantified using a BD J LSR II without removing Dynabeads. The cell surface expressions of CD4 vs CD8 and CD4 vs CD62L were evaluated by surface staining in order to identify the percentage of immune effector cells (CD4+, CD8+ T cells) and activated T cells (CD44+CD62L+).

In Vivo Biodistribution Study. For biodistribution studies, DiR dye-labeled STB-nanoscale supramolecular therapeutics were synthesized using lipid-lymph hydration method. Briefly, 4.3 mg (50 mol %) of i-ω-phosphatidylcholine, 1.9 mg (19 mol %) of STB, 0.12 mg (1 mol %) of DiR dye, and 9.3 mg (30 mol %) of 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)2000] (DSPPEG) were dissolved in 1.0 mL 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 H2O for 2 h at 70 °C. B16-F10 melanoma cells (5 × 106) were inoculated subcutaneously into the flank of each male C57BL/6 mice (4–6 weeks old, weight ~20 g). After the tumor reached ~500 mm3 volume, dye-labeled nanoparticles were injected through the tail vein of the tumor bearing mice. The imaging was performed at 1, 3, 6, 24, and 48 h post-injection using IVIS filter set (excitation 710 nm and emission 760 nm). After the imaging, mice were sacrificed, and major organs were collected and imaged. All the images were captured using Maestro (CR1) small animal in vivo fluorescence imaging system. For the purposes of this study, the exposure times for acquiring the data were kept the same. In addition, the stage settings (which influences the distance from the camera) and the fiber optic adjustable illuminator arms were kept at the same settings for the duration of the study. After the study period, the tumors and other organs were isolated for ex-vivo fluorescence imaging. Fluorescence signals were normalized and quantified using Maestro Software. The Maestro Software was used to conduct spectral unmixing, i.e., separate individual fluorophores from each other and background autofluorescence. This feature was used to produce the spectrally unmixed images. Using a control mouse, we assigned a spectrum for autofluorescence/background. A phantom containing the dye was used to assign a spectrum for signal. Using the Maestro Software, we established a “spectral library” which indicates a spectrum for in vivo signal from the dye, minus autofluorescence. This spectral library, once established, was applied to all raw data to produce the “spectrally unmixed images”. Quantification of fluorescence intensity in the organs was performed by calculating the ratio of relative fluorescent intensity in each organ with the sum of the total intensity accumulated for all organs.

Western Blotting to Determine Liver and Kidney Toxicity. Liver and kidney from all treatment groups stored in −80 °C were homogenized to form a single cell suspension. Protein was extracted using NP40 lysis buffer in the presence of Halt protease and phosphatase inhibitor. Protein quantification was performed using the BCA assay, and equal amounts of protein lysates were electrophoresed on a polyacrylamide gel, then transferred to polyvinylidene fluoride membrane (Bio-Rad), and blocked in 5% milk solution. The membranes were incubated with cleaved-caspase-3, caspase-3, and β-actin primary antibodies (1:1000 dilution) (all antibodies were purchased from Cell Signaling Technology) overnight at 4 °C, followed by horseradish peroxidase-conjugated secondary antibody followed by horseradish peroxidase-conjugated secondary antibody.
incubation for 1 h. Detection was carried out using G-box from Syngene.

**Combination Efficacy Studies.** B16-F10 melanoma cells (5 × 10⁴) were implanted subcutaneously in the right flank of 4-week-old C57BL/6 mice (male). When the tumour volume reached ~50 mm³, animals were randomized into different treatment groups. The animals were administered via tail vain with following treatments: (1) vehicle, (2) selumetinib (5 mg/kg), and (3) STB (at dose equivalent to 5 mg/kg of the selumetinib); (4) PD-L1 antibody (2.5 mg/kg), (5) selumetinib (5 mg/kg) + PD-L1 antibody (2.5 mg/kg), and (6) STB (at dose equivalent to 5 mg/kg of the selumetinib) + PD-L1 antibody (2.5 mg/kg). Separately, 4T1 breast cancer cells (1 × 10⁶) were implanted subcutaneously in the flanks of 4-week-old female BALB/c mice. The drug therapy was started on day 10. Each animal was injected intravenously with three doses of either vehicle (for control group), 2.5 mg/kg of free P103, 2.5 mg/kg of P103 + αPD-1, P103 + αPD-1, or SPI + αPD-1 on each alternate day. For optimizing the dosing schedules, 4T1 tumor bearing female BALB/c mice were injected with either 5 mg/kg αPD-1 on alternate days of administration of SPI (2.5 mg/kg) or 5 mg/kg αPD-1 and administered after the cycle of SPI treatment (2.5 mg/kg) was complete. The tumors were measured every alternate days using a Vernier caliper, and tumor volume (V_t) was calculated using the formula, V_t = L × B²/2, where L is the longest, and W is the shortest dimensions. Tissues were harvested for FACS analysis and further studies. All animal procedures were approved by the Harvard Institutional Use and Care of Animals Committee.

**Tumor Histocytology.** Immunohistochemical analysis was performed on formalin-fixed and paraffin-embedded 5 μm tumor sections. Briefly, the tissue sections were heated to 60 °C and rehydrated in xylene and graded alcohols. Antigen retrieval was carried out in 0.1 M citrate buffer (pH 6.0) for 10 min in a pressure cooker and allowed to cool to room temperature following by rinsing in PBS and PBST (0.1% Tween). The sections were then incubated in 3% hydrogen peroxide for 5 min to ablate endogenous tissue peroxidase activity. Each incubation step was carried out at room temperature and was followed by three 5 min washes in PBST. The tissue sections were then incubated with primary antibodies pERK (Cell Signaling) and CD45 antibody (BD, San Jose, CA) followed by incubations with biotinylated secondary antibody for 30 min. The slides were then stained using DAKO EnVision+ System horseradish peroxidase detection kit and counterstained with hematoxylin. Images were obtained using Nikon 90i microscope.

**FACS Analysis.** The harvested tumor tissues were thoroughly minced and suspended in separate 4 mL type-1 collagenase solution (175 U/mg). A single cell suspension was prepared for each tumor sample and incubated for 1 h at 37 °C and 5% CO₂, and finally passed through 40 μm filter. Washing was carried out using RPMI-1640 media by centrifuging at 2000 rpm for 5 min (2 times). The pellet was resuspended in 1 mL of recommended media for CD90.2+ T cell isolation. The isolation was carried out using EasySep Mouse CD90.2 positive selection kit II according to the manufacturer’s protocol (Stem Cell Technologies). The phenotype of the isolated CD90.2+ T cells from the tumor single cell suspension was quantified using a BD J LSRII cyrometer. The cell surface expression of CD4, CD8, CD44, CD62L, and intracellular Foxp3 was evaluated by respective surface and intracellular staining in order to identify the percentage of immune effector cells (CD4+, CD8+ T cells and Treg) and activated T cells (CD44hiCD62Llow) in the tumor bearing mice.

**Western Blot Analysis of in Vivo Tumor Samples.** Tumor samples were homogenized into single cell suspensions in serum-free RPMI-1640 media containing 175 U/mL of collagenase type I. The cell suspensions were then lysed in NP40 cell lysis buffer containing protease and phosphatase inhibitors. The cellular debris was removed by centrifugation at 15000 rpm, and the supernatant was taken for the experiment. Protein estimation was done by BCA protein assay kit. 20 μg protein was loaded in each well and probed for phospho-ERK1/2, total ERK 1/2, and β-actin (for STB animal study) and phospho Akt, total Akt, phospho mTOR, total mTOR, and β-actin (for SPI animal study). Detection was done using G-box from Syngene, and densitometric quantification was done by image J software.

**Statistics.** The statistical analysis was done using 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**

3 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.6b01600.

Supporting results and figures (PDF)

Movie 1: All-atomic simulation of Selumetinib interacting with colipids in a bilayer (AVI)

Movie 2: All-atomic simulation of Supratininb interacting with colipids in a bilayer (AVI)

Movie 3: All-atomic simulation of P103 interacting with colipids in a bilayer (AVI)

Movie 4: All-atomic simulation of SPI molecular subunit interacting with colipids in a bilayer (AVI)

**AUTHOR INFORMATION**

Corresponding Author

E-mail: shiladit@mit.edu.

Author Contributions

∇These authors contributed equally.

Notes

The authors declare the following competing financial interest(s): S.S. is a cofounder, owns equity, and is a member of the board at Invictus Oncology.

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