Tumor-Targeted Synergistic Blockade of MAPK and PI3K from a Layer-by-Layer Nanoparticle

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Abstract

Purpose: Cross-talk and feedback between the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR cell signaling pathways is critical for tumor initiation, maintenance, and adaptive resistance to targeted therapy in a variety of solid tumors. Combined blockade of these pathways—horizontal blockade—is a promising therapeutic strategy; however, compounded dose-limiting toxicity of free small molecule inhibitor combinations is a significant barrier to its clinical application.

Experimental Design: AZD6244 (selumetinib), an allosteric inhibitor of Mek1/2, and PX-866, a covalent inhibitor of PI3K, were co-encapsulated in a tumor-targeting nanoscale drug formulation—layer-by-layer (LbL) nanoparticles. Structure, size, and surface charge of the nanoscale formulations were characterized, in addition to in vitro cell entry, synergistic cell killing, and combined signal blockade. In vivo tumor targeting and therapy was investigated in breast tumor xenograft-bearing NCR nude mice by live animal fluorescence/bioluminescence imaging, Western blotting, serum cytokine analysis, and immunohistochemistry.

Results: Combined MAPK and PI3K axis blockade from the nanoscale formulations (160 ± 20 nm, –40 ± 1 mV) was synergistically toxic toward triple-negative breast (MDA-MB-231) and RAS-mutant lung tumor cells (KP7B) in vitro, effects that were further enhanced upon encapsulation. In vivo, systemically administered LbL nanoparticles preferentially targeted subcutaneous MDA-MB-231 tumor xenografts, simultaneously blocked tumor-specific phosphorylation of the terminal kinases Erk and Akt, and elicited significant disease stabilization in the absence of dose-limiting hepatotoxic effects observed from the free drug combination. Mice receiving untargeted, but dual drug-loaded nanoparticles exhibited progressive disease.

Conclusions: Tumor-targeting nanoscale drug formulations could provide a more safe and effective means to synergistically block MAPK and PI3K in the clinic.

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Introduction

Combination chemotherapy has been a mainstay of clinical oncology since the mid 1960s (1, 2). While initially proposed to exploit nonoverlapping toxicity profiles of cytotoxic chemotherapeutics, recent advances in cancer cell signaling and the discovery of potent and selective small-molecule inhibitors have led to the development of rational combination approaches to cancer chemotherapy. These strategies can prime cancer cells for apoptosis (3, 4), abrogate mechanisms for resistance (5–9), block cancer cell-cycle progression (10), or dynamically rewire DNA damage response pathways (11, 12)—all leading to enhanced tumor cell killing.

Although combination therapies can be curative in a subset of malignancies (13), inefficient delivery limits the safety and efficacy of cooperative drug combinations that require cellular colocalization for effective cell killing. This can be difficult to achieve with traditional pharmaceutical excipients as synergistic compounds often exhibit vastly different physiochemical properties (e.g., size, charge, lipophilicity, and stability) that necessitate separate drug carriers. Colocalized delivery of cooperative drug combinations from single, multicomponent delivery vehicles can improve both therapeutic index (3) and treatment outcomes (6, 9, 12, 14) compared with combinations of individual carriers; however, tissue-specific delivery remains a significant challenge.
Nanoscale drug carriers that deliver cooperative drug combinations in a tumor-targeted fashion are urgently needed in the clinic. Layer-by-layer (LbL) nanoparticles (15–24) are a new class of self-assembled polymer drug carriers that addresses challenges in the delivery of combination therapeutics. These structures consist of a functional nanoparticle core, a polyelectrolyte multilayer shell, and an exterior tumor-targeting stealth layer. Previously, we developed an LbL nanoparticle architecture that targets solid tumors through 3 independent mechanisms: (i) size-dependent passive tumor targeting, (ii) active, ligand-directed targeting of cell surface CD44 receptor, and (iii) hypoxic tumor pH-responsive cellular delivery (24). Here, we apply multimodal tumor-targeting LbL nanoparticles to drug solid tumors with a synergistic combination of small-molecule inhibitors that addresses in human cancer tumor models.

Nanoparticle synthesis and characterization

LbL nanoparticle assembly was performed as described previously (24). Liposomes were prepared by the sonication/extrusion method. Briefly, a chloroform mixture of DOTAP/DOPE/cholesterol (35:35:30 mol ratio, Avanti) was dried under nitrogen and stored overnight under vacuum. The lipid film was hydrated with a PBS solution of AZD6244 (Selleck) and PX-866 (LC Laboratories) at 18 wt%, each and immersed in a bath sonicator (55°C) for 1 week before use. Drug loading was quantified by HPLC (Agilent Technologies) in 50:50 acetonitrile/water (pH 5). Cryogenic transmission electron microscopy (TEM) was performed using a JEOL 2100 FEG instrument. Photon correlation spectroscopy and laser Doppler electrophoresis measurements were carried out using a Malvern Zetasizer Nano ZS90 particle analyser (λ = 633 nm, material/dispersant RI 1.590/1.330). Chemical properties were computed using ACD/Labs software (pH 7.4).

Cell culture

OVCAR-3, Hep G2, and MDA-MB-231 cells were obtained from ATCC. Murine KPC7B cells were a gift from the laboratory of Tyler Jacks (46); these cells were obtained from the murine KPC model of lung adenocarcinoma (Kras<sup>SL-L12D/wt; p53<sup>fl/fl</sup></sup>).
exhbits conditional activation of oncogenic Ras. Cells were subcultured in DMEM supplemented with 10% FBS and penicillin/streptomycin or the supplier’s recommended basal medium in a 5% CO₂ humidified atmosphere.

In vitro experiments

Viability was assessed by CellTiter-Glo assay (Promega) and normalized to vehicle controls. The Bliss expectation was calculated as (A + B) - (A x B), in which A and B are the fractional growth inhibitions induced by agents A and B at a given dose, respectively (47). Signaling was assessed in MDA-MB-231 cells serum-starved for 24 hours and then treated with drugs and/or particles and serum-stimulated (10% v/v FBS) for the times indicated. Cells were analyzed by flow cytometry or scraped, pelleted, and lysed in RIPA buffer containing protease/phosphatase inhibitor for Western blotting. Densitometry was calculated using ImageJ software. Statistical significance was assessed by the unpaired Student t test.

Flow cytometry

Endocytic inhibition was assessed using a BD LSR II flow cytometer following 1-hour preincubation in complete growth media supplemented with 10 mmol/L sodium azide/50 mmol/L 2-deoxy-D-glucose (ATP inhibition), 200 μmol/L genistein (caveolin inhibition), 10 μmol/L chlorpromazine (clathrin inhibition), 10 μmol/L wortmannin (macropinocytosis inhibition), 5 mmol/L methyl β-cyclodextrin (caveolin- and clathrin-independent inhibition; DMSO < 0.5%v/v), and 1-hour co-incubation in complete growth medium supplemented with inhibitors and 0.1 mmol/L LBL nanoparticles (561 nm ex; 610/20 nm em). Immunofluorescence cytomtery was performed using the FlowCell Pathway Activation Kit (Millipore FCS025100) and 100-nm fluorescent carboxylate-modified polystyrene beads (Life Technologies; Red 580/605) were used as surrogates for dextran sulfate-conjugated liposomes.

In vivo experiments

A total of 5 × 10⁶ MDA-MB-231 cells (1:1 PBS:Matrigel) were injected subcutaneously into the hindflanks of nude mice (NCR nu/nu, Taconic). Tumors were allowed to form over 3 to 4 weeks, after which treatment groups were randomized. Nanoparticles (8.3 × 10¹² NP/kg, 5% glucose) were injected via the tail vein into tumor-bearing nude or immunocompetent mice (BALB/c, Taconic). Whole-animal imaging was performed using a Xenogen IVIS Imaging System (Caliper) and 100-nm fluorescent carboxylate–modified polystyrene beads (Life Technologies; Infrared 715/ 755) as surrogates for dextran sulfate–conjugated liposomes. Free drugs were dosed o.g. or nanoparticle-equivalent drug concentrations (5% n-glucose, 1% polysorbate 80). Albumin, blood urea nitrogen (BUN), and creatinine (Cr) levels were measured by Charles River Laboratories from serum samples obtained via cardiac puncture 24 hours following nanoparticle (i.v.) or free drug (o.g.) administration in 6- to 8-week-old female BALC/c mice. These experiments were approved by the Massachusetts Institute of Technology Committee on Animal Care (CAC).

Histology

Hepatic lesion scores (48) were assigned as follows: grade 0, no evidence of histologic lesions; 1, minimal congestion and mild hepatocellular cytoplasmic degeneration immediately adjacent to the central vein; 2, moderate congestion and degeneration with individual necrotic hepatocytes in the peripoortal region; 3, marked congestion and degeneration with necrotic hepatocytes extending into the midzonal regions; 4, severe congestion and degeneration and necrosis that bridges between most centrilobular zones. Tissue samples were obtained at 24 hours following nanoparticle (i.v.) or free drug (o.g.) administration in 6- to 8-week-old female BALC/c or NCR nude mice for toxicologic or pharmacodynamics analysis, respectively. Tumors were lysed in RIPA buffer containing protease/phosphatase inhibitor and analyzed by Western blotting. Vital organs were formalin-fixed, paraffin-embedded, and processed by the Swanson Biotechnology Center (MIT).

Results

We selected AZD6244 (selumetinib), an allosteric inhibitor of Meki1/2, and PX-866, a wortmannin analogue and covalent inhibitor of PI3K, for their potent activity against MAPK oncogene–addicted solid tumors (33, 49, 50). Interestingly, AZD6244 (logD ~ 5.55) and PX-866 (logD ~ 2.26) exhibit vastly differing lipophilicities and would thus be challenging to entrap within a single polymer nanoparticle matrix such as poly(lactic acid–glycolic acid) (PLGA). Here, the 2 drugs were encapsulated by sonication/extrusion within cationic vesicles (90 ± 11 nm dia) composed of DOTAP with DOPE helper phospholipid (Fig. 1A and B). Polyelectrolyte multilayers were then self-assembled onto the drug-containing vesicle cores by alternating adsorption and centrifugation (24). The strongly ionized polyelectrolyte, dextran sulfate, was used to initially stabilize the carriers and impart anionic charge. Next, a hypoxic tumor pH-responsive and CD44 cancer stem cell marker, CD44, through hyaluronan binding and also selectively underwent a transition in surface charge and swelling at pH values <7, leading to both size-dependent passive
Figure 1.
An engineered LbL nanoparticle for tumor-targeted horizontal blockade of MAPK and PI3K signaling. A, schematic illustrating combined blockade of MEK (AZD6244) and PI3K (PX-866) via multimodal tumor-targeting LbL nanoparticles. B, cryogenic transmission electron microscopy (cryo-TEM) of cationic, drug-encapsulating liposomal cores (left) and tumor-targeting, LbL-coated liposomes (right). Size evolution (C) and corresponding surface charge shift (D) during the LbL assembly process as measured by dynamic light scattering and laser Doppler electrophoresis (zeta potential analysis), respectively. E, in vitro delivery and penetration of tumor-targeting LbL nanoparticles (green) into Hep G2 tumor spheroids (actin, red) as measured by confocal fluorescence microscopy. F, mechanistic analysis of LbL nanoparticle cell uptake (1 hour) as measured by flow cytometry of particle-treated MDA-MB-231 cell monolayers pretreated with inhibitors of various endocytotic pathways indicated. Polydispersity index (PDI) in (C) is reported in parentheses. Error represents (C and D) SD of 3 technical and (F) SEM of 3 biological replicates. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 

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tumor targeting and active targeting through both ligand-directed and hypoxic pH-responsive delivery.

Layer-by-layer assembly onto the drug-loaded particle cores yielded 160/C620 nm dia nanoparticle drug carriers as the final product (Fig. 1B and C; 2.7 wt% AZD6244, 10 wt% PX-866). Hydrodynamic size evolution during the LbL self-assembly process was monitored by cryogenic-transmission electron microscopy (cryo-TEM, Fig. 1B), dynamic light scattering (Fig. 1C), and laser Doppler electrophoresis (Fig. 1D), indicating size increase and corresponding charge shift with each successive layer. In addition to its tumor targeting capabilities (24), the LbL architecture was also used to decrease nonspecific drug leakage from the particles (51).

In vitro, fluorescent LbL nanoparticles penetrated 3-dimensional (3D) tumor spheroids with high efficiency (Fig. 1D), indicating size increase and corresponding charge shift with each successive layer. In addition to its tumor targeting capabilities (24), the LbL architecture was also used to decrease nonspecific drug leakage from the particles (51). In vitro, fluorescent LbL nanoparticles penetrated 3-dimensional (3D) tumor spheroids with high efficiency (Fig. 1E), indicating size increase and corresponding charge shift with each successive layer. In addition to its tumor targeting capabilities (24), the LbL architecture was also used to decrease nonspecific drug leakage from the particles (51).

These results are also in agreement with Hoechlin and colleagues (55) and Ladevaia and colleagues (56) who predicted synergistic cell killing of basal-like (triple-negative) breast carcinoma following dual blockade of MAPK and PI3K. Likewise, Engelman and colleagues (33) demonstrated that combined pathway signaling is required for Ras-dependent tumor initiation, whereas Downward and colleagues found that dual pathway activation is required for Ras-dependent tumor maintenance (42) and angiogenesis (43). Interestingly, LbL nanoparticle encapsulation further improved synergistic cell killing in MDA-MB-231 cells by 2.6-fold, consistent with simultaneous blockade of pErk1/2 and pAkt staining as measured by immunofluorescence cytometry.

Figure 2.
Rational drug combination nanomedicines against MAPK/PI3K signaling synergistically kill tumor cell lines in vitro and target solid tumors in vivo. A, a drug interaction matrix plotting cell killing observed in excess of the additive Bliss expectation between MEK and PI3K inhibition in triple-negative breast and RAS-mutant non–small cell lung carcinoma (MDA-MB-231, KP7B; 0.05–50 μmol/L, 48 hours). B, LbL nanoparticle encapsulation augments drug synergy in vitro and (C) horizontally blocks MAPK and PI3K signaling as measured by phospho-Erk1/2 and phospho-Akt cell staining, respectively. D, at early time points (10-minute serum stimulation), LbL nanoparticle-encapsulated drugs deplete relative pAkt more completely than the equivalent free drug combination. E, in vivo 3D fluorescence tomography of LbL nanoparticles indicates high colocalization with subcutaneous MDA-MB-231 tumor xenografts following systemic administration. B, 11μmol/L AZD6244 and 3.5 μmol/L PX-866, 48 hours. C, 50 nmol/L AZD6244, 160 nmol/L PX-866, 2 hours, PBS; serum stimulation, 1 hour. D, 0.3 μmol/L AZD6244, 1 μmol/L PX-866, 1 hour; serum stimulation, 10 minutes. E, obtained with structured illumination of the lower abdomen (24 hours; 8.3 × 1012 NP/kg, 5% glucose; 715/760 ex/em). Error bars represent (B) SD of 4 technical and (C) SEM of 3 biological replicates.
While studies are currently under way to elucidate the precise mechanism of nanoparticle-enhanced drug synergy, the results here may result from nanoparticle-accelerated or -augmented intracellular transport or simultaneous intracellular delivery of the hydrophobic–hydrophilic drug pair. Delivery of the 2 drugs, directly, may also lead to differential cell uptake for the drug pair that have notably disparate physiochemical properties. Kinetics of uptake and release may also be a factor. Interestingly, we found that at early time points (10-minute serum stimulation following 1 hour of drug treatment), nanoparticle inhibition of pAkt was more complete than from equivalent concentrations of the free drug combination (Fig. 2D), potentially abrogating PI3K pathway-mediated apoptotic suppression, at minimum (57). Using an in vivo mouse model, LbL nanoparticles targeted subcutaneous, CD44-expressing, triple-negative breast (MDA-MB-231) carcinoma tumors with high efficiency (Fig. 2E), suggesting that Mek and PI3K inhibitor–loaded LbL nanoparticles may be effective in treating solid tumors in vivo.

We next investigated LbL nanoparticle–mediated horizontal blockade in mice bearing subcutaneous MDA-MB-231 tumor xenografts. Western blot analyses from excised tumor lysates of untreated and nanoparticle-treated mice bearing subcutaneous MDA-MB-231 xenografts 24 hours following i.v. administration of LbL nanoparticles. C, histologic analysis of corresponding tumor sections indicating decreased phospho-Erk1/2 and phospho-Akt staining, as well as increased cell death as measured by cleaved caspase-3 (CC3). D, in vivo treatment response following i.v. nanoparticle administration on days 0 and 5 (n = 5). Lanes 3 and 4 are technical replicates of lanes 1 and 2 in A. Scale bar in (C) is 200 μm. Error bars represent (B) SD of 2 to 4 technical and (D) SEM of 5 biological replicates. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 3.
Tumor-targeting LbL nanoparticles horizontally block tumor-specific MAPK/PI3K signaling and induce disease stabilization in vivo. A, combined signal blockade as measured by decreased phosphorylation of Akt and Erk1/2 as measured by Western blotting and (B) densitometry from tumor lysates of untreated and nanoparticle-treated mice bearing subcutaneous MDA-MB-231 xenografts 24 hours following i.v. administration of LbL nanoparticles. C, histologic analysis of corresponding tumor sections indicating decreased phospho-Erk1/2 and phospho-Akt staining, as well as increased cell death as measured by cleaved caspase-3 (CC3). D, in vivo treatment response following i.v. nanoparticle administration on days 0 and 5 (n = 5). Lanes 3 and 4 are technical replicates of lanes 1 and 2 in A. Scale bar in (C) is 200 μm. Error bars represent (B) SD of 2 to 4 technical and (D) SEM of 5 biological replicates. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

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LbL nanoparticles improve the therapeutic index of dual MAPK/PI3K pathway inhibition. A, hepatic degeneration score as assessed from hematoxylin and eosin (H&E)-stained liver sections from immunocompetent BALB/c mice 24 hours following combination drug administration as targeted and untargeted LbL nanoparticle formulations, as well as their respective vehicles, in comparison to a lethal equivalent dose of the free drug combination. B, H&E-stained liver sections from MDA-MB-231 tumor xenograft-bearing NCR nude mice (n = 5) 24 hours following administration of free AZD6244 and free PX-866 (1 mg/kg AZD6244, 3.7 mg/kg PX-866 o.g.). Arrowheads indicate damage to tissues surrounding the hepatic central vein and bile ducts (Fig. 4A and B), including fragmented nuclei and erythrocyte extravasation following administration of the free drug combination. In contrast, mice that received drug-containing LbL nanoparticles—both targeted and untargeted—exhibited no significant histopathologic differences with controls or vehicles alone. Interestingly, immunocompetent mice receiving a half-equivalent dose of the free drug combination exhibited a significant decrease in serum albumin (1.5 ± 0.3 g/dl) and a 40 ± 20-fold elevation in serum blood urea nitrogen:creatinine (BUN:Cr) levels (Fig. 4C and D), indicative of impaired hepatic and renal function, respectively, whereas mice receiving a full dose of the tumor-targeted LbL nanoparticle formulation exhibited no significant changes in serum albumin, BUN, or Cr. Collectively, these data indicate that LbL nanoparticle encapsulation can direct tissue disposition of drug combinations in a manner that enhances their therapeutic index—in addition to drug synergy. Biochemical and histopathologic damage observed here from the free drug combination is consistent with a previous phase I clinical trial investigating horizontal blockade in patients with refractory solid tumors that reported dose-limiting hepatic toxicity as a common grade ≥3 adverse event (45), consistent with the hepatic damage we observed in this murine model. Interestingly, mice receiving tumor-targeted LbL nanoparticles exhibited no significant change in body condition and only transient changes in serum cytokine levels (Supplementary Fig. S2). These findings may be attributable to improved tissue distribution profiles of these novel drug carriers, where we previously observed 4-fold improved tumor targeting (ca. 6%ID) and 2-fold decreased liver accumulation in tumor-bearing and immunocompetent mice, respectively, compared with nontargeting LbL nanoparticle architectures (24). Together, these findings suggest that LbL nanoparticle–mediated horizontal blockade could be impactful in improving treatment outcomes in solid tumors driven by MAPK or PI3K axis signaling.

Discussion

Simultaneous blockade of MAPK and PI3K pathway signaling has been shown to synergistically kill a variety of solid tumors in vivo and abrogate resistance-associated signaling (33, 42, 44, 55). Shimizu and colleagues, in a retrospective phase I clinical study of 236 patients with advanced solid tumors receiving small-molecule MAPK or PI3K pathway inhibitors, alone or in combination, found that combined blockade elicited favorable treatment response in the advanced disease population. This favorable response, however, came at the expense of greater toxicity, resulting in a 2.0-fold increased prevalence of dose-limiting toxicity and a 3.0-fold increased prevalence of drug-related grade ≥3 adverse events (45). Nanoscale drug formulations such as Abraxane and Doxil can enhance therapeutic index in patients (5) and improve treatments outcomes in combination delivery approaches (6, 9, 12, 14). Previously, we demonstrated that an emerging class of nanoscale drug carrier, LbL nanoparticles, could improve relative to untreated controls, a response which may be attributable to antiproliferative interactions between high molecular mass hyaluronan (58) on the Lbl particle surface and CD44 receptors expressed highly on triple-negative breast cell lines including MDA-MB-231 (24). Equivalent doses of the free drugs were poorly tolerated, resulting in rapid morbidity and mortality in tumor-bearing mice. Histologic analysis of tissue sections from vital organs (24 hours) indicated significant damage in tissues surrounding the hepatic central vein and bile ducts (Fig. 4A and B), including fragmented nuclei and erythrocyte extravasation following administration of the free drug combination. In contrast, mice that received drug-containing LbL nanoparticles—both targeted and untargeted—exhibited no significant histopathologic differences with controls or vehicles alone. Interestingly, immunocompetent mice receiving a half-equivalent dose of the free drug combination exhibited a significant decrease in serum albumin (1.5 ± 0.3 g/dl) and a 40 ± 20-fold elevation in serum blood urea nitrogen:creatinine (BUN:Cr) levels (Fig. 4C and D), indicative of impaired hepatic and renal function, respectively, whereas mice receiving a full dose of the tumor-targeted LbL nanoparticle formulation exhibited no significant changes in serum albumin, BUN, or Cr. Collectively, these data indicate that LbL nanoparticle encapsulation can direct tissue disposition of drug combinations in a manner that enhances their therapeutic index—in addition to drug synergy. Biochemical and histopathologic damage observed here from the free drug combination is consistent with a previous phase I clinical trial investigating horizontal blockade in patients with refractory solid tumors that reported dose-limiting hepatic toxicity as a common grade ≥3 adverse event (45), consistent with the hepatic damage we observed in this murine model. Interestingly, mice receiving tumor-targeted LbL nanoparticles exhibited no significant change in body condition and only transient changes in serum cytokine levels (Supplementary Fig. S2). These findings may be attributable to improved tissue distribution profiles of these novel drug carriers, where we previously observed 4-fold improved tumor targeting (ca. 6%ID) and 2-fold decreased liver accumulation in tumor-bearing and immunocompetent mice, respectively, compared with nontargeting LbL nanoparticle architectures (24). Together, these findings suggest that LbL nanoparticle–mediated horizontal blockade could be impactful in improving treatment outcomes in solid tumors driven by MAPK or PI3K axis signaling.

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tumor accumulation 4.0-fold and decrease liver accumulation 2.0-fold (24). We hypothesized that the enhanced tissue disposition profiles afforded by these novel drug carriers could improve tumor cell killing from dual MAPK/PI3K inhibition while reducing drug-related hepatotoxicity such as that indicated by increased transaminase levels reported by Shimizu and colleagues. An improved safety profile could potentially augment tolerable dosing levels in patients and further improve response rates to dual MAPK/PI3K pathway inhibition in the clinic.

LbL nanoparticles containing AZD6244 (selumetinib), an allosteric inhibitor of Mek1/2, and PX-866, a covalent inhibitor of PI3K, were 160 ± 20 nm in hydrodynamic diameter, well below the size-dependent splenic clearance threshold, and negative in surface charge (−40 ± 1 mV) to prevent nonspecific cell surface interactions. In previous studies, we found that these architectures could circulate for long periods following i.v. bolus injection (elimination half-life ~ 28 hours; ref. 8) target tumors with high efficiency and decrease liver-specific accumulation (24). In *vivo*, these nanoscale formulations entered cells through various ATP-dependent processes (excluding caveolin-dependent entry), simultaneously blocked MAPK and PI3K pathway activation, and exhibited synergistic cell killing in excess of the additive Bliss expectation. Interestingly, we found that drug synergy was further enhanced following nanoscale encapsulation, a factor that could also further improve the therapeutic index of dual MAPK/PI3K blockade in the clinic. Consistent with patient responders in Shimizu and colleagues harboring co-activation of both MAPK and PI3K pathway signaling, we observed synergistic cell killing in basal-like MDA-MB-231 breast cells by which a harbor an activating mutation in *KRAS* (G12V), high levels of EGFR protein, and *PTEN*-dependent survival following MEK inhibition (55). We further observed drug synergy in KP7B cells derived from an autochthonous mouse model of non–small cell lung cancer which host an activating mutation in *KRAS* (G12D) but not in *OVCAR3* epithelial ovarian cancer cells, which lack any predating mutations on either axis (*PI3KCA, PTEN, KRAS*, and *BRAF*).

As a proof-of-concept, we investigated tumor remediation *in vitro* using a subcutaneous MDA-MB-231 xenograft mouse model. Following a single i.v. bolus injection, we observed significant disease stabilization from our tumor-targeting nanoscale drug formulation but progressive disease in our nontargeting control. This response was accompanied by a reduction in tumor-specific phosphorylation of the terminal kinases Erk and Akt and an increase in apoptosis as histologically measured by cleaved caspase-3 staining. Interestingly, combination therapy with the free drugs was lethal at equivalent dosing and, at sublethal dosing levels, resulted in acute hepatic and renal dysfunction as measured by decreased serum albumin and elevated BUN:Cr levels, as well as gross histopathologic liver damage at the full equivalent dose. These findings are consistent with dose-limiting hepatic toxicity reported in phase I trial patients receiving dual MAPK/PI3K blockade therapy (45). These nanoscale formulations were well-tolerated in tumor-bearing mice and elicited in no acute serum cytokine upregulation following systemic administration in immunocompetent mice, as well as no significant changes in hepatic or renal function as measured from serum blood chemistry and histopathologic analysis of vital organs. Studies are currently under way to identify and profile surviving cell subpopulations, optimize dosing regimens, and investigate therapeutic response in advanced tumor models. While murine xenografts exhibit notable functional and pathologic differences with human breast carcinomas (e.g., stromal compaction, labeling index, and vascularity) these results are particularly promising in the context of prior work investigating related Lbl architecture that demonstrate the ability to target and treat tumors in other sites including orthotopic mammary fat pad (59) and autochthonous lung tumor models (unpublished data). Although here we found these particles capable of rapid diffusion throughout tumor spheroid models, efficient intratumoral distribution and the capacity to target poorly vascularized tumors remains a key challenge for these and other emerging classes of nanoscale drug carriers. Efforts to further engineer modular Lbl nanoparticle delivery platforms that improve therapeutic safety, potency, and durability are currently under way.

In summary, here we developed a tumor-targeting nanoscale drug formulation based on Lbl self-assembly that horizontally blocks MAPK and PI3K signaling *in vitro* and *in vivo*, selectively inhibiting disease progression in tumor-bearing mice. These self-assembled drug carriers co-delivered small-molecule inhibitors of MEK and PI3K with vastly differing physicochemical properties and enhanced synergistic cell killing 2.6-fold following encapsulation. Following systemic administration in breast tumor xenograft–bearing mice, we observed a 3.9- and 9.4-fold reduction in tumor-specific MAPK and PI3K pathway signaling, respectively, accompanied by tumor apoptosis and disease stabilization in the absence of dose-limiting hepatotoxic effects observed from the free drug combination. Mice receiving untargeted, but dual drug–loaded nanoscale formulations, exhibited progressive disease. Tumor-targeting nanoscale drug formulations could provide a more safe and effective means to synergistically block MAPK and PI3K in the clinic.

**Disclosure of Potential Conflicts of Interest**

R. Drapkin is a consultant/advisory board member for Siamah Therapeutics.

No potential conflicts of interest were disclosed by the other authors.

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References


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