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Dual-therapy with $\alpha_v\beta_3$ -targeted Sn2 lipase-labile Fumagillin-prodrug Nanoparticles and Zoledronic Acid in the Vx2 Rabbit Tumor Model

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Abstract

Fumagillin, an unstable anti-angiogenesis mycotoxin, was synthesized into a stable lipase-labile prodrug and incorporated into integrin-targeted lipid-encapsulated nanoparticles ($\alpha_v\beta_3$ -Fum-PD NP). Dual anti-angiogenic therapy combining $\alpha_v\beta_3$ -Fum-PD NP with zoledronic acid (ZA), a long-acting osteoclast inhibitor with proposed anti-angiogenic effects, was evaluated. *In vitro*, $\alpha_v\beta_3$ -Fum-PD NP reduced ($p<0.05$) endothelial cell viability and tube formation without impacting macrophage viability. ZA suppressed ($p<0.05$) macrophage viability at high dosages *in vitro* but not endothelial cell proliferation. 3D MRI neovascular imaging of 17d rabbit Vx2 tumors showed no effect with ZA, whereas $\alpha_v\beta_3$ -Fum-PD NP alone and with ZA decreased angiogenesis ($p<0.05$). Immunohistochemistry revealed decreased ($p<0.05$) microvasculature with $\alpha_v\beta_3$ -Fum-PD NP and ZA and further microvascular reduction ($p<0.05$) with the dual-therapy. However, *in vivo*, ZA did not decrease tumor macrophage numbers nor cancer cell proliferation, whereas $\alpha_v\beta_3$ -Fum-PD-NPs reduced both measures. Dual-therapy with ZA and $\alpha_v\beta_3$ -Fum-PD-NP may provide enhanced neo-adjuvant utility if macrophage ZA uptake is increased.

Graphical Abstract

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Schematic representation of the activated integrin $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticle fumagillin prodrug ($\alpha_v\beta_3$ -Fum-PD) chemistry, contact facilitated drug delivery (CFDD) mechanism, and lipase enzymatic liberation of active fumagillol.

Keywords

fumagillin; zoledronic acid; cancer; nanoparticle; MRI; molecular imaging

Background

Angiogenesis is required for tumor growth beyond a certain size and is an attractive therapeutic target. Current anti-angiogenic agents directly target the tumor endothelium through inhibition of the VEGF pathway. Tumors circumvent anti-VEGF blockade in part through alternative pro-angiogenic factors and endothelial signaling pathways¹. Angiogenesis is a complex process that involves multiple host cell types including endothelial cells, myeloid derived macrophages, myeloid derived suppresser cells (MDSCs) and T-cells^{2, 3}. Tumor associated macrophages (TAMs) promote angiogenesis through soluble factors that increase endothelial cell proliferation and matrix degradation⁴. Macrophage driven angiogenesis significantly contributes to resistance to VEGF pathway inhibitors^{1, 4}. Thus, successful anti-angiogenesis treatment strategies for unresponsive and resistant tumor types will likely require combination therapies targeting multiple pathways driving neovasculature expansion.

Fumagillin, a hydrophobic mycotoxin produced by *Aspergillus fumigatus*, has been demonstrated to suppress angiogenesis by inhibition of methionine aminopeptidase 2 (MetAP2)^{5–7}. Of two methionine aminopeptidase forms in eukaryotes, only MetAP2 is up-regulated during cellular proliferation, providing greater efficiency (1000-fold) catalyzing methionine removal from certain proteins, such as glyceraldehydes-3-phosphate, which can modulate subsequent activity, stability or subcellular localization^{8–10}. TNP-470 is a water-soluble, stable form of fumagillin that was demonstrated in a variety of rodent cancer models,^{11–13} but when tested in human clinical trials,^{14–16} the molecule possessed significant liabilities including a brief systemic half-life and moderately severe symptoms of neurotoxicity¹⁴.

We have reported effective *in vivo* neovascular delivery of native fumagillin with $\alpha_v\beta_3$ -targeted perfluorocarbon (PFC) nanoparticles at a fraction of the dosage that was required systemically for TNP-470 in clinical studies^{17–19}. Fumagillin is very hydrophobic and dissolves easily into the phospholipid membrane component of PFC nanoparticles. When targeted to angiogenic endothelial cells, it was efficiently delivered through a mechanism we described as “contact facilitated drug delivery” (CFDD)^{20–22}. Although $\alpha_v\beta_3$ -targeted perfluorocarbon (PFC) nanoparticle delivery of native fumagillin was effective in preclinical animal models, simultaneous pharmacokinetic tracking of individual nanoparticle components revealed that much of the compound was prematurely lost in blood before the particles bound to the neovasculature²³. To improve the light and chemical stability of fumagillin and to increase its retention in lipid-encapsulated nanoparticles during circulation to target tissues, an Sn-2 lipase labile phospholipid fumagillin prodrug (Fum-PD) was designed, synthesized and incorporated stably into the surfactant of $\alpha_v\beta_3$ -targeted perfluorocarbon (PFC) nanoemulsions (Fig 1)²³. Early data *in vivo* suggest that $\alpha_v\beta_3$ -Fum-PD PFC NP were effective in mouse MatrigelTM angiogenesis plug and in an inflammatory arthritis model, but its effectiveness in cancer, particularly the aggressive rabbit Vx2 tumor model, is unknown^{24, 25}.

Zoledronic acid (ZA) is a long-acting amino-bisphosphonate approved to treat osteoporosis and to prevent cancer-related bone fracture and pain. Recent clinical evidence suggests that ZA may have beneficial effects on primary breast cancer growth and patient survival^{26–29}. Cancer bearing mice treated with ZA have reduced vasculature through an unresolved mechanism that implicates a complex interplay among MDSC, TAMs, tumor cells and T-cells^{30, 31}. In patients, ZA treatment correlated with reduced circulating angiogenic factors including VEGF and PDGF, but a clear effect on the tumor vasculature was not demonstrated. Importantly, myeloid lineage osteoclasts and macrophages have the ability for ZA endocytic uptake and can phagocytose ZA bound to bone or tumor microcalcifications^{30, 32, 33}. We hypothesized that the long-acting nature of ZA via myeloid mediated pathway would complement the anti-angiogenesis benefits of $\alpha_v\beta_3$ -Fum-PD PFC NP nanoparticles in cancer.

The objectives of these studies were: 1) to characterize the anti-angiogenesis effectiveness of $\alpha_v\beta_3$ -Fum-PD *in vitro* and in an aggressive large animal cancer model; 2) to determine and characterize the anti-neovascular effects of intravenous ZA in the Vx2 rabbit model; and 3) to determine if dual therapy with intravenous ZA and $\alpha_v\beta_3$ -Fum-PD nanoparticles offer complementary therapeutic benefit in cancer.

Methods

Primary cell assays

Primary human umbilical vein endothelial cells (HUVECS) obtained from Lonza (07/01/13) expressed CD31/105, von Willebrand Factor VIII, and were positive for acetylated low density lipoprotein uptake. HUVEC were grown to 70% confluence in VasculLife Endothelial Cell Culture Media (Lifeline Cell Technologies, Fredrick, MD, USA) and maintained for <5 passages. Bone marrow macrophage (BMM) cultures were isolated from C57Bl/6J mice, plated and maintained in macrophage media culture for 3 days (MEM alpha,

Life Technologies + 10% FBS + and 100ng/mL M-CSF)³⁴. Osteoclasts were cultured 6 days in macrophage media (described above) + RANKL (50ng/ml) following isolation of BMMs^{35, 36}. Cell viability was measured using MTT assay (Sigma-Aldrich, St. Louis, MO, USA)³⁵. To assess endothelial tube formation, HUVEC (100µl; 30,000 cells/well) were plated in tissue culture plates (96-well) coated with 100µl MatrigelTM (BD Biosciences, San Jose, CA, USA) and incubated at 37°C for 16hr. For nanoparticle binding assays, HUVEC were plated for 24 hours, washed with PBS, then incubated with fluorescent $\alpha_v\beta_3$ -targeted or non-targeted NPs (1hr, 37 C) with gentle continuous shaking. After removal of unbound particles with multiple PBS washes, cells were recovered with trypsin and analyzed by flow cytometry (BD LSRFortessa, BD Biosciences, San Jose, CA, USA).

To assess Vx2 tumor composition, minced tumors were incubated in 1X collagenase/hyaluronidase solution (STEMCELL Technologies, Vancouver, BC, Canada) for 4hr shaking at room temperature. Single cell suspensions were isolated by filtration (BD Falcon 70µM cell strainer, Fisher Scientific, Pittsburg, PA, USA) and incubated with fluorescent anti-mouse pan-cytokeratin (AE1/A13, 1:200, ebioscience, San Diego, CA, USA) and anti-mouse CD11b (M1/70, 1:400, eBioscience, San Diego, CA, USA) antibodies for 30 minutes. Unbound antibody by excess PBS washes then cells were characterized by flow cytometry (BD LSRFortessa, BD Biosciences, San Jose, CA, USA).

Quantitative reverse-transcription PCR

RNA was extracted using RNeasy Mini kit (Qiagen, Venlo, Netherlands) and cDNA generated using iScript (Bio-Rad, Hercules, CA, USA). Quantitative PCR was completed using SsoFast EVA Green Supermix (Bio-Rad). Primer sequences were CD68 forward- CTCCACCTCGACCTGCTCT and CD68 reverse- ATGATGAGGGGCACCAAGAT.

Transwell co-culture assays

BMMs (20,000/well) were cultured as described on 24-well polycarbonate transwell inserts (0.4µm pores, Fisher Scientific, Pittsburg, PA, USA). After 24hr media was aspirated and replaced with fresh media containing mouse IL-4 (5ng/ml) to polarize to an M2 phenotype. Twenty-four hours later this media was replaced with macrophage media + IL-4 (5ng/ml) + LPS (100ng/ml) for 6hr. Transwells were washed with PBS and transferred to 24-well plates containing HUVECs (30,000/well). Viability was measured by MTT assay (Sigma-Aldrich).

Vx-2 rabbit tumor model

Animal studies were performed under a protocol approved and monitored by the Washington University Animal Studies Committee. Male New Zealand White rabbits (~2 kg) were anesthetized with intramuscular ketamine and xylazine. The Vx-2 tumor (Division of Cancer Treatment and Diagnosis Tumor Repository, National Cancer Institute, USA) was implanted as described previously³⁷. All animal care and experimental protocols were approved and conducted in accordance with Washington University guidelines.

Synthesis of Sn2 lipase labile fumagillin-prodrug

An Sn2 fumagillin-prodrug (Fum-PD) was envisioned and the compound was synthesized in a straightforward way in two-steps involving saponification of fumagillin

dicyclohexylamine salt to fumagillol and a subsequent esterification with oxidized lipid 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine²³. (See Supplemental Materials for detail)

$\alpha_v\beta_3$ -Targeted perfluorocarbon nanoparticle synthesis incorporating fumagillin-prodrug

An $\alpha_v\beta_3$ -integrin antagonist coupled to phosphatidylethanolamine-PEG₂₀₀₀ was used for neovasculature homing (gift from Kereos, Inc, St. Louis, MO, USA). This quinolone nonpeptide antagonist developed by Bristol-Myers Squibb Medical Imaging to $\alpha_v\beta_3$ -integrin (US patent 6,511,648 and related patents) was initially reported and characterized as the ¹¹¹In-DOTA conjugate RP748 and cyan 5.5 homologue TA145. The targeted particles presented ~300 ligands/particle with an IC₅₀ of 50 pM for the Mn²⁺-activated $\alpha_v\beta_3$ -integrin¹⁹. Homing specificity to neovascular sprouts was previously demonstrated in a well defined in MatrigelTM plug study³⁸.

$\alpha_v\beta_3$ -Fum-PD perfluorocarbon (PFC) nanoparticles (NP) were prepared as a microfluidized suspension of 20% (v/v) perfluorooctylbromide (Exfluor Inc., Round Rock, TX, USA), 2.0% (w/v) of a surfactant co-mixture, and 1.7% (w/v) glycerin. The surfactant co-mixture of NP included: 97.6 mole% lecithin, 0.15 mole% of $\alpha_v\beta_3$ -ligand conjugated lipid and 2.3 mole% of the Fum-PD. The surfactant components were combined with the PFC, deionized water, and glycerin with pH adjusted to 6.5 with 5% carbonate buffer. The mixture was pre-blended (Tissumizer Mark II, Tekmar, Cincinnati, Ohio, USA) then homogenized at 20,000 psi for 4 minutes (M110s, Microfluidics Inc., Westwood, MA, USA). Routine NP characterization revealed: nominal size of 227.2 nm, polydispersity of 0.072, and zeta potential of -10.74 mV (Brookhaven Instruments Co, Holtsville, NY, USA) (Fig 1).

Synthesis of $\alpha_v\beta_3$ -targeted manganese oleate nanocolloids with gadolinium chelate

For MR imaging, paramagnetic manganese oleate (MnOL) nanocolloid (NC) with a very low concentration of lipid-anchored-chelated gadolinium was synthesized to produce $\alpha_v\beta_3$ -MnOL-Gd NC.^{39, 40} Briefly, Mn-oleate was suspended in almond oil to form the emulsion core, which was encapsulated with phospholipid surfactant via microfluidization to produce the base nanocolloid. The surfactant mixture was comprised of 0.15 mole% of $\alpha_v\beta_3$ -ligand conjugated lipid, 1.25 mole% Gd-DOTA-PE (gadolinium-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid)- phosphatidylethanolamine) and phosphatidylcholine (PC). In selected preparations, the lipid surfactant included Texas Red dye coupled to caproyl-phosphatidylethanolamine (Life Technologies; Avanti Polar Lipids, Alabaster, Alabama, USA) at 0.5 mol % for microscopic fluorescence imaging. Nanocolloids were extensively dialyzed against water using a 20,000 Da MWCO cellulose membrane, passed through a 0.45 μ m syringe filter, and stored under argon atmosphere at 4°C to reduce oxidation and bacterial growth. Nanocolloid characterization revealed a nominal particle size of 162.5nm, polydispersity of 0.071, zeta potential of -21.62mV. NC were preserved under inert gas in sterile sealed vials until use. Full physical-chemical, MR characterization, pharmacokinetics, biodistribution and bioelimination, and demonstration of neovascular imaging with $\alpha_v\beta_3$ -MnOL-Gd NC was previously reported.^{39, 40}

MR neovascular molecular imaging

On day 17 post tumor implantation, rabbits were anesthetized with 1% to 2% Isoflurane in oxygen to effect and imaged in a clinical 3T MR scanner (Philips Achieva, Andover, MA, USA) with an 8-element SENSE coil using a high-resolution, T1-weighted, fat suppressed, 3D gradient echo sequence (TR/TE = 42.6/6.1ms, 45° flip angle, 2 NSA, $0.25 \times 0.25 \times 0.5$ mm resolution, SPIR fat suppression). Images were obtained before and 2.5 hours after IV administration of $\alpha_v\beta_3$ -MnOL-Gd NC (1ml/kg) to compare the influence of the treatments on angiogenesis and tumor volumes.

Signal intensities of MR images were analyzed with custom MATLAB software (The Math Works, Natick, MA, USA). Dynamic T1-weighted signal intensities were normalized to a Gd-DTPA doped water reference standard included within each imaging field-of-view. For each animal, a region-of-interest (ROI) was manually placed around the tumor edge on each baseline slice, and the standard deviation of the average tumor signal at baseline was calculated. Voxels on post-injection MRI images were defined as enhanced if the signal intensity exceeded the mean tumor signal at baseline by more than two standard deviations. The tumor ROI was automatically partitioned into a rim and core region using an automated erosion method (MATLAB). The core region was set to half of the ROI volume. The volume of enhancement was calculated, as well as the relative distribution of enhancing voxels in the periphery versus the core. High-resolution three dimensional (3D) tumor reconstructions were created in MATLAB to map the spatial distribution of the enhancing neovasculature in each treatment group. The overall 3D structure was displayed as a mesh surface plot using iso-surface rendering and a smoothing filter. A surface plot of the enhancing voxels was reconstructed similarly, and overlaid onto the tumor volume.

MicroCT

To corroborate the known effect of ZA on bone, rabbit femurs were suspended in agarose and femoral epiphyses were scanned by micro-computed tomography (mCT-40; Scanco Medical, Wayne, PA, USA). For image acquisition, femurs were scanned in a 30mm sample holder. Fifty 1.5mm image slices of trabecular bone beginning 1.2mm from the epiphyseal growth plate were acquired. A 3D cubical voxel model of each bone was built using contours drawn within the cortical shell, and calculations were made for trabecular thickness (Tb.Th) as described previously^{41, 42}.

Histology

Rabbit tumors were harvested at study endpoint and embedded in OCT compound. Frozen sections were stained for immunohistochemistry (IHC) with mouse anti-human CD31+ (DAKO-M0823, 1:2000, DAKO North America, Carpinteria, CA, USA) and proliferating cellular nuclear antigen (PCNA, ab29, 1:2000, Abcam, Cambridge, MA, USA). Whole tumor slices (n=4) were quantified due to significant morphological heterogeneity. PCNA, proliferating cell nuclear antigen, was quantified as number of positive cells/whole tumor area/slice. CD31 was quantified along the entire tumor rim (defined as non-necrotic counterstained regions adjacent to the surrounding stroma) and reported as area of positive stain/ROI area. Samples were quantified with Visiopharm software (Visiopharm, Broomfield, CO, USA) and threshold levels were set equally across all samples to exclude

background staining. Vx2 tumors were immunostained with anti-mouse CD11b (M1/70, 1:200, eBioscience, San Diego, CA, USA) and quantified along the tumor rim. Three ROI's per sample were randomly selected and the number of CD11b+ cells was counted manually.

Statistical analysis

In vitro experiments were analyzed by t-test or one-way ANOVA with a Bonferroni post-hoc test using PRISM software, (GraphPad, La Jolla, CA, USA) as appropriate. All in vitro experiments were repeated 2–3 times and representative experiments are shown as mean $\pm$ SEM. In vivo imaging results were analyzed with SAS (Cary, NC, USA) using ANOVA and Tukey's range test. Significant differences between mean were declared at $p < 0.05$ or less.

Results

In vitro effects of zoledronic acid and fumagillin on endothelial and macrophage-osteoclast viability

Fumagillin Sn2 prodrug (Fum-PD) and native fumagillin (Fum) were compared in HUVEC culture as equimolar titrations of the active compound. Both Fum and Fum-PD in DMSO produced nearly identical dose-related decreases in HUVEC proliferation in culture. Although Fum-PD required endothelial cell membrane solubility and subsequent cytosolic enzymatic liberation of active drug (API) from its phospholipid backbone, its net bioactivity was unchanged from native free drug. Further, the monotonic decrease in cell proliferation with increasing dosage of Fum-PD indicated that neither the rate of cellular uptake nor cytosolic API liberation limited the prodrug's effectiveness. (Figure 2A) Similarly, Fum-PD in DMSO inhibited endothelial tube formation in 3D culture further corroborating the anti-angiogenic biopotency of the photochemically stabilized compound (Supplementary Figure 1).

Fluorescent nontargeted and $\alpha_v\beta_3$ -Fum-PD NP (i.e., 597 μ M, 0.275mg/ml fumagillin API) were incubated with activated HUVECs. Flow cytometry demonstrated a marked increase in integrin-cell binding whereas the nontargeted control had little cellular affinity. (Figure 2B) Subsequently, $\alpha_v\beta_3$ -Fum-PD NP and control NP were incubated with HUVEC at final doses of 100, 200, and 300 pM, which reduced ($p < 0.01$) endothelial viability in a dose-dependent manner for the targeted therapeutic but not for the nontargeted nanoparticles. (Figure 2C) These results demonstrated that $\alpha_v\beta_3$ -Fum-PD NP drug delivery was efficacious relative to the nontargeted control particles, which remained 100% viable. Unlike the administration of Fum-PD in DMSO, the endothelial cell uptake of $\alpha_v\beta_3$ -Fum-PD NP was dependent on the number of particles binding to the cells, which reflected the density of cell surface activated $\alpha_v\beta_3$ -integrin available. In contradistinction to endothelial cells, the viability of activated primary macrophages was unaffected by Fum-PD in DMSO (Figure 2D). Although activated macrophages express $\alpha_v\beta_3$ -integrin and may be targeted by $\alpha_v\beta_3$ -Fum-PD NP, the mycotoxin did not effect macrophage viability at concentrations that decreased HUVEC viability by greater than 80%.

Titrated levels of ZA had little effect on HUVEC viability except at the highest dosage level (50 μ M). (Figure 3A) Moreover, ZA added to HUVEC cultures did not further decrease the

viability achieved with Fum-PD. (Figure 3B) Analogous results were appreciated for endothelial tube formation. HUVEC proliferation in culture was enhanced by co-culture with M2 polarized macrophages (tumor promoting). (Figure 3C) ZA reduced macrophage viability slightly at concentrations up to 10 μ M ($p>0.05$), but beyond this dose threshold, macrophage viability decreased precipitously ($p<0.05$). Similar findings were noted at higher concentrations in primary osteoclasts in culture, corroborating this result. (Figure 3D, Supplemental Figure 2) These data illustrate the supportive influence of macrophages on proliferating endothelial cell viability. Overall, ZA had minimal effect of HUVEC in culture compared with myeloid cells treated equivalently and the significant effectiveness against macrophage viability observed depended upon the direct exposure at concentrations exceeding 10 μ M.

$\alpha_v\beta_3$ Fum-PD NPs and zoledronic acid decrease tumor vasculature and macrophages in Vx2 rabbit tumors

The potential combination of $\alpha_v\beta_3$ -Fum-PD NP and ZA was evaluated in an aggressive syngeneic immunocompetent rabbit Vx2 squamous cell carcinoma tumor model. This tumor has significant progressive neovascular expansion that was serially documented with MR neovascular molecular imaging using $\alpha_v\beta_3$ -paramagnetic PFC nanoparticles at 3T⁴³. In the present study, rabbits were distributed into 4 treatment groups ($n=8$ /treatment) and received either: intravenous saline, ZA, $\alpha_v\beta_3$ -Fum-PD NPs, and ZA plus $\alpha_v\beta_3$ Fum-PD NPs. Animals were dosed with $\alpha_v\beta_3$ -Fum-PD NP (~0.28 mg Fum/kg) on days 9, 12 and 15 post-implantation. ZA (40 μ g/kg) was given intravenously on days 5 and 12. The fumagillin dose was over 100-fold lower than the TNP-470 treatments used in human clinical studies^{15, 44, 45}. The ZA dosage (40 mg/kg IV) was equivalent to current clinical regimens used for breast cancer patients with metastatic bone disease. MicroCT of rabbit femurs confirmed that the ZA dosing regimen increased trabecular thickness compared to saline controls, as it is clinically intended to do. (Figure 4A,B)

On day 17, $\alpha_v\beta_3$ -MnOL-Gd nanocolloids were used for MR neovascular molecular imaging in cancer for the first time. As previously appreciated with the Vx2 tumor model, 3D MR neovascular map revealed an extensive heterogeneous and asymmetrical distribution of neovascular expansion predominantly around the tumor periphery. (Figure 5A,B) $\alpha_v\beta_3$ -Fum-PD NP markedly decreased ($p<0.05$) Vx2 tumor neovascularity relative to the control, whereas, ZA therapy elicited only a trend ($p>0.05$) toward decreased neovascular expansion. (Figure 5A,B) In combination with $\alpha_v\beta_3$ -Fum-PD NP, ZA did not further decrease ($p>0.05$) the MR molecular imaging signal achieved. While differences in neovascular density are quantifiable with MR molecular imaging at a single point in time, they may not reflect cumulative vascular changes over the treatment course.

Microscopic immunohistochemistry (CD31+) was used to evaluate overall vascular changes in the tumor periphery using CD31+, an endothelial cell marker expressed by forming and established blood vessels. Microscopically, both ZA and $\alpha_v\beta_3$ -Fum-PD NP therapy significantly decreased ($p<0.05$) tumor peripheral microvasculature versus controls. (Figure 6A,B) Further, the combination of ZA and $\alpha_v\beta_3$ -Fum-PD NP additively decreased tumor microvasculature further ($p<0.05$). (Figure 6A)

Decreased tumor vascularity, whether achieved with ZA and $\alpha_v\beta_3$ -Fum-PD NP, was not associated with decreased tumor volume by estimated by MR and corroborated closely by the dissected tumor weight. (Supplementary Figure 3). Vx2 tumor cell proliferation, as reflected by PCNA staining, was reduced by $\alpha_v\beta_3$ -Fum-PD NP ($p<0.05$) (Figure 6C,D). In contradistinction, ZA did not impact cancer cell proliferation despite its measurable diminishment of tumor vascularity. Moreover, the reduced tumor cell proliferation achieved with $\alpha_v\beta_3$ -Fum-PD NP was not increased by ZA despite the additive anti-vascular effects noted microscopically. $\alpha_v\beta_3$ -Fum-PD NP diminished tumor viability more effectively through acute transient depressions of tumor neovascularity or by collateral effects of endothelial apoptosis than the slower chronic reductions in vascularity elicited by ZA through inhibition of macrophage activity.

Control Vx2 tumor tissue was primarily composed of pan-cytokeratin+ tumor cells (75%) and CD11b+ macrophage infiltrate. (19%, Figure 7A) Similar to the prevalence of microcalcifications in a small cohort of human breast tumors examined (7/7), all Vx2 tumor (16/16) stained positively for calcium microdeposits, which could have provided substantial substrate for ZA binding and subsequent TAM phagocytosis. (Figure 7B,C) The density of rabbit tumor microcalcification grossly exceeded the levels observed in the human breast cancer sample and likely did not limit this route of delivery. Nevertheless, IV ZA elicited no significant reduction in macrophage *CD68* expression (Figure 7D), measured by qPCR, while $\alpha_v\beta_3$ -Fum-PD NP decreased ($p<0.05$) in *CD68* expression in the tumors.

Interestingly, ZA reduced CD11b+ leukocyte number at the tumor rim ($p<0.05$), while $\alpha_v\beta_3$ -Fum-PD NP elicited no reduction in CD11b+ cells and possibly an increase. Of note, anti-angiogenesis treatments have been suggested to overcome tumor endothelial anergy, angiogenic factor induced suppression of adhesion molecules on neovascular endothelial cells, which has been observed as a marked increase in tumor associated CD8+ lymphocytes in the Vx2 model^{46–49}, the leukocyte – tumor story remains complex and will require continued focused research.

Discussion

In this study, fumagillin Sn2 lipase-labile prodrug was incorporated into the surfactant of $\alpha_v\beta_3$ -perfluorocarbon nanoparticles and shown to be an effective anti-angiogenic therapy in the Vx2 rabbit tumor model. $\alpha_v\beta_3$ -Fum-PD-NP decreased tumor angiogenesis as demonstrated by MR molecular imaging, microscopic histopathology and also decreased tumor cell proliferation. Importantly, $\alpha_v\beta_3$ -Fum-PD-NP effectiveness was achieved at a fractional dosage of the related clinical analogue, TNP-470. Zoledronic acid, which is commonly administered to prevent cancer induced bone fractures and to ameliorate bone pain, significantly increased bone mineralization in the Vx2 rabbit model, decreased myeloid viability in vitro and in vivo, and reduced overall tumor microvasculature. The long-acting ZA effect on the neovasculature was only a trend when compared to $\alpha_v\beta_3$ -Fum-PD-NP. However, the cumulative reduction in tumor microvasculature along the tumor periphery over the study was equivalent for both treatments. Moreover, $\alpha_v\beta_3$ -Fum-PD-NP and ZA further decreased tumor microvasculature additively.

In the present study, $\alpha_v\beta_3$ -Fum-PD-NP was studied as an anti-angiogenic therapy in cancer. In contradistinction to anti-VEGF therapies that block the cytokine stimulation for endothelial proliferation, $\alpha_v\beta_3$ -Fum-PD-NP directly targets neoendothelial cell sprouts and drives them into apoptosis. A secondary benefit of this direct anti-neoendothelial cell therapy in cancer may be a reduction in TAMs. In arthritis models, endothelial apoptosis induced by fumagillin increased intracellular NO production and local extracellular liberation that modulated synovial macrophage inflammatory responses through increased AMP-activated protein kinase (AMPK) activity. This led to decreased mammalian target of rapamycin (mTOR) activity, increased autophagic flux, increased autolysosome formation, with degradation of IkappaB kinase (IKK), suppression of the NF- κ B-p65 signaling pathway and ultimately decreased inflammatory cytokine release⁵⁰. The present data obtained in the Vx2 cancer model are consistent with elements of this result. In arthritis $\alpha_v\beta_3$ -Fum-PD-NP led to a near full resolution of the pathology, however in cancer, tumor viability was reduced without strong evidence of disease remission

Typically, antiangiogenic treatments, whether fumagillin or anti-VEGF, will be transient unless the cancer and macrophage cells stimulating new vessel formation are killed. Consequently, anti-angiogenic therapies are typically used as an adjuvant in combination with chemotherapy, such as 5-FU in colon cancer. In the present study, we considered the hypothesis that chronic therapy with zoledronic acid could maintain the acute transient anti-angiogenic benefits achieved with $\alpha_v\beta_3$ -Fum-PD-NP, becoming an improved, potent neoadjuvant therapy. Evidence in mice and humans have suggested that ZA reduces macrophage activity, as seen with bone osteoclasts, and an anticipated pleiotropic effect would be to decrease TAM stimulated angiogenesis and reduce tumor progression. ZA did decrease the microvasculature in the Vx2 tumor to similar extent as $\alpha_v\beta_3$ -Fum-PD-NP but through a distinct mechanism of action. Unfortunately, the ZA reduction in vasculature, which was additive to $\alpha_v\beta_3$ -Fum-PD-NP effect, did not reduce tumor volume. This result is consistent with other studies of angiogenesis inhibitors and immunotherapies that stabilize tumor progression and potentiate the anti-cancer cell effectiveness of chemotherapy.

Although ZA was effective as used clinically in strengthening the rabbit skeletal architecture, it appeared that the intracellular TAM levels of ZA achieved in vivo were inadequate to markedly reduce reactive macrophage activity and viability. Further, ZA reduced localized myeloid infiltrate and increased the anti-angiogenesis response but failed to achieve a level that would influence Vx2 tumor growth. Of note, the dramatic suppression of macrophage viability in vitro occurred at ZA dosages of 10 μ M and higher, otherwise the myeloid viability impact of the ZA was nonsignificant. While 10 μ M is achievable in the bone microenvironment in vivo, the Cmax for ZA in serum is 1 μ M⁵¹. However, ZA binds to calcium hydroxyapatite present in peripheral solid tumors and bone tumors alike and may be ingested into macrophages or osteoclasts at significantly higher concentrations. In this work, ZA therapy reduced localized myeloid infiltrate and enhanced the anti-angiogenesis response, However, an effective myeloid intracellular IC50 dose to substantively complement the anti-tumor $\alpha_v\beta_3$ -Fum-PD NP response was not achieved. The intracellular concentrations of the compound by phagocytosis of microcalcifications maybe too low to induce robust macrophage inhibition even though the microcalcification density observed in rabbits was far higher than that observed in the human breast cancer specimens. Numerous

but variable observations of ZA benefit in patients suggests that the drug in combination with $\alpha_v\beta_3$ -Fum-PD-NP has significant neoadjuvant potential if the delivery of ZA into myeloid cells is increased. This concept was illustrated in xenograft prostate cancer mouse models that received IV ZA, ZA PEGylated nanoparticles (ZA-PEG NPs) or ZA PEGylated liposomes (LIPO-ZA). Both particle systems relied on nonspecific macrophage uptake. These ZA delivery platforms offered superior anti-cancer effects when compared to intravenous ZA, which had no benefit in this mouse cancer model. The best treatment results were obtained with ZA-PEG NPs, which reduced TAM numbers in tumor xenografts, decreased tumor vascularity and increased cancer necrosis and apoptosis^{52, 53}. Perhaps a dual therapy, single nanopatform providing $\alpha_v\beta_3$ -targeting of Fum-PD and ZA would provide the potent neoadjuvant benefits sought in the present study.

Supplementary Material

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Figure 1.

Schematic representation of the activated integrin $\alpha_v\beta_3$ -targeted perfluorocarbon nanoparticle fumagillin prodrug ($\alpha_v\beta_3$ -Fum-PD) chemistry, contact facilitated drug delivery (CFDD) mechanism, and lipase enzymatic liberation of active fumagillin.

Figure 2.

Integrin $\alpha_v\beta_3$ -targeted fumagillin prodrug (Fum-PD) nanoparticles ($\alpha_v\beta_3$ -Fum-PD NP) reduced endothelial cell viability. A) Dose-dependent viability of HUVEC exposed to native fumagillin (Fum) or Fum-PD dissolved in DMSO and assessed using the MTT assay after 48hr drug exposure. B) Flow cytometry of HUVEC incubated with fluorescent (Texas Red) $\alpha_v\beta_3$ -NP (black) compared to fluorescent non-targeted NP (grey) and no NP controls (white). C) Viability of HUVEC by MTT assay 48hr after 1hr treatment (** $p < 0.01$, *** $p < 0.001$). D) Viability of primary macrophages by MTT assay after 48hr exposure with Fum-PD in DMSO.

Figure 3.

Zoledronic acid (ZA) reduced viability of myeloid lineage cells. A) HUVEC viability by MTT after 48hr exposure to titrated ZA concentrations. B) MTT assay for HUVEC after 48hr exposure to saline, ZA (25 μ M), Fum-PD (1 μ M) and ZA+ Fum-PD (25 μ M and 1 μ M respectively). C) MTT assay for viability of HUVECs alone or co-cultured with M2 polarized macrophages for 24hr D) Viability of macrophages after 48hr ZA exposure.

* $p < 0.05$ and *** $p < 0.001$.

Figure 4. Zoledronic acid (ZA) increased trabecular thickness. A) Femur trabecular bone thickness (n=5). B) Representative images of microCT reconstruction of rabbit femur exposed to saline versus ZA IV, scale bar = 5mm.

Figure 5. 3D MR neovascular mapping with $\alpha_v\beta_3$ -MnOL-Gd NC in 17d Vx2 tumors following saline, ZA, $\alpha_v\beta_3$ -Fum-PD NP, or $\alpha_v\beta_3$ -Fum-PD NP plus ZA. A) Surface volume percentage of neovascular voxels in the outer tumor (rim) for each treatment group (n=8, * p <0.05). B) Representative images of 3D MRI neovascular reconstructed maps with purple representing neovascular rich voxels overlaid onto chain-mesh surface-rendered Vx2 tumor volume.

Figure 6.

Effect of zoledronic acid (ZA) and $\alpha_v\beta_3$ -targeted fumagillin prodrug nanoparticle ($\alpha_v\beta_3$ -Fum-PD NP) therapy on microvasculature (CD31+) in the Vx2 rabbit tumor model. A) Percent CD31+ stain quantified from tumor immunohistochemistry (n=4, * p <0.05). The area of CD31+ stain was normalized to the total tumor area in the region of interest B) Representative images of CD31+ staining, scale bar = 0.5mm. C) Number of PCNA+ cells in Vx2 tumor tissue by immunohistochemistry (n=4). The number of PCNA expressing cells was normalized to the total area of each tumor. D) Representative images of PCNA stained tumors, scale bar = 11mm (* P <0.05).

Figure 7.

Characterization of Vx2 major pathological features. A) Analysis of relative tumor cell (pan-cytokeratin) and macrophage (CD11b) cell population distribution in the Vx2 tumors using flow cytometry. B) Von Kossa stained (black) microcalcifications in control Vx2 tumor, scale bar = 250µm (top) and 100µm (bottom) and C) in excised human breast cancers, scale bar = 100µm. D) mRNA expression of macrophage marker CD68 in Vx2 tumors by qPCR (* $p < 0.05$). E) Number of CD11b+ cells per field of view (FOV) at the tumor rim in the Vx2 tumor tissue by immunofluorescence ($n = 4$, * $p < 0.05$). F) Representative images of CD11b+ staining, scale bar = 100µm.