Endothelin Inhibition Potentiates Cancer Immunotherapy Revealing Mechanical Biomarkers Predictive of Response

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Immunotherapy efficacy depends on T cell trafficking to tumors and migrating to malignant cells to kill them. One barrier to T cell homing is the tumor blood vessel wall, which inhibits T cell attachment and transmigration through the endothelin B receptor, but antagonizing this receptor has not led to a clinically approved drug. One reason may be tumor hypo-perfusion, which limits the area of perfused vessels for T cell attachment. If collapsed vessels can be decompressed and re-perfused by alleviating tumor stiffness, then endothelin B receptor antagonism can improve immunotherapy. Here, it is tested whether the nonselective endothelin receptor blocker, bosentan, by simultaneously interfering with endothelin A receptor induced fibrosis, can normalize the tumor microenvironment thereby acting as a “mechanotherapeutic.” Tumor stiffness is monitored with ultrasound elastography and nanomechanical properties with atomic force microscopy to find an optimal dose, which reprograms cancer-associated fibroblasts resulting in reduced collagen thereby decompressing vessels. Through this mechanism, T cell association with tumor vessels increases and immunosuppressive hypoxia is reduced. Additionally, bosentan increases the CD8+ T cells proliferating fraction. Ultrasound stiffness measurements correlate well with response to immunotherapy, suggesting the potential role of ultrasound elastography as a predictive biomarker of response to immune checkpoint inhibitors.

1. Introduction

Although immune checkpoint blockade (ICB) has revolutionized treatment for many types of solid tumors, it is currently estimated to benefit less than 20% of cancer patients.\[^1\] Increasing the fraction of patients responding and the length of their response is an urgent unmet clinical need. One major factor impairing ICB efficacy is the lack of T cells in the tumor parenchyma.\[^2,3\] Antitumor T cells must traffic into tumors through blood vessels, bind the endothelium, pass across the vessel wall, and migrate through cancer-associated fibroblasts (CAFs) and extracellular matrix (ECM) before encountering cancer cells.\[^4\] While antagonism of the endothelin B receptor (ET\(_B\)R) increases T cell adhesion to the vessel wall resulting in increased homing to tumors,\[^5\] the accessible area of vessel wall for T cells to migrate across is limited because up to 95% of intratumoral blood vessels are collapsed\[^6-7\] with up to 80% completely lacking perfusion.\[^8\]

Tumors with stroma characterized by excessive amounts of CAFs and ECM, which is in part caused by signaling of the peptide endothelin 1 (ET-1) through the endothelin A receptor (ET\(_A\)R),\[^9-11\] become stiff as they grow and have elevated solid tissue pressure, which compresses vessels impairing blood flow and oxygen delivery.\[^12-15\] Hypo-perfusion and hypoxia mediate resistance to immunotherapy by reducing immune cell trafficking, migration, and function.\[^16\] Strategies that reduce tumor stiffness to decompress vessels by reprogramming CAFs to produce and maintain less ECM potentiate ICB efficacy in ICB-resistant mouse models of metastatic breast cancer.\[^17-19\] If there was a way to decompress tumor vessels while also facilitating vessel adhesion and transmigration of T cells into the tumor parenchyma, the fraction of cancer patients that respond to ICB could increase. To this end, here we identified the nonselective endothelin receptor blocker bosentan as a “mechanotherapeutic”\[^16,20\] capable of reducing stiffness thereby decompressing vessels and also inhibiting signaling that prevents T cells from adhering to tumor vessel walls. In measuring tumor tissue mechanical properties, we found that only moderate doses of daily bosentan could reduce stiffness and hypoxia. Administering ICB after bosentan resulted in breast tumor growth...
delay and enhanced survival in murine models of spontaneous metastasis. Thus, therapies that both reduce tumor stiffness and the barriers to T cell infiltration can increase response rates and survival in ICB-resistant cancer. Translating this strategy to patients is facilitated by the extensive knowledge of bosentan use and safety, its low cost, and the potential use of increased tumor blood flow,[21,22] reduced tissue stiffness, and/or alleviated hypoxia as predictive biomarkers of response.[23] We demonstrate here that response to bosentan and ICB combination therapy is related to ultrasound-based measurements of tissue stiffness.

2. Results

2.1. Bosentan Reduces Tumor Tissue Stiffness and Collagen Levels in a Dose-Dependent Manner

$E_{TAR}$ antagonism inhibits fibroblast activation and collagen synthesis in various disease settings,[9–11] so we hypothesized that bosentan could reduce tumor tissue stiffness. To test this, we treated mice bearing syngeneic, orthotopic triple negative breast cancer with a range of sub-therapeutic doses[24] (i.e., 0.2, 1, 5, and 10 mg kg$^{-1}$) for a period of 10 days and measured tissue stiffness noninvasively and longitudinally using ultrasound elastography (Figure 1A,B). No dose affected tumor growth rate (Figure S1, Supporting Information). We found that a moderate dose of 1 mg kg$^{-1}$ daily bosentan reduced tissue stiffness the most as measured by ultrasound elastography in both E0771 and 4T1 breast tumors (Figure 1C,D). While all doses of bosentan reduced stiffness in E0771, the highest doses (i.e., 5 and 10 mg kg$^{-1}$) were not effective in 4T1. Furthermore, we measured the Young’s modulus distribution at the microscopic, tissue microstructure scale by scanning entire tissue samples with atomic force microscopy (AFM) to derive the nanomechanical fingerprints of the tissue. Low Young’s modulus values in the histograms are attributed to the contribution of cancer cells and higher values to the contribution of collagen[25] (Figure 2A,B and Figure S3, Supporting Information). Control tumors had both contributions from cancer cells and collagen, tumors treated with 1 mg kg$^{-1}$ had a large contribution from cancer cells and little contribution from collagen, and tumors treated with 10 mg kg$^{-1}$ had a heterogenous collagen contribution (Figure S3, Supporting Information). By averaging the microscopic tissue stiffness measurements of the tumor samples, we found that all doses reduced the Young’s modulus in E0771 but the highest dose was ineffective in 4T1 (Figure 2C,D). Over-all, the microscopic AFM data agree with the macroscopic ultrasound elastography measurements indicating that the 1 mg kg$^{-1}$ bosentan dose can effectively reduce tumor stiffness.
Given these changes in stiffness and previous studies demonstrating reduced collagen synthesis by fibroblasts with bosentan treatment,[11] we assessed collagen I and Picosirius red staining (Figure 2E,F) and anti-collagen I antibody (Figure 2G,H). In both cases, only 1 mg kg⁻¹ daily bosentan resulted in reduced collagen I levels compared to control, but hyaluronan levels were unchanged (Figure S4, Supporting Information). We found that, in collagen I levels, we hypothesized that CAFs were reprogrammed with 1 mg kg⁻¹ bosentan. Indeed, α-smooth muscle actin (αSMA) protein expression, which is a marker of fibroblast activation, was reduced with 1 mg kg⁻¹ bosentan (Figure S5A,B, Supporting Information) and the mRNA expression of ACTA2, the encoding gene for αSMA, was also reduced. While αSMA is a broad marker of CAFs and certain perivascular cells, fibroblast activation protein (FAP) identifies particularly immunosuppressive CAFs. We found that mRNA expression of FAP was decreased with 1 mg kg⁻¹ bosentan. As CAFs produce ET-1, we hypothesized and confirmed that ET-1 mRNA expression was reduced in bosentan-treated tumors (Figure S5C, Supporting Information). Therefore, we concluded that 1 mg kg⁻¹ bosentan reprograms CAFs thereby reducing ECM levels and tumor stiffness.

2.2. Bosentan Alleviates Hypoxia and Interstitial Fluid Pressure in a Dose-Dependent Manner

Because reducing tissue stiffness by lowering collagen I levels in tumors decompresses collapsed vessels leading to increased perfusion and hypoxia,[12] we hypothesized that bosentan treatment would decompress vessels resulting in reduced hypoxia. First, we confirmed using immunofluorescence experiments that a higher fraction of tumor vessels had an open lumen (Figure S6, Supporting Information), which is a marker of vessel decompression.[3] Next, we assessed hypoxia as the fraction of tissue area positive for pimonidazole adducts using immunofluorescence staining (Figure 3A-E). Indeed, we found hypoxia to be significantly reduced with bosentan treatment particularly at 1 mg kg⁻¹ in both E0771 and 4T1 models. Given that endothelin receptor signaling plays a role in tumor angiogenesis,[26] we tested whether bosentan has antiangiogenic effects at these doses (Figure S7, Supporting Information). We found no evidence of vessel prun- ing (Figure S7B, Supporting Information), however there was increased pericyte coverage with 1 mg kg⁻¹ bosentan (Figure S7C, Supporting Information) thereby indicating increased pericyte recruitment, which is a potential predictive marker of response in breast cancer.[27] Additionally, increased pericyte coverage reduces plasma leakage from tumor vessels, while extravascular fluid movement and draining is increased by lower ECM levels through lymphatic vessel decompression[12] and hydraulic conductivity,[28] respectively. For this reason, we hypothesized and confirmed that interstitial fluid pressure (IFP) is reduced in both E0771 and 4T1 tumor models with bosentan treatment (Figure 3F,G). This is important because IFP elevation is a hallmark of tumor patho-physiology and a major barrier to the delivery of macromolecules, antibodies, and nanoparticles to tumors.[29,30]

Thus, daily 1 mg kg⁻¹ bosentan decompresses tumor vessels and induces pericyte recruitment thereby alleviating hypoxia and lowering IFP.

2.3. Bosentan Increases T Cell Association with Blood Vessels

After confirming that daily 1 mg kg⁻¹ bosentan treatment for 10 days results in decompression of the vessels and improved oxygenation, indicating an increase in tumor perfusion, we hypothesized that there would be more association between T cells and endothelial cells. To test this, we stained for the pan T cell marker CD3 and the endothelial cell marker CD31 (Figure 4A). We assayed the fraction of colocalization of CD3⁺ and CD31⁺ area as a fraction of total CD3¹⁺ area. We found that only 1 mg kg⁻¹ daily bosentan increased the colocalization of T cells and endothelial cells (Figure 4B) without affecting the area fraction of T cells (Figure 4C) or endothelial cells (Figure 4D). Additionally, this treatment modified the spatial distribution of T cells as there was a higher fraction of T cells in the center of the tumor rather than excluded in the periphery (Figure 4E). We confirmed these findings at an mRNA expression level using real-time quantita-tive polymerase chain reaction of tumor lysates from mice treated with 1 mg kg⁻¹ bosentan (Figure 4F). We found that Glut1 was reduced indicating reduced hypoxia, while vascular adhesion molecules like ICAM1, VCAM1, and P-selectin were increased indicating increased expression of molecules that T cells could use to adhere to the vessel wall. We further noticed that mRNA expression of these adhesion molecules returned to normal val- ues when bosentan was administered for 13 days.

These findings are consistent with the hypothesis that enhanc-ing perfusion increases the amount of tumor vascular surface area for T cells to adhesion, especially with ETₐR inhibition.

2.4. Bosentan Increases T Cell Activity

After investigating the effects of bosentan on T cell spatial dis-tribution, we next assessed T cell function. The immunohis-tochemical analysis of 4T1 tissue sections revealed high accumu-lation of CD8⁺ T cells in ICB and bosentan-ICB treatment groups, while no change was observed after bosentan monotherapy (Figure 5A-C). This high accumulation of CD8⁺ associates

Figure 2. Daily bosentan at 1 mg kg⁻¹ alleviates microscopic stiffness and reduces collagen I levels. A,B) Representative AFM nanomechanical fingerprint histograms of control and 1 mg kg⁻¹ bosentan-treated 4T1 and E0771 tumors. The large peak on the left side of the graph is due to the contribution of the compliant cancer cells, while the tail in the blue box is due to the contribution of the stiffer components, such as collagen. C) Averaged Young’s modulus values from AFM measurements in E0771 tumors. D) Averaged Young’s modulus values from AFM measurements in 4T1 tumors. E) Quantification of the fraction of area positive for Picosirius red staining in E0771 tumors normalized to the control group. F) Representative images of Picosirius red staining in E0771 tumors. Scale bar represents 1 mm. G) Quantification of the fraction of area positive for collagen I staining in 4T1 tumors. H) Representative images of collagen I staining in 4T1 tumors. Scale bar represents 200 μm. Statistical analyses were performed by comparing the treated groups with the control * and the 1 mg kg⁻¹ with all other treatment groups **, p ≤ 0.05, determined by t-test. Data presented as mean ± SEM (n = 4 mice per group, N = 2 image fields per mouse for ultrasound measurements and n = 4 mice per group, N = 3–5 image fields for immunohistochemistry studies, for AFM n = 3 mice per group, N = 10–15 different 20×20 μm² force maps, 16×16 point grids).

Figure 4. A,B) Representative AFM nanomechanical fingerprint histograms of control and 1 mg kg⁻¹ bosentan-treated 4T1 and E0771 tumors. The large peak on the left side of the graph is due to the contribution of the compliant cancer cells, while the tail in the blue box is due to the contribution of the stiffer components, such as collagen. C) Averaged Young’s modulus values from AFM measurements in E0771 tumors. D) Averaged Young’s modulus values from AFM measurements in 4T1 tumors. E) Quantification of the fraction of area positive for Picosirius red staining in E0771 tumors normalized to the control group. F) Representative images of Picosirius red staining in E0771 tumors. Scale bar represents 1 mm. G) Quantification of the fraction of area positive for collagen I staining in 4T1 tumors. H) Representative images of collagen I staining in 4T1 tumors. Scale bar represents 200 μm. Statistical analyses were performed by comparing the treated groups with the control * and the 1 mg kg⁻¹ with all other treatment groups **, p ≤ 0.05, determined by t-test. Data presented as mean ± SEM (n = 4 mice per group, N = 2 image fields per mouse for ultrasound measurements and n = 4 mice per group, N = 3–5 image fields for immunohistochemistry studies, for AFM n = 3 mice per group, N = 10–15 different 20×20 μm² force maps, 16×16 point grids).
Figure 3. Daily bosentan at 1 mg kg\(^{-1}\) alleviates hypoxia and reduces interstitial fluid pressure. A) Representative images of pimonidazole (hypoxia) staining in 4T1 tumors. Scale bar represents 1 mm. B) Quantification of hypoxic area fraction in 4T1 tumors. C) Representative images of pimonidazole staining in E0771 tumors. D) Quantification of hypoxic area fraction in E0771 tumors. E) Quantification of mRNA expression of hypoxia related genes in 4T1 tumors. F,G) Measurement of the interstitial fluid pressure (IFP) in F) 4T1 and G) E0771 tumors after 10 days of daily bosentan treatment. In both cell lines, 0.2, 1, and 5 mg kg\(^{-1}\) doses of bosentan reduce fluid pressure. Statistical analyses were performed by comparing the treated groups with the control * and the 1 mg kg\(^{-1}\) with all other treatment groups **, \(p \leq 0.05\), determined by \(t\)-test. Data presented as mean ± SEM, \(n = 5\) mice per group and \(N = 3–5\) image fields for immunohistochemistry studies.

with improved cytotoxic responses as indicated by the expression of granzyme B (GzmB) protein (Figure 5B,D). Taking into consideration the recently proposed role of T cell proliferation as a biomarker for response to immunotherapy,\textsuperscript{31–33} we examined the population of Ki67\(^+\) CD8\(^+\) T cells to study their functionality. We found that bosentan plus ICB treated tumors contain a significantly higher fraction of Ki67\(^+\) CD8\(^+\) T cells further supporting the role of mechanotherapeutics in potentiating the response to immunotherapy through increased proliferation of CD8\(^+\) T cells (Figure 5E,F).
Figure 4. Daily bosentan at 1 mg kg\(^{-1}\) increases association of T cells with blood vessels independently of angiogenesis. A) Representative images of colocalization of CD\(^3\)\(^+\) T cells and CD31\(^+\) endothelial cells in 4T1 tumors. Scale bar represents 200 µm. B) Quantification of colocalization between CD3\(^+\) T cells and CD31\(^+\) endothelial cells in 4T1 tumors. C) Quantification of fraction of CD3\(^+\) area in 4T1 tumors. D) Quantification of fraction of CD31\(^+\) area in 4T1 tumors. E) Ratio of CD3\(^+\) area normalized to DAPI in the tumor periphery to the tumor center in 4T1 tumor sections. F) Quantification of mRNA expression of genes related to adhesion molecules in 4T1 tumors. Statistical analyses were performed by comparing the treated groups with the control* and the 1 mg kg\(^{-1}\) with all other treatment groups**, \(p \leq 0.05\), as determined by t-test. Data presented as mean ± SEM (\(n = 4\) mice per group, \(N = 3–5\) image fields for CD3 and CD31 immunohistochemistry studies).

2.5. Bosentan Pretreatment Potentiates ICB Efficacy in Resistant Metastatic Breast Cancer

Given that alleviating hypoxia and enhancing T cell transmigration promote antitumor immunity, we hypothesized that the 1 mg kg\(^{-1}\) daily regimen of bosentan, which we identified as the optimized dose, could enhance the efficacy of an ICB cocktail of anti-PD-1 and anti-CTLA-4 antibodies. To test this, we treated mice bearing primary tumors similar to the neo-adjuvant setting, in that we administered therapy before removing the
Figure 5. Bosentan potentiates the antitumor efficacy of ICB by the recruitment of actively proliferative CD8+ T cells. A-B) Representative images of 4T1 paraffin tissue sections stained for CD8 (green) and GzmB (red). Arrowheads indicate CD8+ T cells expressing GzmB (yellow) as a measure of cytotoxicity. Scale bar represents 100 µm. C) Quantification of the fraction of area positive for CD8 staining per total area positive for DAPI signal, all values normalized to the control average. D) Quantification of the cytotoxic CD8+ T cell fraction of area positive for both GzmB and CD8 staining per total area positive for CD8 signal, all values normalized to the control average. E) Representative fluorescence image of a 4T1 tumor section stained with Ki67 proliferation marker (red), CD8 (green), and DAPI (blue). Arrowheads point to the proliferative CD8+ T cells (yellow). Scale bar represents 100 µm (n = 4 mice, N = 3–5 image fields per mouse). F) Quantification of the proliferative CD8+ T cell fraction of area positive for both Ki67 (red) and CD8 (green) signal per total area positive for CD8 signal, all values normalized to the control average. Data presented as mean ± SEM. Statistical analyses were performed by comparing the treated groups with the control *, and bosentan-ICB with all other treatment groups **, p ≤ 0.05 as determined by t-test.

primary tumors surgically to assess mice survival against spontaneous metastases that arose on treatment (Figure 6A). We found that consistent with previous reports, E0771 and 4T1 are resistant to ICB,[3,17,18] but in both tumor models bosentan potentiated primary tumor growth inhibition (Figure 6B,C) and median survival increases after primary tumor excision (Figure 6D,E). In the E0771 study, eight out of ten mice initiated survived, so we sacrificed three mice and found no evidence of macrometastases in their lungs (Figure S8, Supporting Information). The remaining five mice bearing E0771 tumors were rechallenged with a second inoculation of E0771 cells and the tumor growth rates were compared against five healthy, age-matched control mice. Tumors grew in all five control mice but in only two out of the five combination-treated mice that survived the initial experiment (Figure S9, Supporting Information). The two tumors that grew were less than one-third the volume of the smallest tumor grown in a control mouse. Thus, bosentan pretreatment potentiated the efficacy of an ICB cocktail of anti-PD-1 and anti-CTLA-4 antibodies in two ICB-resistant mouse models of metastatic triple negative breast cancer. Finally, we hypothesized that the ultrasound elasticity measurements correlate with the tumor response to ICB. We found that the Young’s modulus of the tumors measured before the initiation of ICB treatment positively and statistically significantly correlated with tumor volume across ICB cocktail monotherapy and combination of bosentan and ICB cocktail treatment groups in E0771 (Figure 6F) and 4T1 tumors.
combination with ETBR using selective inhibitors for both receptor trafficking,[3,17,36,37] are well established in the literature. Here, we also tested whether the addition of ICBr to normalizing 1 mg kg$^{-1}$ bosentan treatment affects stiffness but we did not observe any effect of ICBr on stiffness on control or bosentan-treated tumors (Figure S10, Supporting Information).

3. Discussion

This study highlights the importance of normalizing the TME and increasing tumor vessel functionality and oxygenation for potentiating ICBr efficacy.[16,23] The connections between stiffness and collagen in tumors,[14] collagen and vessel compression in tumors,[8,12] perfusion and hypoxia[8,35] and perfusion and T cell trafficking[1,17,36,37] are well established in the literature. Here, we proposed repurposing bosentan, a nonselective endothelin receptor blocking drug with decades of safe use, to enhance efficacy of ICBr by normalizing the TME and increasing T cell adhesion to the tumor vasculature. The effective daily dose that we determined using ultrasound elastography and AFM reduced tumor fibrosis thereby decompressing vessels and decreasing hypoxia. This finding is consistent with other studies demonstrating reduced fibrosis in various disease settings including cancer after blocking ET$\alpha$R.[9–11] We also observed the most T cell adhesion with the tumor vasculature at the bosentan dose that reduced tumor hypoxia, suggesting that there were more perfused vessels and thus, more vascular endothelium accessible to trafficking T cells. Future work should measure stiffness and perfusion simultaneously, using ultrasound elastography and contrast-enhanced ultrasound to further support the conclusion that the changes in the tumor microenvironment we observe are a result of tissue stiffness. Future studies should also focus on investigating the contribution of perfusion versus adhesion molecules to T cell homing in the tumor by blocking specific adhesion molecules that are upregulated by bosentan.

Previous reports indicated that endothelin signaling through ET$\alpha$R is necessary to stimulate immune cell recruitment to tumors while blocking ET$\beta$R. In these studies, blocking ET$\beta$R in combination with ET$\alpha$R using selective inhibitors for both receptor or the dual inhibitor macitentan negates any potential increases to immune cell recruitment by blocking expression of vascular adhesion molecules.[38] However, in our current study, we observed increased ICAM1 expression. We hypothesize that our use of bosentan, with less ET$\beta$R selectivity, less sustained receptor binding, and less tissue distribution than macitentan, could reduce the negative effects of ET$\beta$R antagonism on immune cell recruitment.[39] Additionally, the differences between our studies underscore the complexity and heterogeneity in endothelin receptor expression across tumor types and cell types.[40]

Regardless, we propose that titrating the bosentan dose to normalize the TME (i.e., ameliorate tissue stiffness, hypo-perfusion, hypoxia, and fluid pressure) was crucial to increasing immune cell recruitment and function through various mechanisms that outweigh any potential negative effects of ET$\beta$R antagonism on recruitment. Furthermore, because depleting CAFs[41,42] and ECM[43] from the tumor can induce disease progression through alternative signaling pathways, we hypothesize that the 10 mg kg$^{-1}$ dose could have induced such an effect causing “rebound fibrosis.”

Mechanotherapeutics that reduce fibrosis in tumors resulting in normalized tissue and fluid mechanics, have high potential to increase the efficacy of immunotherapies.[3,16–20,35] The development of losartan[83] is the most advanced clinically, since addition of losartan to chemoradiation resulted in an improvement in efficacy in locally advanced pancreatic ductal adenocarcinoma patients[44] and is currently being investigated with chemo- and the ICB nivolumab in the same setting (Clinical-Trials.gov identifier: NCT03563248). One advantage of losartan is that, besides acting as a mechanotherapeutic, it reduces immunosuppression by inhibiting TGF$\beta$ signaling and monocyte recruitment to metastases.[45] Here, we show that bosentan is similar, in that it acts as a mechanotherapeutic but also reduces the barrier to T cell homing by antagonizing ET$\beta$R.[3] Also, we show that neither CD3 nor CD8 intratumor densities are changed with bosentan monotherapy, but the spatial distribution is altered. Furthermore, the combination of bosentan with immune checkpoint inhibitors increases the density of CD8$^+$ T cells, stimulates the proliferation and cytotoxic capability of CD8$^+$ T cells. In addition, previous studies have demonstrated that endothelin B receptor blockers can increase the trafficking of both endogenous and adoptively transferred T cells[83] and thus, use of bosentan and the increased T cell trafficking could also work for adaptively transferred T cells. Overall, the preclinical and clinical data

(Figure 6G). The results suggest that tumor response to therapy is noticeably increased when stiffness values drop below 20 kPa for the tumor models considered. These results are consistent with the notion that bosentan-induced normalization of the mechanical tumor microenvironment (TME) can be monitored with ultrasound elastography and suggest that stiffness measurements with ultrasound should be investigated as predictive biomarkers of response to ICBr. We also tested whether the addition of ICBr to normalizing 1 mg kg$^{-1}$ bosentan treatment affects stiffness but we did not observe any effect of ICBr on stiffness on control or bosentan-treated tumors (Figure S10, Supporting Information).
of repurposing bosentan and losartan underscore the potential of “immune-mechanotherapeutics” that both normalize the tumor mechanical microenvironment and inhibit pro-tumor immunosuppressive signaling to increase the efficacy of immunotherapies in solid tumors.

Given the preclinical studies demonstrating that increasing blood flow (and perfusion) and alleviating hypoxia using mechanotherapeutics potentiate the efficacy of immunotherapy,[16,23,37,46,47] perfusion and hypoxia should be evaluated prospectively as potential biomarkers of response to combinations of mechanotherapeutics with ICB and other immunotherapies. Here, we show that tumor stiffness measured with ultrasound elastography systems, similar to those commonly used clinically, correlates with response to ICB. Therefore, we propose that the potential of tumor stiffness to act as an “immunopredictor,” a biomarker of response to ICB, should be investigated.

4. Experimental Section

**Cell Culture:** 4T1 (ATCC CRL-2539) and E0771 (94A001, CH3 Biosystems) mouse breast adenocarcinoma cell lines were purchased from ATCC and CH3 BioSystems, respectively. The cells were maintained at 37 °C/5% CO2 in Roswell Park Memorial Institute medium (RPMI-1640, LM-R1637, biosera) supplemented with 10% fetal bovine serum (FBS, FB-1001H, biosera) and 1% antibiotics (A5955, Sigma).

**Drugs and Reagents:** Bosentan Hydrate (S3051, Selleckchem) was dissolved in ddH2O containing 2% dimethyl sulfoxide (GK2245, Glentham Life Science), 30% PEG300 (S6704, Selleckchem), and 2% Tween 80 (S6702, Selleckchem). The ICBS mouse monoclonal PD-1 (CD279, clone RMPI-14) and mouse monoclonal CTLA-4 (CD152, clone 9D9) antibodies were purchased from BioXCell.

**Syngeneic Tumor Models and Treatment Protocols:** Orthoptopic models for murine mammary tumors were generated by implantation of 5 × 10⁶ 4T1 or E0771 cancer cells in 40 μL of serum-free medium into the third mammary fat pad of 6–8 week-old BALB/c and C57BL/6 female mice, respectively. Mice were purchased from the Cyprus Institute of Neurology and Genetics and all in vivo experiments were conducted in accordance with the animal welfare regulations and guidelines of the Republic of Cyprus and the European Union (European Directive 2010/63/EE and Cyprus Legislation for the protection and welfare of animals, Laws 1994–2013) under a license acquired and approved (No CY/EXP/PR.L2/2018, CY/EXP/PR.L14/2019, CY/EXP/PR.L15/2019, CY/EXP/PR.L03/2020) by the Cyprus Veterinary Services committee, the Cyprus national authority for monitoring animal research for all academic institutions.

For the dose–response studies, bosentan 0.2, 1, 5, 10 mg kg⁻¹ or equal volume of diluent (control group) was administered by intraperitoneal injection (i.p.) once a day for 10 days, starting from once the E0771 and 4T1 tumor volumes reached an average size of 90 and 100 mm³, respectively. E0771 tumors were excised when they reached an average size of 500 mm³ (day 20) and 4T1 tumors when they reached an average volume of 450 mm³ (day 19).

For the combinatorial treatment with bosentan and immunotherapy studies, bosentan 1 mg kg⁻¹ or equal volume of diluent (control group) was administered by intraperitoneal injection (i.p.) once a day for 14 days, starting from once the tumor volumes reached an average size of 5 mm diameter. Immunotherapy was administered as a cocktail of 10 mg kg⁻¹ anti-PD-1 (CD279, clone RMPI-14, BioXCell) and 5 mg kg⁻¹ anti-CTLA-4 (CD152, clone 9D9) following dilution in the recommended InvivoPure pH 7.0 Dilution Buffer (BioXCell).[1,17,18] The immunotherapy cocktail was administered i.p. when E0771 tumors reached an average size of 200 mm³ every 3 days (day 15, 18, 21) for three doses.[18] Mice bearing 4T1 tumors received the immunotherapy cocktail once the tumors reached an average size of 300 mm³ on day 15, 18, and 21. For the immunotherapy studies, animals were also treated with a nontargeting isotype control antibody (BioXCell). E0771 and 4T1 primary tumors were excised when they reached an average size of 600 and 650 mm³, respectively, and the tissue was sutured for the study of the metastatic tumors.

**Planar dimensions (x,y) of tumors were monitored every 2–3 days using a digital caliper and tumor volume was estimated from the volume of an ellipsoid and assuming that the third dimension, z, is equal to √xyz. For the overall survival studies, the ending point was the time to mouse death.**

**Ultrasound Elastography Measurements:** To evaluate the elastic properties of tumors, shear wave elastography was used on a Philips EPIQ Elite Ultrasound scanner with an el18-4 linear array which was approved for clinical scanning. The method generated a 2D elastic modulus color map after applying a shear wave in the tissue with an acoustic push pulse.[43] When performing the elastography imaging, a confidence map highlighting areas of optimal shear wave propagation was produced providing an indication of quality across the stiffness value map. Therefore, elastic modulus values of the areas of the region of interest (ROI) with the highest shear wave quality (Figure S11, Supporting Information) were obtained. The elastic modulus value presented in the results was the average within the tumor region. For the dose–response studies, shear wave imaging of 4T1 tumors was performed prior to bosentan treatment, on day 3, 6, and 9 post-treatment, while imaging of E0771 tumors was performed prior to bosentan administration, on day 3, 7, and 10 post-bosentan treatment. To assess the combined effect of bosentan and ICBS on tissue elasticity, ultrasound was performed prior to any treatment and tumor removal.

**Nanomechanical Properties Measurements using AFM:** During the dose–response studies (bosentan 0.2, 1, 5, 10 mg kg⁻¹), tumors were excised when they reached an average size of 500 mm³. AFM studies were performed using appropriately modified previously published protocols.[51] More specifically, after tumor harvesting, tissue biopsies were obtained with an automatic biopsy tool (16G, MEDAX) and the samples were immediately transferred into ice-cold phosphate-buffered saline (PBS) supplemented with a protease inhibitor cocktail (Complete Mini, Roche Diagnostics GmbH, 1 tablet per 10 mL).[43,50] Then each specimen was immobilized on a 35 mm plastic cell culture petri dish with a thin layer of two-component fast drying epoxy glue. The petri dish was filled with PBS supplemented with a protease inhibitor cocktail and stored at 4 °C to avoid tissue degradation.[49,50] AFM measurements were performed with an automated AFM system (Molecular Imaging-Agilent PicPlus AFM) were performed 1–72 h post tumor removal, so as to prevent any alterations in stiffness profiles.[49,50] The measurements were conducted with silicon nitride cantilevers (MLCT-Bio, cantilever D, Bruker Company with a nominal force constant of 0.02 N/m, resonance frequency in air: 15 KHz). The maximum applied loading force was set to 1.8 nN, the exact spring constant k of the cantilever was determined before each experiment using the thermal tune method and the deflection sensitivity was determined in fluid using petri dishes as an infinitely stiff reference material.[51] AFM measurements were performed by recording 10–15 different 20 × 20 μm² force maps (16 × 16 point grids) per specimen, which were corresponded to 256 force–displacement curves per map (up to 3840 force–displacement curves per specimen) with pixel size of 1.25 μm. Also, for higher spatial resolution, 32 × 32 force–volume maps (1024 force–displacement curves per map and a pixel size of 0.625 nm) were acquired. The collected force maps were analyzed by Atomic[52] so as to calculate the sample’s Young’s modulus using the Hertz model.

**Intestinal Fluid Pressure (IFP):** IFP was measured in vivo using the previously described wick-in-needle technique after mice were anesthetized with i.p. injection of Avertin and prior to tumor excision.[12,53] **Fluorescent Immunohistochemistry:** Tumors were removed, washed twice in 1x PBS for 10 min, and incubated with 4% perfluoroalkoxy alkanes overnight at 4 °C. The fixative was aspirated, and samples were washed twice in 1x PBS for 10 min. Fixed tissues were embedded in optimal cutting temperature compound in cryomolds (Tissue-Tek) and frozen completely at −20 °C. Transverse 30 μm thick tumor sections were produced using the Tissue-Tek Cryo3 (SAKURA). Positively charged HistoBond microslide slides (Marienfeld) were used to bond four tissue sections per...
tumor. Tumor sections were then incubated in blocking solution (10% fetal bovine serum, 3% donkey serum, 1x PBS) for 2 h and immunostained following incubation with primary antibodies; rabbit anti-Collagen I (ab4710, Abcam 1:100), rabbit anti-CD31 (ab28364, Abcam 1:50), and rat anti-CD3 (17A2, BioLegend 1:50) overnight at 4 °C. Secondary antibodies against rabbit or rat conjugated to Alexa Fluor 488 or 647 (Invitrogen) were used at 1:400 dilution. All samples were incubated in secondary antibody solution including 4',6-diamidino-2-phenylindole (DAPI, Sigma, 1:100 of 1 mg mL⁻¹ stock) for 2 h at room temperature (RT) in the dark. Sections were mounted on microscope slides using the ProLong gold antifade mountant (Invitrogen) and covered with a glass coverslip.

**T Cell Proximity to Tumor Vessels:** To demonstrate the presence of T cells in the proximity of tumor vessel capillaries, tissue cryosections of 4T1 and E0771 primary tumors were incubated with primary rabbit anti-CD31 (ab28364, Abcam 1:50) and rat anti-CD3 (17A2, BioLegend 1:50) overnight at 4 °C. CD31 signal was detected with Alexa Fluor-488 anti-rabbit IgG (H+L) (A21247, Invitrogen) and covered with a glass coverslip. Tumor-associated T cell and vessel content were determined by the CD31⁺ and CD3⁺ area fraction normalized to DAPI staining.

**Statistical Analysis:** Data were presented as means with standard errors. Groups were compared using Student’s t-test to study statistical significance. Statistical analyses were performed by comparing the treated and untreated groups with two asterisks, **). Unless otherwise was noted. A p-value of less than 0.05 was considered statistically significant. Statistical analysis was carried out using GraphPad Prism 9 Software.

**Methods for collagen, hyaluronan, pericyte coverage, and hypoxia tissue staining as well as histological image acquisition and RT-PCR analysis are detailed in the Supporting Information.**

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

C.V., M.P., F.M., A.S., J.D.M., and T.S. are named as inventors on the provisional patent application No. 63/124448. C.M and M.A.A. declares no competing interests.

**Data Availability Statement**

Research data are not shared.

**Keywords**

bosentan, endothelin receptor blockers, immune checkpoint blockers, tumor microenvironment, ultrasound elastography

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