Identification of Bone-Derived Factors Conferring De Novo Therapeutic Resistance in Metastatic Prostate Cancer

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Abstract

Resistance to currently available targeted therapies significantly hampers the survival of patients with prostate cancer with bone metastasis. Here we demonstrate an important resistance mechanism initiated from tumor-induced bone. Studies using an osteogenic patient-derived xenograft, MDA-PCA-118b, revealed that tumor cells resistant to cabozantinib, a Met and VEGFR-2 inhibitor, reside in a “resistance niche” adjacent to prostate cancer-induced bone. We performed secretome analysis of the conditioned medium from tumor-induced bone to identify proteins (termed “osteocrines”) found within this resistance niche. In accordance with previous reports demonstrating that activation of integrin signaling pathways confers therapeutic resistance, 27 of the 90 osteocrines identified were integrin ligands. We found that following caboabzatinib treatment, only tumor cells positioned adjacent to the newly formed woven bone remained viable and expressed high levels of pFAK-Y397 and pTalin-S425, mediators of integrin signaling. Accordingly, treatment of C4-2B4 cells with integrin ligands resulted in increased pFAK-Y397 expression and cell survival, whereas targeting integrins with FAK inhibitors PF-562271 or defactinib inhibited FAK phosphorylation and reduced the survival of PC3-mm2 cells. Moreover, treatment of MDA-PCA-118b tumors with PF-562271 led to decreased tumor growth, irrespective of initial tumor size. Finally, we show that upon treatment cessation, the combination of PF-562271 and caboabzatinib delayed tumor recurrence in contrast to caboabzatinib treatment alone. Our studies suggest that identifying paracrine de novo resistance mechanisms may significantly contribute to the generation of a broader set of potent therapeutic tools that act combinatorially to inhibit metastatic prostate cancer.

Introduction

Development of metastasis in bone is the lethal progression of prostate cancer. Although several targeted therapies have improved survival of patients with bone metastasis (1, 2), resistance to targeted therapy invariably develops (3). A distinct feature of metastatic prostate cancer in bone is the induction of new bone formation, resulting in bone-forming lesions. This unique tumor microenvironment likely contributes to resistance to multiple therapies. However, whether factors from prostate tumor-induced bone are involved in therapy resistance is unknown.

Recently, caboabzatinib, an oral multikinase inhibitor with potent activity against p-MET and p-VEGFR-2, demonstrated striking clinical and radiologic responses in patients with castrate-resistant prostate cancer with bone metastasis (4, 5). However, although caboabzatinib increased progression-free survival, it did not lead to improvement in overall survival in a phase 3 clinical trial. Identifying the mechanism(s) of therapy resistance to caboabzatinib and other current drugs for bone metastasis will lead to the development of strategies to treat this fatal disease.

The current theory whereby patients acquire resistance is adaptation of tumor cells in response to therapeutic agents. However, the possibility that prostate tumor-induced changes in the bone microenvironment may confer *de novo* resistance, i.e., resistance that occurs prior to treatment with any therapy, has not been examined previously. In this study, we used caboabzatinib as a study tool to examine the mechanism(s) by which prostate tumor-induced bone formation confers therapy resistance. Our studies indicate that prostate cancer-induced bone provides a “resistance niche” that allows prostate cancer cells to survive under caboabzatinib treatment and perhaps other targeted therapies.
Materials and Methods

Materials

PC3-mm2 and C4-B4, human prostate cancer cells, were maintained in RPMI 1640. HEK-293T cells were maintained in DMEM. All cell lines were verified by polymorphic short tandem repeat loci profiling and tested negative for mycoplasma infection. The patient-derived xenograft (PDX), MDA-PCA-118b (PCA-118b), was derived from a bone lesion of a patient with castrate-resistant prostate cancer (6). Cabozantinib (XL184) was provided by Exelixis. Antibodies were from commercial sources: pTalin-S425 (ECM), Talin (Millipore), FAK-Y397 (Invitrogen), FAK (Cell Signaling), EpCAM (R&D), AE1/AE3 (Dako), penta-His (Qiagen), mouse osteocalcin, and SPARC (Santa Cruz).

Treatment of PCA-118b tumors with cabozantinib in vivo

PCA-118b PDX was maintained by serial passage in SCID mice as described (7). SCID mice bearing PCA-118b tumors were treated with cabozantinib (20 mg/kg) through oral gavage 2×/day, 6 days/week. Control mice were administered with vehicle. Tumor sizes and bone formation were measured by caliper and X-ray, respectively.

Histology and IHC

PCA-118b tumors were fixed in formalin, decalcified with formic acid, and embedded in paraffin. IHC staining was performed as described (8, 9).

Mass spectrometry analysis of conditioned medium from tumor-induced bone

PCA-118b tumor was digested with Accumax enzyme mixture (eBioscience) and cells filtered through a cell strainer. The remaining bone (tumor-induced bone) was cultured in BGJb medium (Thermo Fisher Scientific) for 48 hours. The conditioned medium from tumor-induced bone (Bone-CM) was analyzed by mass spectrometry (MS) as described (10).

qPCR analysis

Total RNA was prepared using TRIzol and RNeasy Mini Kit (Qiagen) and analyzed by qRT-PCR. Human and mouse integrin primers, HINT-1 and MINT-1, respectively, were purchased from RealTimePrimers. GAPDH was used as a control.

Expression and purification of osteoclines

RNA from MC3T3-E1 cells was used to generate cDNAs for osteoclines using primer sequences listed in Supplementary Table S1. Recombinant osteoclines with His8-tag were expressed from HEK293 cells using pcDNA3.1 vectors and purified from conditioned media using Ni-NTA agarose (Qiagen; ref. 11). cDNAs in pBMN-I-GFP retroviral vector (12) were used to generate C4-2B4 cells stably expressed osteoclines.

Soft agar colony assay

Soft agar colony assays were performed as described with modifications (13). In brief, soft agar colony assay was performed in a 6-well plate using low melting point agarose. The base layer was 0.8% agarose and the top layer was 0.4% agarose containing 75,000 C4-2B4-LT cells per well and 10 μg/mL of lumican, osteopontin, SPARC, BIC-H3, or vitronectin in RPMI medium without serum. The cells were grown in soft agar for 14 days and the number of colonies counted. Similarly, in the soft agar assay for the effect of PF562271 on PC3-mm2 cells, various concentration of PF562271 were added to the top layer of agarose containing 0.1 million cells per well in RPMI containing 0.5% FBS. PF562271 was replenished daily, 5 days per week. The cells were grown for 18 days and the number of colonies counted.

Treatment of PCA-118b tumor with PF-562271 in vivo

Mice with PCA-118b tumors grown for 4 to 6 weeks were grouped according to their tumor sizes as small (~30 mm3), medium (~80 mm3), and large (~190 mm3). In each group of four mice, one was treated with vehicle, one with PF-562271 (33 mg/kg), one with cabozantinib (20 mg/kg), and one with both PF-562271 and cabozantinib, all through oral gavage twice a day at 8- and 16-hour intervals for 5 days per week. Tumor sizes were measured weekly. After 2 weeks, treatments were stopped. Mice in the “large” tumor group were sacrificed. Mice in the “medium” and “small” tumor groups were monitored for tumor regrowth for either 1 week or 3 weeks. In another study, the treatments were initiated after the tumors reached palpable sizes.

Statistical analysis

Data are expressed as the mean ± SD unless otherwise stated. Student t test (two-tailed, paired) was used for statistical analyses.

Results

Cabozantinib inhibits the growth of established osteogenic PCA-118b tumors

To study resistance specifically due to osteoblastic metastases, we use a bone-forming PDX, PCA-118b (6), as a model. PCA-118b has been shown to induce bone formation even when grown subcutaneously (6, 7) and PCA-118b tumor has all the cellular elements present in human bone metastases, including osteoblasts (6).

We first determined whether cabozantinib is effective in inhibiting the growth of established PCA-118b tumors. Mice bearing PCA-118b tumors of various sizes were treated with cabozantinib. Tumor size measurements showed that regression occurred in all four tumors at one week after treatment (Fig. 1A, left). Larger tumors exhibited more pronounced decreases in size (Fig. 1A, left). When calculated by percentage of tumor shrinkage, all four tumors showed decreased tumor volume within one week after cabozantinib treatment (Fig. 1A, right) and these decreases persisted throughout the remaining 3 weeks of treatment (Fig. 1A). The decrease in tumor sizes may be an underestimate of tumor shrinkage as there was tumor-induced bone within the tumors (see Fig. 1B).

Tumor cells adjacent to tumor-induced bone are viable after treatment

To further examine the effect of cabozantinib on PCA-118b-induced bone, X-rays were performed at the onset of treatment and 4 weeks after treatment (Fig. 1B). No significant difference was observed in the shape of the bone present in the PCA-118b tumors (Fig. 1B); however, the bone density appeared somewhat intensified in the cabozantinib-treated group (Fig. 1B, right). In addition, decreases in tumor volume were observed in the X-ray images (Fig. 1B), consistent with the tumor size measurements (Fig. 1A). Histologic analysis showed that cabozantinib treatment induced cell death in large areas of the tumors (Fig. 1C). However, small clusters of viable tumor cells were found, most frequently...
adjacent to the tumor-induced bone. Further, osteoblasts rimming the tumor-induced bone were found to be viable (Fig. 1C), suggesting that PCa-118b-induced osteoblasts are resistant to killing by cabozantinib treatment. IHC staining for osteocalcin (an osteoblast marker) and AE1/AE3 (an epithelial cell marker) showed that the viable tumor cells are located adjacent to osteoblasts (Fig. 1C). Interestingly, PCa-118b tumor cells also express osteocalcin (Fig. 1C), consistent with the reported osteomimicry of prostate cancer cells (14, 15). IHC staining of control untreated PCa-118 tumor is shown in Supplementary Fig. S1. These results suggest that osteoblasts are resistant to cabozantinib and may protect tumor cells adjacent to the tumor-induced bone from cabozantinib therapy.

Tumor recurrence after discontinuation of cabozantinib treatment
To examine whether tumor recurrence may occur after discontinuing cabozantinib treatment, PCa-118b tumors were allowed...
to grow until the tumors reached approximately 500 mm$^3$. One group of mice was left untreated (control) and a second group was treated with cabozantinib. Cabozantinib not only halted tumor growth but also led to reductions in tumor sizes (Supplementary Fig. S2A). Histologic analyses showed that small clusters of viable tumor cells were present along with considerable cell death in cabozantinib-treated tumors at the time cabozantinib treatment was discontinued (Supplementary Fig. S2A). However, tumors resumed growth after treatments were discontinued. In tumors collected 1 or 2 weeks after cabozantinib treatments, robust tumor regrowth was observed that completely replaced the area of cell death from prior cabozantinib treatment (Supplementary Fig. S2B and S2C). IHC analysis showed that cells in the recurrent tumors are positive for AE1/AE3 epithelial cell markers (Supplementary Fig. S2B), indicating regrowth of cabozantinib-resistant tumor cells.

Secretome analysis of conditioned medium from tumor-associated bone

Because small clusters of viable tumor cells were found adjacent to the bone after cabozantinib treatment (Fig. 1C), we hypothesized that prostate tumor-induced osteoblasts secrete factors that induce signaling pathways in tumor cells, leading to therapy resistance. We termed these bone-secreted factors that modulate cellular function in a paracrine fashion as "osteocrines" ("osteo" = bone; "crine" = paracrine). To identify osteocrines, PCA-118b tumor was digested with Accumax enzyme mixture to dissociate tumor cells (Fig. 2A). The conditioned media of tumor-associated bone (Bone-CM) from two independent PCA-118b tumors were resolved by SDS-PAGE (Fig. 2B, left). SPARC (also known as osteonectin), a major bone secreted factor, was detected in Bone-CM (Fig. 2B, right). MS analyses of Bone-CM identified 65 and
75 secretory proteins in each sample. Fifty proteins were found in both samples (Fig. 2C). Together, 90 secretory proteins were identified (Supplementary Table S2). The most frequently identified group of proteins is ligands for integrins (27/90; Supplementary Table S2). We (16) and others (17–20) have shown that integrin activation plays a critical role in prostate cancer cell survival. Additionally, integrin activation has been associated with resistance to chemotherapy, radiotherapy, and targeted agents (21). Thus, the secreted proteins that are integrin ligands are candidate osteocrines that may confer therapy resistance to prostate cancer cells.

We employed mouse-specific primers in qRT-PCR analysis to confirm the expression of several of these integrin ligands, including fibronectin, laminins, SPARC, SPP1 (osteopontin), BIG-H3 (TGFβ induced, TGFβ3), tenascin C (TNC), lumican (LUM), and vitronectin (VTN) in PCa-118b tumors. High levels of messages for mouse SPARC, SPP1, and lumican and medium levels for mouse fibronectin, laminins (LAMA3, B1, and C1), and BIG-H3 were observed (Fig. 2D). Because PCa-118b tumors contain both human PCa-118b cells (22) and mesenchymal cells from mouse osteoblasts, endothelial cells, and fibroblasts, we employed human- and mouse-specific primers to determine the origin of the osteocrines. High levels of mouse SPARC and SPP1 messages were found in PCa-118b tumors but not in purified PCa-118b epithelial cells (Fig. 2E), consistent with previous reports that osteoblasts in PCa-118-induced bone are of mouse origin (6, 7), and the presence of SPARC in the Bone-CM (Fig. 2B). These observations demonstrate that SPARC, SPP1, and lumican are secreted from tumor-associated osteoblasts.

Integrins and integrin ligands in PCa-118b tumors

Because integrin ligands interact with different integrins, we next determined which integrins are expressed in PCa-118b tumor cells. qRT-PCR analysis using human-specific primers for 16α and 8β integrin subunits showed that PCa-118b tumor cells mainly express αv, αv, and β1 integrins (Fig. 3A). Low levels of other integrins, including α1, α2, αv, β1, β3, and β8, were also detected. To confirm that αv is expressed in PCa-118b tumor, we performed RT-PCR using human- or mouse-specific integrin αv primers on RNAs from isolated PCa-118b cells or PCa-118b tumors. Human-specific primers detected αv in both isolated PCa-118b cells and total tumor (Fig. 3B). Mouse-specific αv primers also detected αv in total tumor, suggesting that mouse cells that were recruited into PCa-118b tumor also expressed αv. Interestingly, αv is much more abundantly expressed in PCa-118b cells relative to PC3-mm2, DU145, and C4-2B cells (Fig. 3B, right).

The ligands for αvβ1 are fibronectin, SPP1, and vitronectin, among others (23, 24). The ligands for α6β1 are laminins (23) and TNC (25), and others. Fibronectin, TNC, laminins (α1, α4, β1, and γ1), SPP1, lumican, and vitronectin are also detected by MS (Supplementary Table S2) and by qRT-PCR (Fig. 2D). These results demonstrate that PCa-118b cells express receptors for the integrin ligands identified from the tumor-induced bone.

FAK and Talin phosphorylation are associated with integrin activation

To examine whether the integrin ligands secreted by the tumor-induced bone activate integrin signaling in PCa-118b tumor, we examined the phosphorylation of two integrin signaling mediators, focal adhesion kinase (pFAK-Y397) and pTalin-S425 (Fig. 3C; ref. 9), in cabozantinib-treated tumors. Intense staining of both pFAK-Y397 and pTalin-S425 was observed in viable tumor cells that are adjacent to the bone (Fig. 3D). These results
suggest that osteoblast-secreted integrin ligands likely activate integrin signaling in tumor cells.

**Integrin expression in C4-2B4 and PC3-mm2 prostate cancer cells**

To investigate whether integrin signaling confers therapy resistance, we examined the effects of integrin ligands on prostate cancer cells. Because the PCA-118b cells isolated from PCA-118b tumors do not survive for more than 2 days in standard culture conditions (22), C4-2B4 and PC3-mm2 cells were used for this analysis. qRT-PCR using human-specific integrin primers showed that PC3-mm2 cells mainly expressed $\alpha_5$, $\alpha_6$, and $\beta_1$ integrins (Fig. 4A). C4-2B4 cells expressed low message levels of integrins compared with PC3-mm2 and PCA-118b cells, with $\alpha_5$, $\alpha_6$, $\beta_1$, and $\beta_5$ being the major integrins expressed (Fig. 4A). Therefore, expression of integrins in prostate cancer cells is heterogeneous and this may lead to varied responses to different integrin ligands present in the tumor microenvironment.

**Effect of integrin ligands on C4-2B4 cell survival in vitro**

To examine the effects of integrin ligands on C4-2B4 cell survival, cDNAs encoding SPP1, SPARC, BIG-H3, lumican, and vitronectin with His$_8$-tags were generated and recombinant proteins were expressed in HEK293 cells (Fig. 4B). Treatment of C4-2B4 cells with recombinant SPP1 and lumican increased phosphorylation of FAK-Y397, indicating that these osteocrines activate integrin signaling (Fig. 4C). Addition of SPP1 or lumican increased the colony formation of C4-2B4 cells compared with vector control (Fig. 4D). SPP1 is a ligand for $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_\beta_3$, $\alpha_\beta_5$, $\alpha_\beta_1$, and $\alpha_\beta_1$, and lumican has been shown to be a ligand for $\alpha_5\beta_1$, $\alpha_6\beta_1$, and $\alpha_2\beta_1$. Thus, the effects of these two osteocrines on C4-2B4 cells are consistent with the expression of $\alpha_5$ and $\beta_1$ integrins in C4-2B4 cells. In contrast, addition of BIG-H3 (a ligand for $\alpha_\beta_3$, $\alpha_\beta_5$, and $\alpha_\beta_4$ integrins) and vitronectin (a ligand for $\alpha_\beta_1$ and $\alpha_\beta_5$ integrins) did not have a significant effect on C4-2B4 cell colony formation, likely due to the low to undetectable levels of expression of $\alpha_\beta_1$ and $\beta_1$ in C4-2B4 cells (Fig. 4A). SPARC (osteonectin), which is known as a counter-adhesive protein (26–29), also did not have a significant effect on C4-2B4 cell colony formation (Fig. 4D). Additionally, we expressed SPARC, SPP1, lumican, BIG-H3, and vitronectin in C4-2B4 cells through retroviral transduction. Soft agar colony assays showed that overexpression of SPP1 or lumican in C4-2B4 cells led to an increase in cell survival (Supplementary Fig. S3), similar to the results observed with exogenous addition of purified recombinant proteins.

**Effect of FAK inhibitor PF-562271 on PC3-mm2 in vitro**

A consequence of integrin activation is phosphorylation of FAK, which is implicated in cell survival (30). PF-562271 is a competitive inhibitor for ATP binding to FAK (31), and is undergoing clinical trial (32). We have shown previously that the integrin signaling pathways are constitutively activated in PC3-mm2 cells and that FAK is constitutively activated (16). Therefore, we examined the effect of FAK inhibition on cell survival. To characterize the effect of PF-562271 on prostate cells, we treated C4-2B4 cells with increasing concentrations of PF-562271 and measured cell survival using an MTT assay. As shown in Figure 5A, PF-562271 inhibited cell survival in a dose-dependent manner. Western blot analysis of C4-2B4 cells treated with PF-562271 showed decreased phosphorylation of FAK-Y397 (Fig. 5B). These results suggest that FAK inhibition led to decreased cell survival in C4-2B4 cells. To confirm the role of FAK in regulating cell survival, we used an siRNA approach to knock down FAK expression in C4-2B4 cells and measured cell survival using an MTT assay. As shown in Figure 5C, knockdown of FAK expression led to a significant decrease in cell survival, indicating that FAK is essential for the survival of C4-2B4 cells.
cancer cells, PC3-mm2 cells were first treated with PF-562271. Decreased FAK-Y397 phosphorylation was observed as early as 3 hours and persisted for 24 hours (Fig. 5A), with no change in total FAK expression. A dose–response analysis showed that treatment for 24 hours with PF-562271 at greater than 0.1 μmol/L was sufficient to decrease pFAK-Y397 by 80% in PC3-mm2 cells (Fig. 5B). Immunofluorescence showed that a 3-hour treatment with 5 or 10 μmol/L PF-562271 reduced pFAK-Y397 in focal adhesion sites as well as in the cytosol in PC3-mm2 cells (Fig. 5C, inset). Although Talin is not a direct target of PF-562271, phosphorylation of Talin-S425 was similarly inhibited, albeit for a shorter duration and requiring a higher concentration of PF-562271 (Fig. 5A and B). These results suggest that PF-562271 effectively inhibits the phosphorylation of FAK-Y397 and Talin-S425 in PC3-mm2 cells. We further examined the effect of PF-562271 on the survival of PC3-mm2 cells. We observed a dose-dependent inhibition of anchorage-independent growth by PF-562271 in soft agar colony assays (Fig. 5D).

We next examined the effects of PF-562271 on C4-2B4 cells, in which the integrin/FAK pathway is not constitutively activated. C4-2B4 cells were treated with 10 μg/mL SPARC, SPP1, lumican, BIG-H3, or vitronectin with or without 5 μmol/L PF-562271 and their growth in soft agar examined. Incubation of C4-2B4 cells with integrin ligands increased C4-2B4 cell survival (Fig. 5E). Treatment with PF-562271 significantly inhibited the osteocrine-induced C4-2B4 cell survival (Fig. 5E).

Recently, several second-generation FAK inhibitors, including defactinib (VS-6063 PF-04554878), are being tested in clinical trials. We thus examined the effects of defactinib on pFAK inhibition in PC3-mm2 cells. Treatment of PC3-mm2 cells with defactinib led to a time- and dose-dependent inhibition of FAK phosphorylation similar to that, which was observed with PF-562271 (Supplementary Fig. S4A and S4B). Defactinib treatment also resulted in a dose-dependent inhibition of PC3-mm2 cell survival in soft agar colony formation (Supplementary Fig. S4C). Together, these results suggest that inhibition of FAK activity decreases prostate cancer cell survival in vitro.

Effect of PF-562271 on tumor growth

Because the expression of pFAK-Y397 was increased in the resistant tumor cells (Fig. 3D), we reasoned that: (1) inhibition of FAK activation alone might lead to the decrease of tumor growth; and (2) a combination of a FAK inhibitor with cabozantinib might reduce the number of resistant cells resulting from integrin ligand-mediated FAK activation. We first examined the effect of PF-562271 on FAK phosphorylation in vivo. SCID mice bearing PCA-118b tumors were treated with or without PF-562271 for 1, 2, or 5 days. Western blot and immunostaining showed that pFAK-Y397 was inhibited in the tumor as early as 1 day after treatment with PF-562271 (Fig. 6A and B).

Next, we tested whether PF-562271 is able to inhibit tumor growth. Mice bearing PCA-118b tumors were grouped according to tumor sizes: one group with tumors approximately 30 mm³, a second group with tumors approximately 80 mm³, and a third group with tumors approximately 190 mm³. Each mouse in the group was treated with vehicle, PF-562271, cabozantinib, or cabozantinib in combination with PF-562271. Mouse body weights in all groups except the cabozantinib alone group remained similar throughout the course of treatment, suggesting that there was limited treatment toxicity (Supplementary Fig. S5). Tumor growth over 14 days was calculated as the fold increase...
over the initial tumor size (Fig. 6C). We found that PF-562271 treatment alone resulted in an approximately 40% decrease in tumor size when compared with control, regardless of initial tumor sizes (Fig. 6C), suggesting that PF-562271 can inhibit tumor growth. Treatment with cabozantinib or cabozantinib plus PF-562271 resulted in complete tumor growth inhibition regardless of the initial tumor size (Fig. 6C).

Histologic analyses of the tumors showed that a majority of tumor cells were still viable after treatment with PF-562271 (Fig. 6D, hematoxylin and eosin (H&E)). In contrast, significant tumor cell death was observed in cabozantinib-treated tumors (Fig. 6D, H&E). Mice treated with both cabozantinib and PF-562271 had fewer viable tumor cells compared with those treated with cabozantinib alone (Fig. 6D, H&E). Immunofluorescence analysis showed that pFAK-Y397 levels in PCa-118b tumor are heterogeneous, with some tumor areas expressing a higher level of pFAK-Y397 than other areas (Fig. 6D, control). PF-562271-treated tumors have decreased levels of pFAK-Y397 (Fig. 6D). Cabozantinib-resistant tumor islets have increased level of pFAK compared with those in untreated tumors (Fig. 6D), supporting FAK Figure 6.
activation in the survival of tumor cells. The combination of cabozantinib with PF-562271 reduced the levels of pFAK-Y397 in the survived tumor cells compared with cabozantinib alone (Fig. 6D), although staining was heterogeneous. Importantly, we found that the tumor cells resistant to PF-562271, cabozantinib, or combination treatments were Ki67 positive (Fig. 6D), indicating that these tumor cells have the potential to resume their growth upon cessation of treatments.

Effects of PF-562271 on reducing therapy resistance in PCa-118b tumor

Next, we examined tumor regrowth after treatment cessation. After 2 weeks of treatments, tumors in one group of mice were allowed to re-grow for 1 week and another group for 2 to 3 weeks after stopping the treatments. Upon cessation of treatments, cabozantinib-treated tumor re-grew rapidly (Fig. 6E), consistent with the presence of resistant tumor cells. Treatment with cabozantinib plus PF-562271 delayed tumor regrowth compared with the tumor regrowth with cabozantinib alone (Fig. 6E), consistent with the reduction in resistant tumor cells by PF-562271 (Fig. 6D). Similar results were observed in another set of studies in which treatments were started as soon as the tumors were palpable (Supplementary Fig. S6). Together, these results support our interpretation that cabozantinib eradicates most of the tumor cells except those adjacent to the bone (Fig. 6F). Cabozantinib-resistant cells have higher levels of pFAK, which are sensitive to PF-562271 treatment, leading to a delayed tumor recurrence after treatment cessation in combination therapy compared with those with cabozantinib alone.

Discussion

Our results suggest that a novel form of "preexisting" or de novo therapy resistance stems from the osteoblastic nature of prostate cancer metastasis, in which osteoclines secreted from tumor-induced bone contribute to therapy resistance. We propose a working model of how the newly formed bone can induce "de novo" resistance in prostate cancer cells prior to the initiation of therapy (Fig. 6G). Within the tumor-induced newly formed woven bone, osteoblasts secrete factors (osteocrines), many of which are integral ligands that activate integrin signaling in prostate cancer cells, thus increasing cell survival and "de novo" therapy resistance. Inhibition of FAK activity reduces therapy resistance, suggesting that osteocrine-mediated FAK activation through integrin signaling is one of the de novo resistance mechanisms.

Increasing evidence demonstrates that integrin-mediated survival pathways play a major role in tumorigenesis and therapy resistance (17, 20, 33, 34). As a result, several integrin antibodies are being tested in clinical trials for various cancers (21, 35), on PCa-118b tumor growth. We found that C4-2B4, PC3-mm2, and PCa-118b cells express different panels of integrins. These observations raise concern that the heterogeneity of integrin expression in prostate cancer cells may limit the therapeutic efficacy of integrin antibodies. Our unpublished data support such a possibility. We examined the anti-integrin αβ antibody mAb 33B6 (16) in combination with DI17E6, an anti-integrin αβ antibody that has been tested in phase 1 clinical trials (35), on PCa-118b tumor growth. We found that, when tested in established PCa-118b tumors, the combination of mAb 33B6 and DI17E6 did not show a significant inhibition of tumor growth (data not shown). These antibodies also did not significantly reduce resistance to cabozantinib treatment in the PCa-118b model (data not shown). These results may be due to a failure in blocking α6 integrin that is highly expressed in PCa-118b cells. As most integrins signal through FAK, inhibiting pFAK may be a strategy to overcome the heterogeneity in integrin expression in tumor cells. In addition to PF-562271 (38), several FAK inhibitors, e.g., VS-6063 (defactinib, PF-04554878) and VS-4718, are currently being tested in clinical trials. Although our studies showed that PF-562271 is able to reduce PCa-118b growth as a single agent and improve the therapy outcomes when combined with cabozantinib, PF-562271 may not be suitable for clinical application. PF-562271 exhibits potent inhibition of CYP3A, leading to dose- and time-dependent nonlinearity in PF-562271 pharmacokinetics (32). As a result, second-generation FAK inhibitors VS-6063 and VS-4718 may be better agents than PF-562271 in clinical application.

Although integrin signals through FAK, FAK is also known to be activated by many growth factor receptors in various types of human cancer (for review, see ref. 39). Thus, FAK activation in the tumor cells that are resistant to cabozantinib treatments may also be due to integrin-independent signaling pathways other than by integrin activation. As a result, FAK inhibitors may be used in therapy resistance arising from pathways other than integrin.

Besides integrin activation, osteocrines may also be involved in other resistance mechanisms. Several of the identified osteocrines, e.g., periostin and tenasin C, have been shown to regulate maintenance of cancer stem cell-like properties in other solid tumor types (40, 41). Interestingly, FAK is also a survival factor for cancer stem cells (42). FAK has been shown to play a role in self-renewal, tumorigenicity, and maintenance of mammary cancer stem cells (42). Shapiro and colleagues (43) showed that FAK inhibitor treatment preferentially eliminates cancer stem cells. Thus, the combination of a FAK inhibitor with chemotherapy may also eliminate cancer stem cells that are resistant to chemotherapy. Other nonintegrin osteocrines may also confer resistance through different mechanisms. These osteocrines together may contribute to therapy resistance in human prostate cancer bone metastasis. Recently, it was reported that vascular heterogeneity may represent another source of primary therapy resistance (44). Systematic dissection of tumor–microenvironment interactions may reveal other important mechanisms underlying therapy resistance. Investigations into the various mechanisms of resistance from tumor microenvironment may lead to different therapy combinations that further overcome resistance.

In addition to targeting the osteocrine-mediated resistance pathways in the tumor cells, another approach would be to target the release of osteocrines from osteoblasts. Thus, it is possible that the bone-targeting agents, e.g., radium-223 (Alpharadin), could be considered for preventing therapy resistance from tumor-induced bone. Radium-223 dichloride is a bone-seeking, α-emitting radionuclide that has been shown to result in increased overall survival, i.e., 3.6 months (45, 46), and has received FDA approval. As the mechanism of resistance we have identified is therapy-independent, we predict that these bone-targeting agents may also be effective in preventing therapy resistance from the bone microenvironment by blocking the release of osteocrines when used in combination with numerous therapeutic agents approved or in clinical trial for bone-metastatic prostate cancer.

In summary, we identify for the first time that factors secreted from tumor-induced bone constitutes one type of microenvironment-mediated therapy resistance. Although cabozantinib was
used in this study, our studies identify a novel mechanism of de novo therapy resistance from tumor-induced bone that leads to resistance prior to any therapy application. Our observations suggest that pathways mediating such resistance need to be targeted simultaneously with therapeutic regimens for bone metastasis. As bone metastasis is the lethal progression of prostate cancer, strategies that combine inhibitors of preexisting therapy resistance with currently approved drugs may improve therapy outcomes and prolong patient survival.

Disclosure of Potential Conflicts of Interest

C. Logothetis reports receiving a commercial research grant from Astellas, BMS, Karyopharm, Sanofi, J&J, Exelixis, Pfizer, Novartis, Coag Biotechnologies, Medivation, Bayer, and Glaxo Smith Kline; has received speakers bureau honoraria from Bayer, J&J, AstaZeraeca, Pfizer, Novartis, Helsinn HC, Astellas, and BMS; and is a consultant/advisory board member for Astellas, BMS, J&J, Pfizer, Novartis, Bayer, AstaZeraeca, Helsinn, and Exelixis. No potential conflicts of interest were disclosed by the other authors.

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Yu-Chen Lee, Song-Chang Lin, Guoyu Yu, et al.


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