Phenylboron acid is a more potent inhibitor than boric acid of key signaling networks involved in cancer cell migration

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Previous studies from our lab have shown that both boric (BA) and phenylboronic acid (PBA) inhibit the migration of prostate cancer cell lines, as well as non-tumorigenic prostate cells. Our results indicate that PBA is more potent than BA in targeting metastatic and proliferative properties of cancer cells. Here we focus on the impact of BA and PBA on Rho family of GTP-binding proteins and their downstream targets. Treatment with 1 mM PBA and BA decreases activities of RhoA, Rac1 and Cdc42 in DU-145 metastatic prostate cancer cells, but not in normal RWPE-1 prostate cells. Furthermore, ROCKII activity and phosphorylation of myosin light chain kinase decrease as a result of either PBA or BA treatment in DU-145 cells, suggesting these compounds target actomyosin-based contractility.

Introduction

Cell migration plays a critical role during embryonic growth and differentiation, as well as during routine tissue maintenance and wound healing. Aberrations in cell migration are an important property of most metastatic solid tumors.1 Understanding how tumor cells migrate could aid in the development of treatments that specifically target the metastatic cascade. Indeed, some of the most promising current cellular targets for metastasis are members of the Rho family of GTP-binding proteins, such as RhoA, Rac1, and Cdc42, which are often over-expressed in many forms of malignant cancer and play an important role in focal adhesion regulation, actin stress fiber formation, and cell migration.2,3

Presently, there are no curative treatments available for patients with metastatic cancer, as the efficacy of standard cancer treatments decreases once a tumor becomes metastatic.4 Consequently, downstream steps of the metastatic cascade—including migratory behaviors such as escape from the primary tumor, intravasation, and extravasation—represent attractive targets for additional therapeutic intervention.

Therefore it is crucial to identify highly selective and less toxic compounds in order to stunt the formation and spread of secondary metastases. Interestingly, naturally occurring compounds are a rich source for novel anticancer agents. Specifically, a promising new category of candidates is boron and its derivatives. Boric acid is a mild organic Lewis acid with structural features similar to carbon, allowing it to act as a competitive inhibitor for many carbon containing substrates. This characteristic of BA makes it a promising pharmaceutical agent, as it serves as an effective inhibitor of enzymes such as peptidases, proteases, proteasomes, arginase, nitric oxide synthase and transpeptidases. Previous studies from our lab have shown that phenylboronic acid (PBA) is a more potent and selective inhibitor of cancer cell migration and viability than its parent compound, boric acid. Additionally we have shown that both compounds did not affect cell adhesion or viability at 24 h but did alter cell morphology, decreasing cancer cell viability at 8 d. The ability of BA and PBA to elicit a short-term selective, anti-migratory response, while decreasing cancer cell viability overall, makes it a promising candidate for a novel anti-cancer treatment.5,6 PBA can serve as an ideal cancer treatment, which should boast high selectivity to target cancer cells over regular cells, low sensitivity for a wide array of malignant phenotypes and favorable pharmacokinetic properties.7

In this study we further investigate the potential of PBA as a natural, prophylactic, anti-cancer treatment by examining the specific signaling mechanisms that are altered upon treatment of metastatic prostate cancer cells, DU-145, with boric and phenylboronic acid. Our hypothesis is that BA and PBA inhibit the function of the pathways linking RhoA, Rac1 and Cdc42 with ROCK. Our results show that both BA and PBA alter actin distribution in DU-145 cells grown on fibronectin, as well as decrease the activity of several proteins that play key roles in cell migration and cell spreading, including the Rho family of GTPases and their corresponding downstream targets.

Results and Discussion

One millimolar BA and PBA alter actin distribution in DU-145 prostate cancer cells. Cell morphology is determined primarily by the arrangement of the actin cytoskeleton and can
offer insight into the signaling mechanisms of an attached or migrating cell. As shown in Figure 1, BA and PBA (Fig. 1B and C, respectively) induce a flattened, spread morphology in DU-145 prostate cancer cells at 1 mM, a concentration that inhibits cell migration. All images were collected at the same magnification, and with the same exposure settings. Thus, the differences in cell size and actin distribution are a direct effect of the drug exposure, as we reported previously. The relatively weak fluorescence in Figure 1C illustrates a net reduction in F-actin (green channel) induced by PBA. While some of the nuclei in Figure 1C stain brightly, most do not, consistent with the finding that exposure to 1 mM of PBA decreases proliferation of these cells without decreasing their adhesion or viability. These data suggest that these compounds selectively target actin polymerization and force generation. Specifically, these morphological changes may be due to the role of RhoA in stress fiber formation as well as generation of the force required for cell adhesion, spreading, and migration.

To further investigate the effects of BA and PBA on cellular migratory pathways, we examined key components of cell signaling pathways that regulate actin dynamics.

RhoA, Rac1, and Cdc42 activity is decreased in prostate cancer cells but not in normal prostate cells. RhoA, Rac1, and Cdc42 regulate changes in the actin cytoskeleton that control cell migration. The cycling between an active GTP bound and inactive GDP bound state is the basis of the Rho GTPase activity assay, which binds only the activated form. Using this assay, we found that both 1 mM PBA and 1 mM BA induced a significant decrease in RhoA activity in DU-145 cells, relative to RWPE-1 cells, as demonstrated in Figure 2A and B. Additionally, the decrease in RhoA activity induced by 1 mM PBA was significantly greater than that of 1 mM BA.

BA and PBA decreased Rac1 activity in DU-145 cells, but not in RWPE-1 cells, as shown in Figure 2C and D, respectively. Rac1 has been found to accumulate in the rear of migrating cells. Additionally, Rac1 is required for JNK MAP kinase signaling, which may contribute to cell migration by phosphorylating paxillin. This leads to focal adhesion turnover at the cell rear, suggesting PBA and BA treatment slow migration and increase spreading by inhibiting this turnover.

In contrast, both BA and PBA increased Cdc42 activity in DU-145 cells (Fig. 2E), with no significant difference between the two compounds. Neither compound altered activity of Cdc42 in RWPE-1 normal cells (Fig. 2F). In a migrating cell, Cdc42 controls the formation of filopodia, primarily through the GTPase Ral and its effector sec5. Cdc42 can be activated by various external stimuli, such as release of extracellular chemoattractants or loss of cell to cell contacts.

Interestingly, Cdc42 activation and function is tightly connected to Rac1 signaling. Upon its activation, Cdc42 initiates signaling pathways that determine active Rac1 localization. One potential target of Cdc42 is the p21 activated kinase (PAK) family, critical effectors that link Rho GTPases to cytoskeleton reorganization and nuclear signaling through their role as scaffolding for the Rac-specific GEF, α-PIX. This cellular polarization is demonstrated in the organization of the actin cytoskeleton as well as the formation of adhesion sites in a migrating cell: small focal complexes form at the leading edge in response to Rac1 activity and mature into larger focal contacts as the cell moves over them. Our data suggest that the decrease in cellular migration as a result of BA and PBA treatment may be a result of inhibition of Rac1 activity, which disrupts its function in actin mediated protrusion of the membrane in the leading edge of the cell.

In conjunction with their effects on RhoA, Rac1, and Cdc42, both PBA and BA selectively inhibited ROCK activity in the tumor cells, providing additional evidence in support of our hypothesis. This was observed in DU145 cells treated as per Figure 3 (compare Fig. 3A with control cells in Fig. 3B). Both ROCK and one of its downstream targets, MLC, are effectors of the actomyosin contractility required for cells to migrate. ROCK can increase phosphorylation of MLC by directly phosphorylating MLC, as well as phosphorylating the myosin binding subunit.
of myosin phosphatase, inhibiting its activity and allowing for sustained MLC phosphorylation. Reducing levels of LIMK-1 activation by inhibition of ROCK activity leads to excessive protrusions at the leading edge of a migrating cell, similar to our observation of increased spreading in BA and PBA treated cells.

We observed a significant decrease in phosphorylation of MLC as a result of treatment with PBA and BA (Fig. 3C) with no concomitant decrease in overall MLC amounts (Fig. 3D). MLC predominantly controls non-muscle myosin II, which is involved in the generation of the contractile force for cell migration. Myosin II primarily controls the last step of migration by contraction of the rear of the cell, supported by localization studies of phosphorylated MLC. Because MLC-K is often overexpressed in malignant cancer cells, PBA and BA may be attractive treatments for inhibiting the effects of this phosphorylation.

This is, to our knowledge, the first report describing the mechanism of action of BA and PBA on cancer cell migration. Our findings on proliferation are entirely consistent with previous studies primarily focused on cell proliferation, including cyclin expression, senescence, and apoptosis. In a previous study, Barranco and Eckhart, using DU-145 prostate cancer cells, showed that BA inhibited cell proliferation without inducing apoptosis; instead, BA induced conversion to a senescent-like cellular phenotype. BA treatment also inhibits proliferation of prostate tumors in nude mice.

These studies also offer additional clues as to how BA may impact migration. For example, BA inhibits mitogen-activated protein kinases including the ERK1/2, which regulates integrin-mediated migration in most human cell types. Additionally, a 2009 study found that higher BA blood levels lower the risk of prostate cancer by reducing intracellular Ca²⁺ signals and storage. This may impact migration by interfering with activation of the calcium-dependent protease calpain, which cleaves integrins and focal adhesion proteins in the rear of migrating cells. Calpain activity is implicated in metastatic progression of multiple cancers.

**Figure 2.** RhoA, Rac1, and Cdc42 activity is decreased in DU-145 prostate cancer cells but not RWPE-1 normal prostate epithelial cells. (A, C, E) Treatment with 1 mM BA or 1 mM PBA of DU-145 cells serum starved overnight and plated on fibronectin for 15 min in the presence of 10% FBS caused a significant inhibition of each protein compared to untreated cells (*). One millimolar PBA caused a significant decrease in protein activity compared to untreated (*) and BA treated cells (**). (B, D, F) Neither compound inhibited RhoA, Rac1, or Cdc42 activity in the normal cells. Results expressed as mean ± SEM for three replicates.
and is a promising target in new cancer therapies.\textsuperscript{20}

Together, these data and our current studies suggest that high levels of boric and phenylboronic acid induce growth arrest and inhibit migration in rapidly proliferating cells through an unknown signaling mechanism. Before we can address this conclusively, several unanswered questions remain, including how boronic acid enters cells. Boron has a cell-surface receptor, but it is not clear if PBA can utilize this same route of entry. Additionally, as observed through a phosphorylation-specific ELISA, both BA and PBA do not decrease activity of FAK or Src in DU-145 prostate cancer cells (data not shown). As such, the direct target(s) of either BA or PBA compound is also unknown.

**Materials and Methods**

**Tissue culture.** All cultures were maintained at 37°C in a humidified atmosphere of 5% CO\textsubscript{2}. The cells were harvested during the exponential growth phase using phosphate buffered saline (PBS) and 0.25% trypsin-EDTA (Invitrogen). DU-145 cells were obtained from the American Type Culture Collection (ATCC), number HTB-81; RWPE-1 cells are ATCC number CRL-11609. DU-145 cells were grown in RPMI-1640 medium supplemented with 10% FBS, 15 mM HEPES buffer (pH 7.4), 50,000 units penicillin and 50 mg Streptomycin. RWPE-1 cells were grown according to manufacturer’s instructions in Keratinocyte Serum Free Media (K-SFM), Kit Catalog Number 17005-042 (Invitrogen).

**Real time RT-PCR (quantitative RT-PCR).** Total RNA was prepared from cell culture samples according to the RNeasy Mini Kit instructions (Qiagen) and quantified using a NanoDrop spectrophotometer. Only RNA with a 260/280 ratio > 2.0 was used for qRT-PCR. Quantitative real time RT-PCR was performed using the LightCycler 480 Real-Time PCR System (Roche) and the QuantiTec SYBR Green RT-PCR Kit (Qiagen). Primers used for gene amplification were designed using OligoPerfect (Invitrogen) and purchased through IDT Technologies. Quantitative RT-PCR was performed according to the protocol recommended by the manufacturer.

**Immunofluorescence staining for morphological imaging.** Cells were plated and imaged as previously described.\textsuperscript{6}

**GTPase activity assays.** The enzyme-linked immunosorbent assay (ELISA)-based G-LISA absorbance based kits (Cytoskeleton) were used to determine endogenously activated (GTP bound) levels RhoA (#BK124), Rac1 (#BK035), and Cdc42 (#CN02) in DU145 and RWPE-1 cells according to the manufacturer’s instructions. DU-145 or RWPE-1 cells were serum starved overnight on FN-coated plates and treated with 1 mM BA or PBA. The next day, cells were activated with 10% FBS and were lysed in 250 μl of G-LISA lysis buffer (supplemented with protease inhibitors). Cell lysate was clarified and snap-frozen in liquid nitrogen. Protein concentration was determined using the Precision RedTM Advanced Protein Assay provided with the kit and normalized between the various time points. Equal total protein amounts were added to a 96-well dish coated with the GTP binding domain of Rho GTPase proteins and incubated at 4°C for 30 min on an orbital shaker. Active protein levels were determined by subsequent incubations with anti-Rho.
Racl or Cdc42 antibody, respectively, and secondary horseradish peroxidase-conjugated antibody for 60 min, each with vigorous shaking at room temperature. Protein activity was determined by measuring absorbance at 490 nm using an ELISA plate reader after subtracting background (only lysis buffer). Treated samples were compared to the untreated sample, and positive and negative controls were provided.

**ROCK activity assay.** ROCK activity was measured by Cell Biolabs’ 96-well ROCK Activity Assay Kit (STA-406) run according to manufacturer’s instructions (positive control provided). Samples were treated and collected according to the same protocol for the Rho GTPase assays. Reaction kinase buffer was prepared fresh prior to use. The substrate wells were incubated with ROCK samples at 10 ng and the phosphorylated MYPT1 (Thr696) antibody. Final absorbances were read on a spectrometer at 450 nm and results were graphed relative to the untreated control.

**Statistical analysis.** All experiments were repeated a minimum of three times. The representative data is presented as mean ± SEM. Statistical analyses were performed using Student unpaired t-test and a p value of less than 0.05 was considered significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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