Original Paper

Istaroxime Inhibits Motility and Down-Regulates Orai1 Expression, SOCE and FAK Phosphorylation in Prostate Cancer Cells

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Key Words

Istaroxime • DU-145 prostate cancer cells • Orai1 • SOCE • FAK • Migration

Abstract

Background/Aims: Istaroxime is a validated inotropic Na⁺/K⁺ ATPase inhibitor currently in development for the treatment of various cardiac conditions. Recent findings established that this steroidal drug exhibits potent apoptotic responses in prostate tumors *in vitro* and *in vivo*, by affecting key signaling orchestrating proliferation and apoptosis, such as c-Myc and caspase 3, Rho GTPases and actin cytoskeleton dynamics. In the present study we examined whether istaroxime is affecting cell motility and analyzed the underlying mechanism in prostate tumor cells. **Methods:** Migration was assessed by transwell and wound healing assays, Orai1 and Stim1 abundance by RT-PCR and confocal immunofluorescence microscopy, Fura-2 fluorescence was utilized to determine intracellular Ca²⁺ and Western blotting for FAK/pFAK measurements. **Results:** We observed strong inhibition of cell migration in istaroxime treated DU-145 prostate cancer cells. Istaroxime further decreased Orai1 and Stim1 transcript levels and downregulated Orai1 protein expression. Moreover, SOCE was significantly decreased upon istaroxime treatment. Furthermore, istaroxime strikingly diminished phosphorylated FAK levels. Interestingly, the efficacy of istaroxime on the inhibition of DU-145 cell migration

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was further enhanced by blocking Orai1 with 2-APB and FAK with the specific inhibitor PF-00562271. These results provide strong evidence that istaroxime prevents cell migration and motility of DU-145 prostate tumor cells, an effect at least partially attributed to Orai1 downregulation and FAK de-activation. **Conclusion:** Collectively our results indicate that this enzyme inhibitor, besides its pro-apoptotic action, affects motility of cancer cells, supporting its potential role as a strong candidate for further clinical cancer drug development.

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Introduction

Istaroxime is a novel drug candidate derived from a new generation of steroidal Na⁺/K⁺ ATPase enzyme inhibitors that has successfully finalized phase II clinical trials in patients with cardiac failure [1-3]. Recent experimental evidence points to a novel role of ion channels and especially of Na⁺/K⁺ ATPase as potential anti-cancer targets. Indeed, expression of Na⁺/K⁺ ATPase subunits has been observed in various cancer types, while different, recently developed steroid-inhibitors of this enzyme have shown potent inhibitory activities in various previous studies [reviewed in [4, 5]].

Based on these reports, our group addressed previously the anti-cancer potential of various Na⁺/K⁺ ATPase inhibitors [6, 7], showing strong activity in multiple cancer cell lines *in vitro*, as well as in prostate and lung cancer xenografts *in vivo* [8, 9]. Focusing on istaroxime we demonstrated in a more recent study strong anti-cancer activity of this compound acting through caspase-3, c-Myc, actin reorganization and RhoA signaling in various cancer cell lines *in vitro*, as well as in prostate cancer xenografts *in vivo* [10].

However, although pro-apoptotic responses to istaroxime were clearly demonstrated, the profound anti-tumorigenic action of this compound has not been functionally correlated to other key tumor specific mechanisms, including Orai1 expression as well as cell motility and invasiveness. Indeed, cell proliferation, migration and cell death are frequently controlled by alterations of cytosolic Ca²⁺ activity [11-13], which in turn is regulated by the pore forming Ca²⁺ channel subunit Orai1 accomplishing store operated Ca²⁺ entry (SOCE) [14-19]. In line with this, Orai1 expression seems to be crucial for several tumor cell responses including cell proliferation, migration and apoptosis in various tumors [18, 20-22]. Based on these reports, in the present work we examined the migratory potential of prostate tumor cells following treatment with istaroxime and we evaluated the molecular signaling pathways involved. Our findings indicate that istaroxime blocked migration and invasiveness of DU-145 prostate tumor cells, an effect mainly associated with Orai1/Stim1 down-regulation, SOCE inhibition and FAK de-phosphorylation. These results provide novel mechanistic insights into the regulation of the anti-migratory potential of istaroxime in prostate tumors.

Material and Methods

Cell lines and culture conditions

The cell line DU-145 was cultured in RPMI 1640, supplemented with 1% penicillin/streptomycin (Biochrom, Berlin, Germany) and 10% fetal bovine serum (FCS, Biochrom, Berlin, Germany) in a humidified atmosphere containing 5% CO₂ at 37 °C. All cells were tested to be mycoplasma negative.

RNA isolation and Real time PCR

Real-time PCR (RT-PCR) experiments were performed in six-well plates with 3x10⁵ DU-145 cells. Cells were harvested after treatment without or with istaroxime (1.25 and 2.5 μM) for 4 and 6 hours. RNA was isolated using the RNeasy mini kit by following the manufacturer's instructions (Qiagen). cDNA synthesis was performed using High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, Massachusetts, USA).

Polymerase chain reaction (PCR) amplification of the respective genes was set up in a total volume of 20 μl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x GoTaq® qPCR Master Mix (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Cycling conditions were as

follows: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 58°C for 30 seconds and 72°C for 20 seconds. For the amplification the following primers were used (5'-3' orientation):

Orai1, fw TGATGAGCCTCAACGAGCACTCCATG;

Orai1, rev TGCTGATCATGAGCGCAAACAGGTG;

STIM1, fw CCTGTGGAAGGCATGGAAGT;

STIM1, rev CTGAGGCAGCTCCACATATGT;

TBP, fw GCC CGA AAC GCC GAA TAT;

TBP, rev CCG TGG TTC GTG GCT CTC.

Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad). All experiments were done in duplicate. Amplification of the house-keeping gene *tbp* (TATA binding protein) was performed to standardize the amount of sample RNA. Relative quantification of gene expression was achieved using the Δct method as described earlier [22-25].

Western Blotting

After the required treatment with istaroxime (1.25 and 2.5 μM), cells were washed with ice cold phosphate-buffered saline (PBS), followed by lysis in cell lysis buffer (Cell Signaling Technology, Inc., New England Biolabs). The extracts were centrifuged at 13,000 rpm for 20 min at 4°C, the protein concentration of the supernatant was determined and the sample buffer for SDS-PAGE was added. 30 μg protein per lane were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane (VWR). Nonspecific binding was blocked by incubation with 10% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween-80. Subsequently, membranes were incubated with a rabbit monoclonal FAK antibody (1:1000, Cell Signaling Technology, Inc., New England Biolabs, 125 kDa) or with rabbit polyclonal phospho-FAK (Y397) antibody (1:500, Cell Signaling Technology, Inc., New England Biolabs, 125 kDa). Incubation with rabbit monoclonal GAPDH antibody (1:1000, Cell Signaling Technology, Inc., New England Biolabs) served as a loading control. Phospho FAK and total FAK bands were detected using horseradish peroxidase-labeled anti-rabbit antibody (1:3000, Goat Anti-Rabbit IgG peroxidase conjugate (Pierce) and a Western Sure Premium Chemiluminescent Substrate (LI-COR, USA). Specific bands were quantified by LI-COR Image Studio software (Licor, USA). Levels of each protein were expressed as the ratio of signal intensity for the target protein relative to that of GAPDH [24, 26].

Confocal laser scanning immunofluorescence microscopy

For immunofluorescence laser scanning microscopy DU-145 cells, incubated with 1.25 or 2.5 μM istaroxime, or DMSO respectively for the indicated time scales, were grown on glass chamber slides (Sarstedt, Germany), washed twice with PBS and fixed with 4% PFA for 15 min at room temperature. Subsequently, the cells were incubated for 1 hour at room temperature in blocking buffer containing 3% BSA (in PBS) and exposed overnight at 4°C with rabbit polyclonal Orai1 antibody (1:200, Alomone labs). After three washing steps with PBS the cells were incubated with FITC labeled goat anti-rabbit secondary antibody (1:1000, Invitrogen) for 1h at room temperature. Following three washes with PBS all slides were mounted with ProLong Gold antifade reagent (Life Technologies, USA). Images were subsequently taken on a Zeiss LSM5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss Micro Imaging) with an A-Plan 40 $\times$ ocular.

Ca²⁺ measurements

Fura-2 fluorescence was utilized to determine intracellular Ca²⁺ after treatment with and without istaroxime. Therefore cells were loaded with Fura-2/AM (2 μM , Invitrogen, Goettingen, Germany) for 20-30 min at 37°C and were excited alternatively at 340 nm and 380 nm through an objective (Fluor 40 $\times$ /1.30 oil) built in an inverted fluorescence microscope (Axiovert 100, Zeiss, Oberkochen, Germany). Emitted fluorescence intensity was recorded at 505 nm and the data were acquired using specialized computer software (Metafluor, Universal Imaging, Downingtown, USA). Cytosolic Ca²⁺ activity was estimated from the 340 nm/380 nm ratio. SOCE was determined by extracellular Ca²⁺ removal and subsequent Ca²⁺ re-addition in the presence of thapsigargin (1 μM , Invitrogen) [27]. For quantification of Ca²⁺ entry, the slope ($\Delta\text{ratio/s}$) and peak (Δratio) were calculated following re-addition of Ca²⁺ [28, 29].

Experiments were performed with Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 MgSO₄, 2 CaCl₂, 2 Na₂HPO₄, 32 HEPES, 5 glucose, pH 7.4. To reach nominally Ca²⁺-free conditions, experiments were performed using Ca²⁺-free Ringer solution containing (in mM): 125 NaCl, 5 KCl, 1.2 MgSO₄, 2 Na₂HPO₄, 32

HEPES, 0.5 EGTA, 5 glucose, pH 7.4. In the DU-145 experiments, cells were treated with istaroxime (1.25 μ M) for 4 hours before the experiment.

In vitro migration assays

For wound healing assays, 3×10^5 cells per well were plated onto 12-well dishes. A single scratch wound was inflicted using a micropipette tip in each confluent monolayer. Cells were washed with PBS to remove cell debris, supplemented with vehicle (control) or istaroxime (5 μ M) and monitored. Images were captured by phase microscopy using a 10x objective lens at 0, 4h, 7h and 10 h post wounding. Wound width was measured using image J analysis software and expressed as percentage of the initial wound width.

For transwell migration assays, transwell inserts with a pore diameter size of 8 μ m (BD Bioscience) were used. The transwells were placed in a 24-well cell culture plate containing cell culture medium with 10% fetal bovine serum as chemoattractant (750 μ l). The upper chambers were filled with 500 μ l of cell culture serum-free medium containing DU-145 cells in a concentration of 8×10^4 cells/well. The cells were incubated during experiment with istaroxime (1.25 and 2.5 μ M) with and without 2-APB (5 μ M) or PF-00562271 (2 μ M) for 24 h in a humidified atmosphere of 37°C and 5% CO₂. Cells that did not migrate through the pores of the transwell membrane were removed by a cotton swab and washing with PBS. The transwells were moved to 70% ethanol, incubated for 10 min at room temperature for fixation. After staining with 0.2% crystal violet for 10 min the membranes were washed twice with water. Membranes were removed by scalpel, placed on slides and analyzed. The migrated cells bound on the lower surface to the membrane were then counted at 3 different representative areas using Axio Vision Release 4.8 software (Carl Zeiss Vision, Oberkochen, Germany).

Statistics

GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses. All data were tested for significance using unpaired Student *t*-test or ANOVA (Bonferroni test, Dunnett test). Only results with $p < 0.05$ were considered statistically significant. Data are presented as means $\pm$ standard deviation (SD) unless otherwise specified. All experiments were performed in at least 3 independent replications.

Results

Istaroxime inhibits migration of DU-145 prostate cancer cells

We first addressed the efficacy of istaroxime to influence the migration potential of DU-145 prostate cancer cells by using two distinct techniques, the wound healing- and transwell-assays. As shown in Figure 1A, treatment of the cells with 5 μ M istaroxime significantly inhibited cell motility, manifested by the diminished wound width as compared to control untreated cells (Fig. 1B). This finding was fully supported by the transwell assay, showing

Fig. 1. Istaroxime inhibits cell motility. A uniform scratch was made in each confluent monolayer culture in the absence (control) or presence of istaroxime (5 μ M) (A, B). Wound area, at the indicated time points, was expressed as a percentage of the initial wound area (B). Transwell assay: Arithmetic means $\pm$ SD ($n = 4$) of the percentage migrated DU-145 cells in the absence (white bar) and presence of istaroxime (1.25 and 2.5 μ M, 24 h) (black bars) and solvent DMSO (grey bar) (C). Significantly different from control (* $p \leq 0.05$, ** $p \leq 0.01$).

potent inhibition of 24 h cell migration potential, even at lower istaroxime concentrations (Fig. 1C).

Istaroxime down-regulates Orai1/Stim1 expression and store operated Ca^{2+} entry

Having established the anti-migratory potential of istaroxime we further addressed possible mechanisms that may contribute to this effect. We first focused on the impact of istaroxime on Orai1, Stim1 expression and store operated Ca^{2+} entry (SOCE). As illustrated in Figure 2A, B, RT-PCR analysis showed significant decrease in Orai1 and Stim1 transcript levels in istaroxime treated DU-145 cells that was evident after 4 hours and reached statistical significance after 6 hours treatments. In line with this finding, confocal laser scanning microscopy revealed significantly diminished expression of Orai1 protein levels in DU-145 cells treated with 1.25 and 2.5 μ M istaroxime for 6 hours (Fig. 3A, B). As illustrated further in Figure 4, 4 hours istaroxime-treatment significantly decreased the peak of store operated Ca^{2+} entry (SOCE) that was triggered by depletion of Ca^{2+} stores with thapsigargin, a well-established inhibitor of sarcoendoplasmic reticulum Ca^{2+} ATPase.

Istaroxime controls FAK de-phosphorylation

In addition to the aforementioned analyses, we have also addressed the influence of istaroxime on Focal Adhesion Kinase (FAK) phosphorylation. FAK and its downstream signaling activators PI3K/Rac1/Akt/SGK1 regulate cell survival maintenance [30-35] while deactivation of FAK induces loss of substrate adhesion, triggers apoptotic responses and inhibits migration [36-41]. As shown in Figure 5, the ratio of phosphorylated FAK to the total FAK protein decreased significantly after 2h treatment of DU-145 cells with 1.25 and 2.5 μ M istaroxime, while longer incubation time revealed total de-phosphorylation of this protein, indicating strong kinase deactivation. Although degradation of p-FAK or phosphatases

Fig. 2. Istaroxime down-regulates Orai1/Stim1 transcript levels. Arithmetic means $\pm$ SD (n=3) of Orai1 (A) and STIM1 (B) transcript levels relative to TBP in DU-145 cells treated with 1.25 or 2.5 μ M istaroxime for 4 or 6 hours. Significantly different from control (* $p \leq 0.05$, ** $p \leq 0.01$).

Fig. 3. Istaroxime down-regulates the Orai1 protein abundance of DU-145 cell line. A) Confocal microscopy of Orai1 protein abundance in DU-145 cells after treatment without and with 1.25 or 2.5 μ M istaroxime and DMSO for 6 hours. Scale bar represents 10 μ m. (B) Statistical analysis of Orai1 immunofluorescence abundance without (white symbol) and with (black symbols) 1.2 or 2.5 μ M istaroxime and solvent DMSO (grey bar) after 6 hours treatment. **($p < 0.01$); ****($p < 0.0001$) indicates statistical significance from untreated cells.

Fig. 4. Istaroxime down-regulates store operated Ca^{2+} entry. A) Representative tracings of Fura-2 fluorescence-ratio in fluorescence spectrometry before and following extracellular Ca^{2+} removal and addition of thapsigargin ($1 \mu\text{M}$), as well as re-addition of extracellular Ca^{2+} without (white symbols) and with (black symbols) after 4 hours with $1.25 \mu\text{M}$ istaroxime treatment B, C. Arithmetic means ($\pm$ SEM, $n = 5$) of peak (left) and slope (right) increase of fura-2-fluorescence-ratio from intracellular stores (B) and upon SOCE (C) following addition of thapsigargin ($1 \mu\text{M}$) in DU-145 cells without (white bars) and with (black bars) prior istaroxime treatment for 4 hours. ***($p < 0.001$) indicates statistically significant difference from untreated cells (two-tailed unpaired t -test).

Fig. 5. Istaroxime controls FAK de-phosphorylation. A) Original Western blot of the expression of phosphorylated FAK (upper panel), total FAK protein (middle panel) and GAPDH protein (lower panel) in DU-145 cells treated with 1.25 and $2.5 \mu\text{M}$ istaroxime for 2, 4 and 6 hours. (B) Arithmetic means $\pm$ SD ($n = 4$ independent experiments) of phosphorylated (Y397) over total FAK protein abundance in DU-145 cells treated without (white bar) or with istaroxime (1.25 or $2.5 \mu\text{M}$) for 2 hours (black bars). Significantly different from control (***) ($p \leq 0.001$).

induction by istaroxime were not evaluated in this study, the dramatic decrease of phosphorylated protein fraction indicates rather a significant deactivation of this kinase that is controlling the observed anti tumorigenic effects of this compound.

Orai1/SOCE and FAK inhibition promote anti-migratory effects of istaroxime

We further evaluated the significance of Orai1/SOCE and FAK on the istaroxime-initiated inhibition of cell motility. To this end, DU-145 cells were pre-treated with the specific Orai1 and FAK inhibitors 2-APB and PF-00562271 respectively. As illustrated in Figure 6, the

Fig. 6. Orai1/SOCE and FAK inhibition promote anti-migratory efficacy of istaroxime in DU-145 prostate cancer cells. Arithmetic means $\pm$ SD (n=3) of the percentage migrated DU-145 cells in the absence (white bars) and presence of istaroxime (1.25 and 2.5 μ M, 24 h) (black bars) and solvent DMSO (grey bars) with and without (A) 2-APB (5 μ M) or (B) PF-00562271 (2 μ M). * (p<0.05), ** (p<0.01); *** (p<0.001) indicates statistical significance; # indicates statistically significant difference from untreated cells *** (p<0.001).

inhibitory effect of istaroxime on DU-145 cell migration was further substantially enhanced by blocking Orai1 and FAK by the specific inhibitors 2-ABP and PF-00562271 respectively.

Discussion

Apart from its well documented cardiac role, Na⁺/K⁺ ATPase is also emerging as a promising novel anti-cancer target [reviewed in [4]]. Previous studies from our group established effective anti-cancer activity of various Na⁺/K⁺ ATPase inhibitors in multiple cancer cell lines [8, 9, 42], while *in vivo* studies in prostate and lung cancer xenografts showed potent, dose dependent tumor inhibition properties [9]. In line with these reports, istaroxime, the lead molecule of this class of Na⁺/K⁺ ATPase inhibitors [1-3, 6], showed strong activity in multiple cancer cell lines derived from a large variety of tumor panels *in vitro* as well as in prostate cancer xenografts *in vivo* [10]. In this study it was demonstrated that istaroxime was inducing apoptosis, caspase-3 activation, down-regulation of c-Myc oncoprotein, actin reorganization and rapid activation of RhoA signaling [10]. These findings recognized a crucial anti-cancer potential of this compound. In agreement with these reports, the results presented here disclose an additional functional role of istaroxime in other key tumor specific mechanisms, namely migration and cell motility. These effects were documented by using two distinct approaches, confirming the anti-tumorigenic activity of this compound. They are also in accord with recently published observations, demonstrating inhibition of breast cancer cell migration by the cardiac glycoside digoxin, a classical Na⁺/K⁺ ATPase inhibitor [43].

Furthermore, looking for molecular mediators triggered by istaroxime and controlling inhibition of migration, we recognized for the first time istaroxime-induced down-regulation of Orai1/Stim1 expression and significant decrease of store operated Ca²⁺ entry (SOCE), assessed by the Fura-2 fluorescence of Fura-2-AM loaded DU-145 cells that was taken as a measure of cytosolic calcium concentration ([Ca²⁺]_i) [21]. These findings imply the involvement of cytosolic Ca²⁺ activity, regulated by the pore forming Ca²⁺ channel subunit Orai1 in controlling prostate tumor cell migration upon istaroxime treatment, further confirming previous observations in various cancer cell types [5, 11, 44-48].

FAK signaling has also been reported to control cell survival and migration in various tumor cells [36-38, 49, 50]. Our findings showing strong de-phosphorylation of this kinase by istaroxime fully support these reports and imply that FAK deactivation, besides Orai1/SOCE down-regulation, may contribute to the observed istaroxime-induced inhibition of DU-145 prostate cell migration. This assumption is further supported by the enhanced istaroxime-induced inhibition of migration in the presence of the Orai1 and FAK inhibitors.

We have previously profiled istaroxime against a panel of 22 different cancer cells from 9 tumor panels [10]. The average GI_{50} (compound concentration to suppress growth by 50%) in those cell lines was 4 μ M, while in the prostate cell lines the compound's average GI_{50} was 2.5 μ M. Based on these findings we have used concentrations of 1.25, 2.5 and 5 μ M in all our assays, i.e. concentrations that fit the compound's GI_{50} . In addition, taking into account istaroxime's Na^+/K^+ ATPase inhibitor IC_{50} of ~ 0.4 μ M, our applied concentrations both, in previously published cancer studies as well as in this work, are up to 10x fold higher than the compound's target IC_{50} ; this is in agreement with standard practice in order to sufficiently hit the molecular target and induce a possible effect over the time course of a given assay.

It was previously reported that the Na^+/K^+ ATPase inhibitor istaroxime may exert functional cross talk with mAR [10], a membrane androgen receptor mediating rapid, non-genomic anti-cancer effects of androgens in multiple cancer cells [34, 40, 51-59]. Although it was supposed that Na^+/K^+ ATPase and mAR are not identical [10], based on the similarity of the downstream signaling events triggered by istaroxime [9, 10] and mAR activation [summarized in [55] and [23]] it was assumed that Na^+/K^+ ATPase and mAR may be functionally interlinked [10]. The results of the present study further support this notion. Indeed, mAR activation by testosterone conjugates in prostate, breast and colon tumor cells was previously reported to inhibit migration and induce potent pro-apoptotic responses, effects that were regulated -among others- by Orai1 expression [20], SOCE [20] and FAK deactivation [38, 50, 60]. Interestingly, in the present study these signaling effectors are shown to be implicated as well in the istaroxime-induced anti-migratory effects, implying once again, a possible functional cross-talk between Na^+/K^+ ATPase and mAR. Further studies are now required to analyze this interaction in more detail.

To conclude, in the present study we demonstrate for the first time that istaroxime, a clinically validated Na^+/K^+ ATPase inhibitor that is functionally interlinked with mAR, exerts potent inhibition of cell migration in prostate cancer cells, by and at least partially due to down-regulation of Orai1/Stim1 expression, decrease of store operated Ca^{2+} entry (SOCE) and de-phosphorylation of FAK. These findings reinforce the anti-tumorigenic properties of this compound providing additional insights in the molecular signaling involved.

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Discloser Statement

The authors declare that they do not have any conflicts to state.

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