Induction of Neuroendocrine Differentiation in Prostate Cancer Cells by Dovitinib (TKI-258) and its Therapeutic Implications

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Abstract
Prostate cancer (PCa) remains the second-leading cause of cancer-related deaths in American men with an estimated mortality of more than 26,000 in 2016 alone. Aggressive and metastatic tumors are treated with androgen deprivation therapies (ADT); however, the tumors acquire resistance and develop into lethal castration resistant prostate cancer (CRPC). With the advent of better therapeutics, the incidences of a more aggressive neuroendocrine prostate cancer (NEPC) variant continue to emerge. Although de novo occurrences of NEPC are rare, more than 25% of the therapy-resistant patients on highly potent new-generation anti-androgen therapies end up with NEPC. This, along with previous observations of an increase in the number of such NE cells in aggressive tumors, has been suggested as a mechanism of resistance development during prostate cancer progression. Dovitinib (TKI-258/CHIR-258) is a pan receptor tyrosine kinase (RTK) inhibitor that targets VEGFR, FGFR, PDGFR, and KIT. It has shown efficacy in mouse-model of PCa bone metastasis, and is presently in clinical trials for several cancers. We observed that both androgen receptor (AR) positive and AR-negative PCa cells differentiate into a NE phenotype upon treatment with Dovitinib. The NE differentiation was also observed when mice harboring PC3-xenografted tumors were systemically treated with Dovitinib. The mechanistic underpinnings of this differentiation are unclear, but seem to be supported through MAPK-, PI3K- and Wnt-signaling pathways. Further elucidation of the differentiation process will enable the identification of alternative salvage or combination therapies to overcome the potential resistance development.

Introduction
Prostate cancer remains the second leading cause of cancer-related deaths in American men with 26,120 estimated deaths in 2015 [1]. Radical prostatectomy, with or without radiation, comprises the primary line of curative treatment. The metastatic tumors are treated with androgen-lowering agents and androgen-receptor inhibitors. However, the tumors eventually acquire resistance to such androgen deprivation therapy (ADT) and develop into lethal castration resistant prostate cancer (CRPC). The development of resistance is, in part, due to the reactivation of androgen-receptor (AR) signaling [2], which prompted the development and subsequent Food and Drug Administration (FDA) approval of enzalutamide, an improved androgen-receptor antagonist, and abiraterone, a local androgen biosynthesis blocker [3,4]. However, not all patients respond to the new therapies, and 50% of those who respond eventually develop resistance to the new therapies within 2 years [5–8]. Importantly, more than 25% of the therapy-resistant patients end up with a more aggressive and treatment resistant form of the disease neuroendocrine prostate cancer (NEPC) [9].

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Received 26 January 2017; Accepted 31 January 2017
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http://dx.doi.org/10.1016/j.tranon.2017.01.011
Neuroendocrine differentiation (NED) includes focal NED and pure NED, also known as small cell PCa. Pure NED (cancer of the NE cells) is very rare (0.5–2%) and carries very poor prognosis with the probability of survival being less than 1 year [10]. Focal NED can be classified into four sub-groups, a) adenocarcinomas with focal NE differentiation, b) Paneth-cell-like NE differentiation, c) large cell NEPC, and d) small cell NEPC [10–12]. NE cells are present in small numbers in the normal prostate and are thought to support the basal and luminal cells, during normal development and malignancy, through the secretion of various neuropeptides [13]. The number of NE cells present within the tumor has been shown to increase with disease aggressiveness [11,13] and thus, has been purported to play a role in resistance development [14]. Indeed, prolonged androgen deprivation therapy (ADT) or radiation therapy increase NE differentiation of PCa cells [15,16]. Moreover, stimulation with factors such as cAMP and interleukin-6 (IL-6) leads to the differentiation of PCa cells into a NE phenotype in vitro and in vivo [17–20]. Studies looking at the mechanism of various ligand-induced NE differentiation processes have implicated the JAK/STAT pathway specifically through STAT3 [21], the PI3K- [22,23], MAPK- [23], and Wnt- signaling pathways [24].

Dovitinib (TKI-258/CHIR-258) is a pan receptor tyrosine kinase (RTK) inhibitor that primarily targets fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), fms-like tyrosine kinase 3 (FLT3) and Proto-Oncogene Receptor Tyrosine Kinase (c-KIT) [25]. It has shown anti-tumor activity in pre-clinical models of several cancers and is presently in clinical trials for prostate, renal, gastrointestinal, adenoid cystic, glioblastoma, urothelial, thyroid, pancreatic, breast, and non-small lung cancers [26,27]. Here we show that both AR-positive and AR-negative cells differentiate into a NE phenotype when treated with Dovitinib, presumably as a modality of resistance development. Elucidation of the exact mechanism will enable the identification of alternative salvage or combination therapies to overcome the resistance-development.

Material and Methods

Cell Lines and Reagents

Human prostate cancer cell lines LNCaP, CWR22Rv1, Du145 and PC3 were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin (100 U/mL) and streptomycin (100 μg/mL). Media, FBS, Penicillin, Streptomycin, and 0.25% trypsin/EDTA were purchased from Gibco-Invitrogen Corporation (Carlsbad, CA, USA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Dovitinib treatment: Cells were cultured in 10-cm dish until they reached ~70–80% confluence. Thereafter, they were treated with media containing Dovitinib (SelleckChem, S2769) at 6 μM concentration. Media with drug was replaced every 3 days.

Cell Viability Assay/WST

Cell viability was assessed using the cell impermeable water-soluble tetrazolium (WST-1) assay where cellular mitochondrial dehydrogenases of living cells cleave the tetrazolium salt to form purple colored formazan. After a three-day day Dovitinib treatment, cell proliferation was determined using the WST-1 reagent (Clontech, MK4000) according to the manufacturer’s instructions. Absorbance was read at 440 nm and 690 nm using a SpectraMax M5 Multimode Plate Reader. Actual sample absorbance values were calculated by subtracting sodium dodecyl sulfate (SDS)-treated negative control wells (at the time of setting up experiment) to eliminate noise from dead cells. Prism6 was used to plot and calculate IC₅₀ values. All the assays were performed three independent times in triplicates.

Fluorescence Microscopy

LNCaP and PC3 cells were cultured in 6-cm dishes with uncoated, circular-glass coverslips and treated with Dovitinib (6 μM) for 3 weeks. Cells were fixed with 4% w/v formaldehyde in phosphate buffered saline (PBS) (pH 7.4) for 1 hour at room temperature, and then washed three times with PBS. Cells were permeabilized with 0.2% w/v Triton-X-100. Cells were washed with PBS again, and blocked in PBS containing 1% w/v bovine serum albumin (BSA) (1 hour at room temperature). Primary antibodies, anti-chromogranin A + B and anti-synaptophysin (kind gift from Dr. Michael Donovan, Department of Pathology, Icahn School of Medicine at Mount Sinai (ISMS)), were diluted to 1:5000 and 1:200 in PBS containing 1% BSA, respectively. Cells were incubated in primary antibodies at 4°C overnight. Subsequently, the cells were washed three times in PBS containing 1% BSA (5 minutes per wash), and incubated in receptor for temperature for 1 hour with anti-mouse immunoglobulin-G-tagged fluorescein isothiocyanate (IgG–FITC) antibody (Sigma, F0257) and anti-rabbit IgG antibody (Life Technologies, A-11008) respectively, at room temperature. Cells were washed three times using PBS with 1% BSA and mounted in antifade mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA; H-1200). Labeled cells were analyzed by Leica DM5500B fluorescence microscopy equipped with a 2-megapixel Leica monochrome camera (model DFC345FX) and Leica application suite (LAS) software.

Cell Lysate and Immunoblotting

The protein concentration of the samples was determined using a detergent-compatible bicinchoninic acid (BCA) protein assay (Quick Start Bradford Protein Assay Kit, Bio-Rad, Catalog number 5000201). The samples were diluted to the same quantity of protein (20 μg) using loading buffer (100 mM Tris–HCl pH 6.8, 2% SDS, 0.02% bromophenol blue, 20% glycerol, 2% β-mercaptoethanol), separated by SDS–polyacrylamide gel electrophoresis (PAGE) on 10% gels, and electro-transferred using a Mini Trans-Blot wet transfer system (Bio-Rad Catalog number 1703930) onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad, catalog number 162–0177). Membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% non-fat milk for 1 hour. After blocking, the membranes were probed with following primary antibodies for immunodetection: mouse monoclonal anti-neuron specific enolase (NSE) [ENO2] (MRQ-55) antibody (1:5000; Ventana Medical Systems, Inc., catalog number 760–6786), rabbit monoclonal anti-phospho-AKT (Ser473) antibody (1:1000; Cell Signaling, catalog number 9601), mouse monoclonal anti-phospho-MAPK antibody (1:1000; Cell Signaling, catalog number 9145), rabbit polyclonal anti-MAPK antibody (1:1000; Cell Signaling, catalog number 9102), rabbit polyclonal anti-androgen receptor (AR) (N-20) antibody (1:1000; Santa Cruz Biotechnology, catalog number sc-816), and mouse monoclonal anti-β-actin antibody (1:1000; Santa Cruz Biotechnology, catalog...
cells were harvested with 0.25% Trypsin and incubated with the following secondary antibodies for 1 hour on a rocking platform: anti-mouse IgG horseradish peroxidase (HRP)-linked antibody (1:10,000; Cell Signaling, catalog number 7076) or anti-rabbit IgG HRP-linked antibody (1:10,000; Cell Signaling, catalog number 7074). After a final step of washing, detection of antibody reactivity was performed using the Western Bright enhanced chemiluminescence (ECL) western blotting detection kit (Advansta Inc., catalog number K-12045-D50) and signals were captured using the ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences).

**Quantitative Real-Time PCR**

Total RNA was extracted from cells using Qiagen RNAeasy kit (Qiagen catalog number 74104) and cDNA generated using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, catalog number 1708881), according to the manufacturer’s instructions. The cDNA were then amplified and transcripts detected using SYBR Green Supermix (Bio-Rad Laboratories, catalog number 1708880) in real-time PCR detection system (Bio-Rad CFX96, catalog number 1855196) using specific primers. Each sample was examined in triplicates and Cq values were collected for β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the target genes during the log phase of the cycle. The levels of expression for the genes of interest were normalized to β-actin and GAPDH for each sample, and compared with the values obtained for the different samples. Each experiment was repeated at least 3 times in triplicates.

**Mouse Xenograft Studies**

All mice experiments were carried out at the Mount Sinai vivarium in accordance with institutional guidelines and approved by the Mount Sinai Medical Center Institutional Animal Care and Use Committee (IACUC) (protocol number 2014-0176). PC3 cells (1 × 10⁶ cells, 1:1 mixed with Matrigel™) (Corning, catalog number 356234) were injected subcutaneously into both flanks of 8-week-old male nude mice (Jackson laboratories, catalog number 007850) and tumors were allowed to grow without treatment. Tumor volume was measured every other day through direct measurement with calipers and calculated by the formula: 1/2(length x width²). When tumor volumes reached 200mm³, mice were treated with Dovitinib dissolved in saline (50 mg/kg per day), or same volume of saline for control, until the endpoint of experiment (1000 mm³) through oral gavage. At the end of the study, mice were sacrificed and tumors collected and stored at −80°C for protein extraction and Western blot analysis.

**RTK Array**

The screen for the presence of phosphorylated RTKs was carried out using the Human Phospho-RTK Array (R&D Systems, catalog number ARY001B) as per the manufacturer’s instruction. Briefly, cells were harvested with 0.25% Trypsin–EDTA (Gibco, catalog number 25200056) and rinsed with PBS prior to lysis. Cells were solubilized in lysis buffer (1×10⁷ cells/mL, R&D Systems with protease inhibitors, catalog number 895561) by gently rocking for 30 minutes at 4°C. Following blocking, 300 μg of proteins were added to the membranes and incubated overnight at 4°C with gentle agitation. After washing, horseradish peroxidase (HRP)-conjugated anti-phospho-tyrosine antibodies (1:2000) (supplied in the kit) were added to each membrane and incubated at room temperature for 2 hours on a gentle shaker. After another series of washes, proteins were visualized using the ECL detection reagent (Advansta Inc., catalog number K-12045-D50) and ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences).

**Statistical Analysis**

Statistical analysis was performed using Prism6 (GraphPad Software) and Student’s t test (two-tailed) was used for the comparison of differences; P values less than 0.05 were considered significant.

**Results**

Dovitinib is a pan RTK inhibitor targeting FGFR, VEGFR, PDGFR, and Kit [25]. To determine whether PCa cells are sensitive to Dovitinib, we tested the cytotoxicity effect of the drug on PCa cell lines LNCaP and PC3 using the WST assay. For this, cells were treated with increasing amounts of Dovitinib and the number of live cells left after 3-day treatments were quantified and IC₅₀ values plotted. Both the lines exhibited sensitivity to Dovitinib treatment with IC₅₀ in the lower μM range, Supplementary Figure S1. In an effort to uncover the mechanism of resistance development, we set out to generate Dovitinib-resistant clones through the long-term treatment of PCa cell lines. However, we observed striking morphological changes in the PCa cells upon prolonged Dovitinib treatment.

**Dovitinib-Treated PCa Cells Display Morphological Transformations Consistent with NE Differentiation (NED) and Express Transcripts Associated with NE Differentiation**

In the case of the AR-positive prostate adenocarcinoma cell line LNCaP, treatment with Dovitinib induced strong morphological alterations of reduced cell-body content and pronounced, elongated neurite-like processes, Figure 1A (left column). Phenotypically, these were consistent with previous reports of morphological alterations in LNCaP cells after anti-androgen treatment with cAMP or IL6 [15,17,28]. To test whether this effect extended to other PCa cell lines, we similarly treated the AR-negative CRPC cell line, PC3. Since PC3 cells have been reported to share NEPC characteristics [29], we were surprised to observe that similar to the observation in LNCaP cells, treatment of PC3 cells with Dovitinib resulted in the production of marked neurite-like processes, Figure 1A (right column). These observations were similar to previously reported outcomes of PC3 cells treated with cAMP [17]. Perhaps PC3 cells are at an intermediate stage where they are not completely terminally differentiated into the NEPC phenotype. To test whether this phenotype further extended to other PCa cell lines, we Dovitinib-treated Du145 (AR negative PCa cell line) and 22Rv1 (AR positive PCa cell line) cells with Dovitinib and looked for the formation of neurite-like structures. Upon treatment with Dovitinib, both these lines also exhibited pronounced branched neurite-like formations suggesting it to be a universal phenomenon for PCa cells lines, Supplementary Figure S2.

NE differentiation is characterized by the varying levels of expression of neuronal markers such as neuron specific enolase (NSE), chromogranin A (CHGA) or synaptophysin (SYP) [30,31]. To test whether the morphological changes observed with Dovitinib treatment also resulted in the expression of the above NE markers, both LNCaP and PC3 cells were treated with Dovitinib and their
cellular RNA subjected to qPCR analysis using gene-specific primers. Dovitinib treatment induced a 10-fold increase in NSE mRNA expression in LNCaP cells when compared to untreated controls, and a 5-fold increase in PC3 cells compared similarly, Figure 1B. The level of chromogranin A was observed to be around 15-fold higher in LNCaP cells treated with Dovitinib when compared with untreated control, and was more than 3-fold higher in the case of treated PC3 cells when compared to untreated PC3 controls Figure 1B (right panel). The expression of SYP was 2-fold higher in Dovitinib-treated LNCaP cells and more than 15-fold higher for Dovitinib-treated PC3 cells. Taken together, treatment of prostate cancer cells with Dovitinib led to morphological trans-differentiation accompanied

Figure 1. Induction of the neuronal morphology in PCa cells upon treatment with Dovitinib and expression of NE markers. A, Representative images of LNCaP and PC3 cells treated with 6 μM Dovitinib for 3 weeks (bottom row) or not (top row). Images captured at 20× magnification using a bright-field microscope. Arrow heads point to neurite-like cellular outgrowth. Scale bar = 100 μM. B, Increased expression of NE markers upon Dovitinib treatment in LNCaP (left) and PC3 (right) cells. C. Levels of REST transcript in LNCaP (left) and PC3 (right) cells. B and C, Cells were treated with 6 μM Dovitinib for 3 weeks and the levels of mRNA of the indicated genes quantified using qRT-PCR using specific primers (See Methods). Values were normalized to GAPDH, and results plotted as fold change over control cells. Plot is representative of 3 independent experiments conducted in triplicates. (Error bars: S.E.) *P < .05, ** P < .01.
by the expression of the transcripts encoded by these three widely used NE-markers.

The repressor element (RE)-1 silencing transcription factor (REST), which represses neuronal gene expression in non-neuronal tissues, has been shown to be suppressed in NEPC [32–34]. To test whether treatment of PCa cells with Dovitinib resulted in suppression of REST, we compared the level of REST transcripts in 3-week Dovitinib treated cells to control cells. As shown in Figure 1C, we observed a significant suppression of REST levels in Dovitinib treated LNCaP (Figure 1C, left) and PC3 (Figure 1C, right) cells. Taken together, the above observations suggest that treatment of PCa cells with Dovitinib induces expression of markers associated with NE differentiation and suppression of REST.

Detection of Proteins Associated with NE Differentiation Upon Dovitinib-Treatment of PCa Cells

To test whether the observed increase in mRNA expression levels resulted in protein expression, both LNCaP and PC3 cells were treated with Dovitinib and their cell lysates subjected to western blot analysis using NSE antibody. MAPK was used as loading control and NCI-H660, a NEPC cell line, as positive control. B, Western blot analysis of the LNCaP lysate, treated as in B with Dovitinib, with AR antibody. Actin was used as loading control. C, Indirect immunofluorescence-based detection of chromogranin and synaptophysin in LNCaP (left) and PC3 (right) cells upon 6 μM Dovitinib treatment, using antibody against synaptophysin (top row) and chromogranin A + B (bottom row), and FITC-labeled secondary. DAPI was used to stain the nucleus (blue). Arrows point to neurites-like cellular extensions. Scale bar: 100 μM.

Figure 2. Expression of proteins associated with NE differentiation A, LNCaP and PC3 cells were treated or not with 6 μM Dovitinib for 3 weeks, and their cell lysates subjected to western blot analysis using NSE antibody. MAPK was used as loading control and NCI-H660, a NEPC cell line, as positive control. B, Western blot analysis of the LNCaP lysate, treated as in B with Dovitinib, with AR antibody. Actin was used as loading control. C, Indirect immunofluorescence-based detection of chromogranin and synaptophysin in LNCaP (left) and PC3 (right) cells upon 6 μM Dovitinib treatment, using antibody against synaptophysin (top row) and chromogranin A + B (bottom row), and FITC-labeled secondary. DAPI was used to stain the nucleus (blue). Arrows point to neurites-like cellular extensions. Scale bar: 100 μM.
and compared to control cells. As depicted in Figure 3, expression of NSE was induced, as early as the second week of treatment, and continued through 4 weeks for both the cell lines. However, despite an increase in NSE expression in the 4-week treated samples when compared to 3-week treated samples, the cells began to look unhealthy and started to lift off the plates. Thus the time for complete transdifferentiation was taken to be around 3 weeks.

**Dovitinib Induces NE Differentiation In Vivo**

To test whether the NED-effect of Dovitinib extended to in vivo systems, we utilized the PC3 xenograft tumor model as it is an advanced model of CRPC and physiologically relevant in terms of therapy response leading to NEPC development. Nude mice were bilaterally injected with 1 million PC3 cells and tumors were allowed to form. When the tumors reached 200 mm$^3$ (around 2 weeks) mice were treated with 30 mg/kg Dovitinib daily and changes in tumor volume monitored. When compared to saline treated controls, which continued to grow rapidly and reached experimental end point (1000 mm$^3$), mice treated with Dovitinib had slower-growing tumors. However, after an initial lag-phase, these tumors appeared to rapidly increase their growth-rate past day-9 of treatment, reaching endpoint by Day 15, Figure 4A. To test whether tumors from Dovitinib-treated mice had increased level of neuronal markers, we carried out western blot analysis of lysates prepared from various stages of the tumorigenesis process. These included untreated saline control, suppressed (i.e. slow-growing phase) from the 1-week Dovitinib treatment and resistant phase from the 2-week Dovitinib treatment. When compared to PC3 cells, the levels of NSE were higher in all the tumor samples. Notably, there was a marked and progressive increase of NSE expression from control to suppressed and finally resistant tumors, Figure 4B. Taken together, the increased level of NSE expression suggests that the NED can be achieved with PC3 cells in vivo as observed in vitro.

**Mechanism of NE Differentiation: the Role of PI3K- and MAPK-Pathways**

Previous studies have identified the PI3K-pathway to play a major role in driving prostate tumorigenesis, including NE differentiation [35,36]. Furthermore, studies have also suggested the role of the MAPK-pathway in promoting NE differentiation [22,37,38]. To test whether up-regulation of the PI3K/AKT/mTOR pathway and/or MAPK-pathway played a role in the acquisition of Dovitinib-induced NED process, we probed cell lysates of Dovitinib-treated PCa cells with phospho-AKT and phospho-MAPK-specific antibodies. Both the cell lines showed increased levels of p-AKT upon Dovitinib-treatment, Figure 5, suggesting that perhaps up-regulation of the PI3K-pathway plays an important role in coordinating the differentiation process. However, when Dovitinib-treated cell lysates were tested for levels of MAPK-activation only, LNCaP cells showed a robust increase in phosphorylation of MAPK compared to controls, Figure 5. This increase in MAPK phosphorylation is consistent with a

![Figure 3](image-url)  
**Figure 3.** Time course of NE-differentiation of prostate cancer cells treated with Dovitinib. LNCaP (Left) and PC3 (right) were treated or not with 6 μM Dovitinib in 6-well plates. Cells were harvested at each week time point and frozen. After the 4-week time point, qPCR was carried out and analyzed as in Figure 1.

![Figure 4](image-url)  
**Figure 4.** Induction of NE differentiation in PC3 xenografted tumors upon Dovitinib treatment. A, Nude mice were bilaterally injected with PC3 cells to induce xenograft tumor formation. When the tumors developed to around 200 mm$^3$, mice were treated daily with 30 mg/kg Dovitinib or saline (control) through oral gavage. Changes in tumor volume were measured every day, and at around 1000 mm$^3$, mice were sacrificed and tumors harvested. Mice were also sacrificed and tumors harvested at day 9. n = number of tumors and Error bars = S.E. B, Western blot analysis of representative samples collected at various points of tumorigenesis. Control (saline treated), suppressed (tumors from mice treated for 9 days), and resistant tumors were harvested, lysates prepared and the levels of the indicated proteins detected using neuron specific enolase, NSE antibody. Actin was used as loading control.
Changes in PI3K and MAPK pathways upon Dovitinib treatment. LNCaP or PC3 cells were treated or not with 6 μM Dovitinib for 3 weeks and the cell lysates subjected to western blot analysis using phospho-AKT specific antibody to detect changes in PI3K pathway alterations. Total AKT was used as loading control (top rows). Phospho-MAPK specific antibody was used to probe for activation of MAPK pathway. Total MAPK was used as loading control (bottom rows). The same membrane was stripped after phospho-specific antibody probing and the membrane was re-probed using antibody targeting the total protein. Figure is representative of 3 independent experiments with similar results.

Figure 5. Changes in PI3K and MAPK pathways upon Dovitinib treatment. LNCaP or PC3 cells were treated or not with 6 μM Dovitinib for 3 weeks and the cell lysates subjected to western blot analysis using phospho-AKT specific antibody to detect changes in PI3K pathway alterations. Total AKT was used as loading control (top rows). Phospho-MAPK specific antibody was used to probe for activation of MAPK pathway. Total MAPK was used as loading control (bottom rows). The same membrane was stripped after phospho-specific antibody probing and the membrane was re-probed using antibody targeting the total protein. Figure is representative of 3 independent experiments with similar results.

Role of Various RTKs in Dovitinib-Mediated Neuroendocrine Differentiation

Since both MAPK- and PI3K-pathway can be regulated through activated RTKs, we reasoned that perhaps the sustained treatment of the cells with the pan-RTK inhibitor Dovitinib induces a compensatory response from some of the 50 or so other RTKs, or perhaps even a re-activation or desensitization of the targeted receptors themselves. To address these possibilities we used a human RTK array that measures the amount of activated RTK in cell lysates. The lysates of 1 day (short term) and 3-week Dovitinib-treated cells, from both LNCaP and PC3, were compared to determine the differential regulation of RTK activation in the two conditions. Notably, in both the cell lines, 1-day treatment with Dovitinib displayed suppression in the phosphorylated form of its target RTKs (FGFR, VEGFR and PDGFR), Figure 6 and Supplementary Figure S3.

Further analysis of the RTK strip treated with LNCaP lysates revealed a specific activation of EGFR in the 3-week Dovitinib treated cells when compared to 1-day treated cells, Figure 6 (left panel). EGFR has been shown to signal through various pathways including the MAPK-pathway [40], and EGF treatment has been demonstrated to promote neuroendocrine-like state in LNCaP cells [41]. Taken together, this adds support to role of the MAPK pathway seen previously (Figure 6) in the NED process.

Amongst other activated RTKs anaplastic lymphoma kinase (ALK) and receptor-like tyrosine kinase (RYK) were prominent. Whereas ALK is expressed specifically in the nervous system [42], RYK is part of the Wnt signaling pathway shown to influence axonal outgrowth [43,44]. Furthermore, we also observed suppression of receptor tyrosine kinase-like orphan receptor 2 (ROR2) activation, which is also a part of the Wnt-signaling pathway, and its suppression has been shown to increase axon length [45,46]. Thus, AKL, RYK and ROR2 perhaps act in concert to control neuron morphogenesis and regeneration [47,48]. Taken together, the alterations in the above RTKs support the modulation of not only MAPK- and PI3K-pathways, but also the Wnt-signaling in driving the transdifferentiation of LNCaP cells upon Dovitinib treatment.

In the case of PC3 cells however, we observed a constitutively active EGFR in both the 1-day as well as the 3-week Dovitinib treated cells, which is consistent with previous reports of EGF up-regulation in PC3 cells [49,50]. Similar to LNCaP cells, we observed suppression of ROR2 activity upon prolonged Dovitinib treatment. Furthermore, we observed loss of Ephrin receptor activation, particularly Eph A4 (ephrin type-A receptor 4) and Eph A7 (ephrin type-A receptor 7) in the 3-week treated PC3 cells. Regulations through Ephrin receptors have been shown to guide axon elongation of neuronal cells [51,52] and their suppression in certain contexts promotes tumorigenesis [53]. Remarkably, Eph A4 loss has been shown to promote axon regeneration [54,55], Eph A7 suppression has been shown to help complex dendrite formations and axon elongation [56]. Taken together, they suggest the involvement of the Wnt signaling pathway and provide additional support to the differentiation of PCa cells to neuron-like cells with highly complex neurite-like processes.

Discussion and Conclusions

Treating prostate cancer remains a major challenge as around 33% of the treated patients eventually develop resistance to their drug regimen, and progress to metastatic CRPC for which there are limited treatment options [57]. Nevertheless, drug development efforts have produced more FDA-approved drugs in the last three years than in the previous 30 years [58]. However, resistance development to the newly approved therapies has impeded success [59]. Although the occurrence of de novo NEPC is rare, the development of resistance through NE differentiation, during ADT, docetaxel chemotherapy and radiation therapy, have been documented [14,16,59–61]. Further corroborating this, the analysis of tumors resistant to more potent AR-pathway targeted therapies (enzalutamide and abiraterone) suggests that around 39% of the resistant tumors display either pure or intermediate NEPC [62]. Thus, understanding the mechanism of resistance development through the NE trans-differentiation process is crucial towards determining alternative therapy regimens.

Therapeutically-targeting multiple receptor tyrosine kinases (RTKs) that play an important role in promoting tumor progression, such as angiogenesis and growth factor signaling, presents an attractive approach. Dovitinib (TKI-258/CHIR-258) fulfills this niche by inhibiting multiple RTKs such as FGFR, PDGFR, cKIT and FLT3, and is being evaluated in clinical trials for several cancers [27,63].

We serendipitously observed that treatment of LNCaP cells with Dovitinib resulted in a uniform transformation of the cells to a distinct neuronal morphology. This alteration of was concomitant with expression of NE markers such as NSE, SYP and CHGA, loss of AR expression and suppression of REST.

It is challenging to address why the cancer cells differentiate into NE type during therapy and how this trans-differentiation benefits
the cancer cells. Perhaps, through the acquisition of characteristics of both neuronal and endocrine cells, the cells gain the ability to withstand therapy and maintain survival signaling. Our in vitro experiments suggest that within 2 weeks of treatment, cells start to express high levels of NE marker NSE. Similarly, mice harboring PC3 xenografted tumors when treated systemically with Dovitinib showed high levels of NSE expression in the resistant tumors. The short-term nature of NED both in vitro and in vivo suggests that perhaps epigenetic regulation drives the differentiation process.

Previous studies have shown that PI3K and MAPK play a role in the differentiation process [35,36]. Supporting these observations, Dovitinib-induced NE differentiation suggests the involvement of both PI3K and MAPK pathways. PC3 and LNCaP cells show up-regulation of PI3K signaling upon Dovitinib treatment and LNCaP cells show additional activation of MAPK. Further biochemical studies are needed to uncover the exact mechanistic links between AR and MAPK-pathway and their role in NED upon Dovitinib treatment.

Using human RTK arrays, we identified not only activation of receptors that are known to play a role in neuronal signaling, such as the various Ephrin receptors, but also those that are involved in driving MAPK- and PI3K-signaling, such as EGFR and Insulin R. We also identified RYK and ROR2, which have been shown to play a role in WNT-signaling in neuronal tissues, in line with previous reports that showed WNT signaling to promote NE differentiation of PCa cells [24,64].

Our study for the first time demonstrates that Dovitinib-treated PCa cells develop NE phenotype, perhaps as a way of developing resistance. Analyzing the data of an ongoing clinical trial for Dovitinib in prostate cancer [27], not only in terms of survival benefit but also in terms of the number of patients developing treatment-induced NEPC, would be highly informative in making future treatment decisions. Finally, the study establishes a robust method to bring about rapid NED in vitro and could be a valuable tool to understand the biology and identify targets for therapeutic interventions.

**Figure 6.** Activation status of various RTKs during Dovitinib mediated NE differentiation of PCa cells. LNCaP (left) and PC3 (right) cells were treated with 6 μM Dovitinib for 1 day or 3 weeks, and the cell lysates subjected to analysis using the human Phospho-RTK array kit that only detects the activated or phosphorylated form of the receptor using a pan phospho-tyrosine antibody (see Methods for details). Each receptor is spotted in duplicate and 3 sets of positive controls added at three corners (top and bottom right) and 1 set of negative control on the bottom left corner (see Fig. S3 for more explanation and individual receptor locations). Epidermal growth factor receptor (EGFR), receptor-like tyrosine kinase (RYK), anaplastic lymphoma kinase (ALK), receptor tyrosine kinase like orphan receptor 2 (ROR2), Ephrin type-A receptor 4 (EPH A4), and Ephrin type-A receptor 7 (EPH A7) were identified to be differentially expressed and are shown in red-boxes below the strips.

**Acknowledgements**

We are thankful to the Department of Hematology and Oncology for use of their equipment and resources. We also would like to extend our thanks to the faculty of the Department of Urology for helpful discussions and suggestions. Thanks also to
David Mulholland, PhD and Roseanne Wick for critical reading of the manuscript. KKY thanks Dr. Nora M Navone, MD Anderson Cancer Center for insightful discussions. The research work was supported by funds from Deane Prostate Health. Both SSY and KKY are Prostate Cancer Foundation Young Investigator Awardees.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2017.01.011.

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