Relaxin receptor antagonist AT-001 synergizes with docetaxel in androgen-independent prostate xenografts

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Abstract

Androgen hormones and the androgen receptor (AR) pathway are the main targets of anti-hormonal therapies for prostate cancer. However, resistance inevitably develops to treatments aimed at the AR pathway resulting in androgen-independent or hormone-refractory prostate cancer (HRPC). Therefore, there is a significant unmet need for new, non-androgen anti-hormonal strategies for the management of prostate cancer. We demonstrate that a relaxin hormone receptor antagonist, AT-001, an analog of human H2 relaxin, represents a first-in-class anti-hormonal candidate treatment designed to significantly curtail the growth of androgen-independent human prostate tumor xenografts. Chemically synthesized AT-001, administered subcutaneously, suppressed PC3 xenograft growth by up to 60%. AT-001 also synergized with docetaxel, standard first-line chemotherapy for HRPC, to suppress tumor growth by more than 98% in PC3 xenografts via a mechanism involving the downregulation of hypoxia-inducible factor 1 alpha and the hypoxia-induced response. Our data support developing AT-001 for clinical use as an anti-relaxin hormonal therapy for advanced prostate cancer.

Key Words

- prostate cancer
- anti-hormone therapy
- angiogenesis
- relaxin
- relaxin antagonist
- androgen independence
- docetaxel
- tumor xenografts

Introduction

Interfering with the androgen receptor (AR) pathway has been the main focus of therapeutic interventions in prostate cancer (Heinlein & Chang 2004). Despite recent advances in clinical development of second-generation anti-androgens, namely, cytochrome P450 17A1 (CYP17A1) inhibitors such as abiraterone acetate (Zytiga)
and AR antagonists such as enzalutamide (Xtandi) and ARN-509 (currently in clinical development), resistance to these therapies still develops rapidly (Joseph et al. 2013, Korpal et al. 2013, Yuan et al. 2013). As the cancer progresses, there is a shift from androgen dependence to pathways that favor proliferation-, survival-, and angiogenesis-promoting signals, often in crosstalk with the AR pathway (Reis 2012). More than 40% of prostate cancer patients will develop hormone-refractory prostate cancer, with the average survival estimated at just 18 months (Gulley et al. 2003). Therefore, targeting other non-androgen pathways in advanced prostate cancer represents an unmet clinical need, and the relaxin hormone pathway is one such pathway that is implicated in the progression of advanced prostate cancer (Hombach-Klonisch et al. 2006, Feng et al. 2007).

Relaxin increases prostate tumor growth by virtue of its ability to induce cellular proliferation and increase tumoral blood vessels, which are critical for tumor growth and viability (Silvertown et al. 2003, Hombach-Klonisch et al. 2006, Feng et al. 2007). The role of relaxin in prostate cancer progression is increased upon transition to androgen independence. Three potential pathways may be involved: i) mutations in the tumor-suppressor gene p53 (TP53), which facilitates androgen-independent tumor growth – the relaxin pathway has been implicated in driving this process (Thompson et al. 2006, Vinall et al. 2006); ii) relaxin stimulates cell proliferation, angiogenesis (via upregulation of VEGF), extracellular matrix remodeling, tumoral blood flow, and apoptosis (Silvertown et al. 2003, 2007, Hombach-Klonisch et al. 2006); and iii) relaxin can drive the activation of the AR signaling pathway via an intracellular mechanism that is independent of external stimulation by androgens. In the absence of androgen, relaxin promotes downstream AR signaling via crosstalk with the Wnt signaling pathway (Vinall et al. 2006, Liu et al. 2008). Therefore, blocking relaxin would engender a first-in-class anti-hormonal therapy with both anti-relaxin and anti-androgen functional characteristics. Besides a well-characterized role in prostate cancer, relaxin has also been implicated in a number of other human cancers, including breast (Tashima et al. 1994), thyroid (Hombach-Klonisch et al. 2006), endometrium (Kamat et al. 2006), as well as bone, liver, and esophagus.

We and others have shown that modification of the H2 relaxin hormone in its receptor-binding domain (RBD) renders it a receptor antagonist, thereby neutralizing endogenous H2 relaxin signaling (Silvertown et al. 2007, Hossain et al. 2010). We showed that recombinant human H2 relaxin receptor antagonist expressed from prostate cancer xenografts results in significantly impaired tumor growth (Silvertown et al. 2007). The current work describes the development and characterization of chemically synthesized AT-001, a relaxin receptor antagonist, and the effect of its systemic administration on the growth of aggressive, androgen-independent human prostate cancer tumor xenografts. We further show that AT-001 treatment synergistically combines with standard first-line chemotherapy, docetaxel (Garmey et al. 2008, Carles et al. 2012), and that hypoxia-induced response plays a key role in the underlying mechanism.

Subjects and methods

Cell culture and other reagents

Human prostatic adenocarcinoma PC3 (CRL-1435), human embryonic kidney 293 (HEK293) (CRL-1573), and the HEK293-derived 293T (CRL-3216) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured as described previously (Hombach-Klonisch et al. 2006). PC3 cell line was authenticated by STR profiling by The Centre for Applied Genomics (The Hospital for Sick Children, Toronto, ON, Canada) using the AmpFLSTR Identifier PCR Amplification Kit (Life Technologies) (Amelogenin: X; CSF1PO: 11; D13S317: 11; D16S539: 11; D18S51: 14,15; D19S433: 14; D21S11: 29,31,2; D2S1338: 18,20; D3S1358: 16; D5S818: 13; D7S820: 8; D8S1179: 13; FGA: 24; THO1: 6,7; and TPOX: 8,9; vWA: 17; 92% match with the ATCC STR Database). HEK293.RXFP1 and 293T.RXFP1 stably transfected cell lines were obtained from parental HEK293 and 293T cell lines, respectively, by transfection of the pCDNA3.1/Zeo-LGR7 (relaxin/insulin-like family peptide receptor 1 (RXFP1)) plasmid (Silvertown et al. 2006a). HEK293.RXFP1.mGL.2 (clone #1) cell line was derived by stable transfection of HEK293.RXFP1 cells with the pCRE-mGL.2 plasmid (Xactagen, Shoreline, WA, USA), carrying membrane-anchored Gausia luciferase (Verhaegent & Christopoulos 2002) (see Supplementary Materials and Methods, see section on supplementary data given at the end of this article). Docetaxel (Cayman Chemical, Ann Arbor, MI, USA) was formulated in anhydrous ethanol, combined with polysorbate 80 to give a 50 mg/ml concentrated stock solution, and, before injection, diluted in 5% dextrose in water to a final concentration. Lyophilized H2 relaxin (Bachem, Torrance, CA, USA) and AT-001 were reconstituted in a 20 mM sodium acetate, pH
Radioligand-binding assay (RBA) was performed to determine $K_i$ binding constants for H2 relaxin and AT-001, respectively, as following: $10^5$ 293T.RXFP1 cells were seeded per well of a 24-well tissue culture plate and cultured overnight. Before the assay, culture media were removed, cells were washed with HEPES binding buffer (HBB, 100 mM NaCl, 5 mM KCl, 1.3 mM MgSO$_4$, 1 mM EDTA, 10 mM glucose, 15 mM NaOAc, 100 mM HEPES, 1% BSA, and pH 7.4), and replaced with warm HBB. $^{125}$I-labeled H2 relaxin, and ‘cold’ H2 relaxin or AT-001 were added together at a range of concentrations and incubated for 2 h at room temperature. A saturating concentration of ‘cold’ H2 relaxin (500 nM) was utilized to evaluate non-specific binding. Following the incubation, cells were washed twice with HBB and lysed with 1 M NaOH for 30 min at room temperature. Lysates were collected and analyzed on a GammaMaster 1277 gamma counter (LKB, Wallac, Finland).

Relaxin bioactivity assay and competitive inhibition assay: Gaussia luciferase-based reporter assay

Stimulation of cAMP and competitive inhibition of H2 relaxin-mediated cAMP stimulation by AT-001 were assessed as following: $5 \times 10^4$ HEK293.RXFP1.mGL. Two cells were seeded per well of a 96-well tissue culture plate and cultured overnight. Media were changed 30 min before the addition of treatment samples. Pre-mixed, concentrated treatment samples were then added to each well to yield a final concentration of 50 nM 3-isobutyl-1-methyl-xanthine (IBMX), and the vehicle or desired treatment concentration of H2 relaxin, AT-001, or H2 relaxin and AT-001. All treatments were performed in at least duplicate. Following stimulation for 6 h at 37 °C in a humidified atmosphere with 5% CO$_2$, cells were allowed to equilibrate to room temperature and were immediately analyzed on a Luminoskan Ascent Microplate Luminometer (Thermo Fisher Scientific) equipped with an auto-dispenser by addition of coelenterazine substrate to a final concentration of 10 μM in a flash-assay, kinetic format. Integration time was 2000 ms, with two to three measurements performed for each well.

Tumor xenograft animal models in mice

Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice (NOD.CB17-Prkdcscid, males, 6–8 weeks old) were purchased from and maintained at the Animal Resource Centre (University Health Network, Toronto, ON, Canada).

For the efficacy study, animals were injected subcutaneously on day 0 into their right dorsal flank with $2.5 \times 10^6$ PC3 cells, suspended in 100 μl PBS and 100 μl Matrigel Basement Membrane Matrix (354234, BD Biosciences, San Jose, CA, USA) per injection. Animals were treated starting on day 1. Animals were randomized into four treatment groups. Animals in drug-treated groups received a three-times a week (TIW) dose of 3, 0.3, or 0.03 mg/kg of AT-001 (in 20 mM sodium acetate buffer, pH 5.0) subcutaneously, for 8 weeks starting at day 1. Animals in vehicle-treated group received an equivalent volume of the 20 mM sodium acetate, pH 5.0, buffer by s.c. injection. Tumor volume was assessed by caliper measurements weekly and approximated using the formula: length $\times$ width $\times$ height $\times \pi / 6$. Animals were killed on week 8; tumors were then harvested, photographed, measured with a caliper, weighed, and processed for subsequent analyses.

For the combination treatment study, animals were injected as above with $3 \times 10^5$ PC3 cells, and randomized into four treatment groups. Animals in the AT-001- and AT-001/docetaxel-treated groups received a TIW dose of 0.3 mg/kg AT-001 (in 20 mM sodium acetate buffer, pH 5.0) subcutaneously, for 5 weeks starting at week 2.5 (middle of week 3). Animals in the docetaxel- and AT-001/docetaxel-treated groups received four weekly (Q7D×4) i.v. injections of 10 mg/ml docetaxel. Animals in vehicle-treated group received an equivalent volume of the 20 mM sodium acetate, pH 5.0, subcutaneously. Animals were killed on week 8 and processed as above.

Immunohistochemistry

A portion of each harvested tumor was fixed in neutral buffered formalin, paraffin embedded, sectioned, and several sections from each tumor were stained with a 1:1000 dilution of the rabbit anti-mouse CD31 (PECAM-1) antibody (SC-1506-R, Santa Cruz Biotechnology, Inc.) to
visualize vascular endothelium. Slides were stained on the Ventana Benchmark XT stainer (Ventana Medical Systems, Tucson, AZ, USA) using the iVIEW DAB detection system (Ventana Medical Systems). Intratumoral and extratumoral average microvessel areas (MVAs) were determined for each tumor by identifying four intratumoral and four extratumoral regions of increased density of vascular endothelial cells, termed ‘hot spots’, from two to three serial tumor cross sections, and measuring, under a 200× magnification, the cross-sectional areas of all microvessels in each region. The areas of three largest microvessels were averaged for each region analyzed, and the average across analyzed regions yielded the respective MVAs. Intratumoral and extratumoral microvessel densities (MVDs) were determined by counting the number of stained microvessels in each ‘hot spot’ analyzed (normalized to unit area). Total MVAs and MVDs for each treatment group were calculated by averaging the MVAs and MVDs, respectively, from each tumor within the group. All sections were examined using a Leica DM1000 phase-contrast microscope (Leica Microsystems, Concord, ON, Canada), and images were captured at 200× magnification using a Leica EC3 camera and analyzed using the Leica Application Suite Software (ver. 4.0.0).

Western blotting
A portion of each tumor was flash frozen, and a small portion was resected and lysed by sonication in RIPA lysis buffer (Thermo Fisher Scientific) containing a protease inhibitor cocktail (Complete Ultra Mini Tables, EDTA-free, Roche) and 1 mM phenylmethanesulfonyl fluoride (Sigma–Aldrich). For each treatment group, an equivalent amount of protein lysate was pooled by total protein content from each tumor for a total of 150 μg/group. Pooled lysates were resolved by 7% SDS–PAGE in duplicate and blotted onto a PVDF membrane (Immobilon-P, EMD Millipore, Billerica, MA, USA). Membranes were blocked in 5% non-fat dried milk (NFDM) in 0.05% Tween 20–PBS (T–PBS) at 4°C overnight. Membranes were probed in 1% NFDM in T–PBS for either hypoxia-inducible factor 1 alpha (HIF1α) with 10 μg/ml monoclonal mouse anti-HIF1α antibody (clone #241809, R&D Systems, Minneapolis, MN, USA) or a 1:5000 dilution of the monoclonal mouse anti-α-tubulin (Sigma–Aldrich) as a loading control for 2 h at room temperature. Subsequently, membranes were probed with a 1:3000 dilution of the secondary goat anti-mouse IgG–HRP conjugate antibody (Bio-Rad) in 1% NFDM in T–PBS for 1 h at room temperature. Membranes were developed using the SuperSignal West Pico Substrate Kit (Pierce, Thermo Fisher Scientific) and exposed on film. Densitometry was performed on a film with moderate exposure using the Image Lab Software (ver. 3.0, Bio-Rad).

RNA disruption assay
A portion of each harvested tumor, ∼10 mg in size, was preserved in the RNAlater fixative (Life Technologies). Following permeation and stabilization in RNAlater overnight at room temperature, samples were frozen for storage until analysis. Total RNA was isolated using the Qiagen miRNeasy Kit (Qiagen) from individual samples and analyzed using an Agilent Bioanalyzer 2100 (Agilent, Pleasanton, CA, USA). Data from the resulting RNA electropherograms were analyzed using proprietary algorithms and an RNA disruption index (RDI) value was established for each RNA isolate.

Ethics statement
All animal experimental procedures followed a protocol approved by the Animal Care Committee of the University Health Network (Toronto, ON, Canada). Animals were maintained at the Animal Resource Centre (University Health Network) colony at the Canadian Blood Services Building.

Statistical analysis
Statistical significance between groups was evaluated by Student’s t-test or one-way ANOVA analyses, with Dunnett’s post hoc comparison test, as applicable, using GraphPad Prism (ver. 5.0, GraphPad Software, San Diego, CA, USA). The log-rank (Mantel–Cox) test was used for the comparison of survival curves.

Results
Synthesis and characterization of AT-001’s binding and antagonist properties
AT-001 is a synthetic analog of human H2 relaxin with Arg13 and Arg17 in the RBD within the B-chain substituted with lysine residues (Silvertown et al. 2007, Hossain et al. 2010). To confirm that the chemically synthesized AT-001 retained its binding ability to the primary relaxin receptor, RXFP1, H2 relaxin was labeled with 125I using the Pierce iodination reagent (Millar & Smith 1983), which radioiodinates the phenol aromatic ring of Tyr residues (Tyr3 on the A-chain). In an in vitro cell-based assay utilizing the 293T.RXFP1 cell line (engineered to overexpress RXFP1), we
showed that H2 relaxin binds to RXFP1 with a $K_d$ of 17.0 nM in a homologous competitive RBA format (Fig. 1A and B). Similarly, AT-001 was shown to retain the binding capacity for RXFP1 in a competitive RBA, inhibiting binding of 125I-labeled H2 relaxin with a $K_i$ of 9.242 µM (Fig. 1A and B).

To confirm that AT-001 exhibits impaired signaling properties, we evaluated its ability to stimulate cAMP signaling, the standard measure of activation of the relaxin pathway. H2 relaxin was able to stimulate cAMP signaling in a dose-dependent manner with an EC$_{50}$ of 24.9 pM (Fig. 1C and D). In contrast to H2 relaxin, AT-001 exhibited limited activity up to micromolar concentrations, with an EC$_{50}$ of 2.17 µM in the same assay (Fig. 1C and D), confirming previous findings about this modification of
the RBD (Silvertown et al. 2007, Hossain et al. 2010). Partial agonist properties of AT-001 can result in a basal level of signaling that would be dependent on the relaxin receptor density and sensitivity of the stimulated cells, consistent with the in vitro observation on the HEK293.RXFP1 cells (Fig. 1C), which are engineered to express supra-physiological levels of RXFP1. The ability of AT-001 to act as a competitive antagonist to human H2 relaxin on these cells was evaluated by measuring cAMP stimulation by 100 pM H2 relaxin (~80% of maximal cAMP response) in the presence of increasing concentrations of AT-001 (Fig. 1E). AT-001 suppressed signaling induced with 100 pM H2 relaxin by ~25% at AT-001 concentrations >1 nM (Fig. 1E). Taken together, these results confirmed in vitro that AT-001 binds to RXFP1 but exhibits significant functional impairment in signaling capacity.

AT-001 suppresses growth of aggressive, androgen-independent PC3 prostate carcinoma-derived xenografts in vivo

We have previously shown that a recombinant form of AT-001, expressed in PC3 tumor xenografts, suppressed tumor growth (Silvertown et al. 2007). PC3 cells express RXFP1 (Silvertown et al. 2006b, 2007), secrete H2 relaxin in vivo (Silvertown et al. 2006b), and express H2 relaxin transcripts (Gunnersen et al. 1995, Feng et al. 2007). To support AT-001 as a drug candidate for tumor growth suppression to be delivered subcutaneously, a similar tumor xenograft model was employed. NOD/SCID mice were inoculated with the androgen-independent PC3 human prostate cancer cell line in their right flank and treated with either vehicle control or increasing doses of AT-001 delivered by s.c. injection three times a week. Pharmacokinetic studies of i.v. vs s.c. H2 relaxin have shown that
s.c. administration results in a sustained release with a significantly longer serum half-life (Moore & Wroblewski 1992). At all doses evaluated, AT-001 was able to significantly suppress tumor growth compared with vehicle-treated control by up to 60% (Fig. 2A), with a % T/C of up to 40% (Fig. 2B) and a tumor growth delay of ~2 weeks. The lowest dosing of AT-001 examined, 0.03 mg/kg TIW, was sufficient to achieve a close-to-maximum tumor growth suppression in this model (Fig. 2A and B). In vivo caliper-based tumor volume measurements were consistent with in vivo measurements of tumor volumes (Fig. 2C) and weights (Fig. 2D) on harvested tumors. All tumors were photographed, and representative tumors are shown (Fig. 2E), demonstrating the significant reduction in tumor volumes observed in the AT-001-treated groups compared with vehicle-treated controls. No toxicity or adverse events were observed due to AT-001 injections.

Anti-angiogenic properties of AT-001 contribute to impairment of tumor growth

We have previously shown that antagonizing the relaxin signaling pathway in prostate tumor xenografts exerts an anti-angiogenic effect as one of the contributing mechanisms to impairing tumor growth (Silvertown et al. 2007). To examine the impact of AT-001 on tumoral microvasculature, AT-001- and vehicle-treated tumors were harvested and analyzed by immunohistochemistry to evaluate the intratumoral and extratumoral (immediately surrounding the tumor boundary) microvasculature by staining for mouse CD31 (PECAM-1), a marker for endothelial cells. Both average extratumoral and intratumoral mean MVAs (Fig. 3A and B respectively) of the AT-001-treated tumors were significantly reduced compared with tumors from the vehicle-treated group. However, no significant reduction was detected in MVD of the tumors (extratumoral) and (D) within the tumors (intratumoral). Data represent mean ± S.E.M. (n = 9–10). Statistical significance is indicated (*P < 0.0001, treatment vs vehicle control). (C and D) Mean microvessel density (MVD) of the extratumoral and intratumoral microvasculature (Fig. 3A and B respectively) of the AT-001-treated tumors were significantly reduced compared with tumors from the vehicle-treated group. However, no significant reduction was detected in MVD of the tumors (extratumoral) and (D) within the tumors (intratumoral). Data represent mean ± S.E.M. (n = 9–10). Statistical significance is indicated (*P < 0.0001, treatment vs vehicle control). (C and D) Mean microvessel density (MVD)
AT-001 synergizes with chemotherapeutic agents acting on the hypoxia-induced response

The hallmark of anti-angiogenic therapy is the creation of a hypoxic environment in the tumor, which triggers the hypoxia-induced response (Rapisarda & Melillo 2009). This leads to an adaptation to hypoxic growth by a number of different mechanisms, including compensatory angiogenesis (Gu et al. 2006), autophagy (Hu et al. 2012), metabolic changes (Keunen et al. 2011), and increased invasiveness (Pennacchietti et al. 2003). Transient effectiveness and resistance to anti-angiogenic therapy have been documented in numerous models, including in PC3 cells (Voss et al. 2010, Weisshardt et al. 2012). Therapeutics with an anti-angiogenic mechanism of action can greatly benefit from agents that specifically target the hypoxia-induced response. The transcription factor subunit HIF1α (HIF1A) is a master regulator of the hypoxia-induced response (Semenza 2002), is upregulated by androgen (Mabjeesh et al. 2003), and plays an important role in the progression and aggressiveness of androgen-insensitive prostate cancer (Jeong et al. 2012). Several agents achieve significant inhibition of HIF1α and the hypoxia-induced response, including a variety of microtubule-targeted drugs such as docetaxel (Escuin et al. 2005, Forde et al. 2012). This study determined that the combined treatment of androgen-insensitive xenografts with AT-001 and docetaxel results in synergistic efficacy. NOD/SCID mice were treated with vehicle-treated control, AT-001, docetaxel, or both AT-001 and docetaxel at 2.5 weeks after inoculation with PC3 cells. Guided by the observed efficacy of AT-001 in suppressing tumor growth (Fig. 2), a 0.3 mg/kg dose level was employed. AT-001 and docetaxel treatments alone significantly suppressed tumor growth by ~40 and 95% respectively (Fig. 4A and C). The combination of AT-001 and docetaxel treatment (>98% suppression of tumor growth) resulted in a significantly more impaired tumor growth when compared with the docetaxel only-treated tumors (Fig. 4B), suggesting that AT-001 and docetaxel acted in concert. Endpoint ex vivo tumor volume measurements on harvested tumors confirmed significantly smaller tumors in the combination treatment group (27.03 ± 9.73 mm³) compared with the docetaxel-treated group (48.31 ± 4.63 mm³).

To determine whether AT-001 and docetaxel synergized in impairing prostate cancer xenografts in the PC3 model, we needed to delineate the relative contribution of the different mechanisms of action of each treatment. Expression of HIF1α in tumor lysates from each group was examined. HIF1α is rapidly degraded under normoxic conditions, but is upregulated under hypoxic conditions (Cheng et al. 2007). Vehicle-treated PC3 tumors had detectable HIF1α expression (Fig. 5A), consistent with a hypoxic tumor environment that would trigger the hypoxia-induced response in these cells, which are also

![Figure 4](image-url)

**Figure 4**
AT-001 enhances the efficacy of docetaxel chemotherapy in PC3 tumor xenografts. (A) Xenografts were established by implantation of 3 × 10⁶ PC3 cells into the right dorsal flank of male NOD/SCID mice (n = 10–11). Starting in the middle of weeks 3, animals were treated TIW for 5 weeks with vehicle (open circles), or 0.3 mg/kg AT-001 subcutaneously with (semi-closed squares) or without (closed squares) docetaxel. Animals in docetaxel only (open squares) and docetaxel + AT-001 (semi-closed squares) treatment groups were treated intravenously with four-times weekly (Q7D) injections of 10 mg/kg docetaxel. Tumor dimensions (length, width, and depth) were measured weekly with a caliper to estimate the volume. Data represent mean ± SEM. Statistical significance is indicated (*P < 0.05 or **P < 0.001, treatment vs vehicle control). (B) Tumor volumes in the docetaxel-treated groups, with (closed squares) or without (open squares) AT-001 treatment. Data represent mean ± SEM. Statistical significance is indicated (**P < 0.01 or ***P < 0.001, treatment vs vehicle control). (C) Percentage of treatment over control (% T/C) for each treatment group, calculated as the mean relative tumor volume (RTV) of the treatment group over the mean RTV of the control group multiplied by 100%. Data represent mean ± SEM. (D) Kaplan–Meier survival analysis of mice bearing tumors in each treatment group (mice bearing tumors of >1000 mm³ in volume were considered as moribund). Statistical significance is indicated (**P < 0.01 or ***P < 0.001, treatment vs vehicle control).
known to express high HIF1a even under normoxic conditions (Zhong et al. 1998). AT-001-treated tumors exhibited a threefold increase in HIF1a expression over vehicle-treated controls (Fig. 5A), suggesting that the anti-angiogenic activity of AT-001 has triggered an increased hypoxia-induced response. Docetaxel treatment significantly downregulated the expression of HIF1a in treated tumors (Fig. 5A), reducing the expression of HIF1a by >65% compared with the vehicle-treated control group. Moreover, docetaxel acted synergistically with AT-001 by decreasing HIF1a expression in the combination treatment group by ~90% compared with the AT-001-treated group (Fig. 5A). Docetaxel likely increased the sensitivity of treated tumors to the anti-angiogenic effects of AT-001, while concomitantly downregulating the compensatory angiogenesis promoted by HIF1a.

To delineate the contribution of anti-angiogenic and other effects mediated by AT-001 from the cytotoxic activity of docetaxel, we evaluated the disruption of rRNA in the harvested tumors by the RNA disruption assay. RNA disruption is associated with tumor response to chemotherapy, including such cytotoxic agents as docetaxel (Parissenti et al. 2010). Consistent with this, we observed a significant increase (greater than threefold) in the RDI, a measure of rRNA disruption, in the docetaxel-treated tumors (Fig. 5B). No increase in RDI was induced by AT-001, as evidenced from tumors treated with AT-001 alone (Fig. 5B), indicating that RDI is only sensitive to the effects of docetaxel. The RDI value increased by more than sevenfold in the combination treatment group of AT-001 and docetaxel (Fig. 5B) compared with the vehicle-treated control groups or AT-001-treated groups. AT-001 treatment clearly potentiated the effects of docetaxel, as reflected in the results of this assay. Taken together, this indicates that AT-001 synergized with docetaxel to result in increased RNA disruption in tumors concomitantly treated with both agents. These data parallel the finding that a greater decrease in tumor mass was observed in the combination treatment group compared with either treatment alone (Fig. 4A and B).

**Discussion**

In the previous work, we and others have demonstrated that suppressing the relaxin signaling pathway in androgen-sensitive (LNCaP) and androgen-insensitive, AR-negative (PC3) prostate cancer xenografts impairs their growth in vivo (Silvertown et al. 2007, Feng et al. 2010). In this study, we functionally confirmed the in vivo efficacy of a new anti-hormonal drug candidate, AT-001, targeting the relaxin hormone signaling pathway in a prostate cancer model. AT-001, an antagonist of the human H2 relaxin receptor, exhibits significant potency in impairing tumor growth in an aggressive model of androgen-insensitive prostate cancer. PC3 tumor xenografts are devoid of AR expression (van Bokhoven et al. 2003) and represent a model of an advanced, androgen-
independent form of human prostate cancer for which few treatment options are currently available. Owing to the lack of AR expression in PC3 cells, other anti-hormonal drugs targeting the AR pathway (anti-androgen class), such as CYP17 inhibitors (e.g. abiraterone and galeterone) and AR antagonists (e.g. enzalutamide and ARN-509), have or are expected to have demonstrated limited to no efficacy in androgen-independent cells or tumor xenografts (Mostaghel et al. 2011, Zhang et al. 2011, Clegg et al. 2012). The efficacy demonstrated by AT-001 in the PC3 model (tumor reduction by 60%), which is highly resistant to hypoxia-induced apoptosis (Coffey et al. 2005), surpasses the efficacy observed in this model with other types of anti-angiogenic agents, such as bevacizumab (Newman et al. 2007, Xu et al. 2012), likely owing to the fact that AT-001 is antagonizing multiple pathways downstream of relaxin receptor signaling (Fig. 6) (Silvertown et al. 2007). Treatment with increasing doses of AT-001 appeared to reach a plateau in the current prostate cancer model, suggesting that, even at the lower dose levels in this study, AT-001 may have completely antagonized the contribution of the relaxin signaling pathway to promoting the growth of PC3 tumors. AT-001 synergized with docetaxel chemotherapy to curb PC3 tumor growth via a mechanism that involved the down-regulation of hypoxia-induced response. When combined with AT-001, docetaxel dosing regimens could be lowered, reducing the risk of chemotherapy-associated toxicity in prostate cancer patients. It is conceivable that AT-001 could be successfully combined with other chemotherapeutic agents that downregulate HIF1α to synergistically curb the growth of aggressive tumors.
Other strategies targeting RXFP1 have also demonstrated suppression of tumor growth (Feng et al. 2010); however, targeting cells expressing RXFP1 by conventional approaches, such as mAbs therapeutics, is not an optimal therapeutic strategy given the broad pattern of RXFP1 expression in a multitude of normal tissues beyond the prostate (Hsu et al. 2000, Ivell et al. 2003). Compared with other biological therapeutics, such as mAbs, AT-001 is sufficiently small to result in good tumor penetration. Development of small molecule-based RXFP1 antagonists would not be trivial given the lack of X-ray crystal structure and appropriate bioassays, and the general difficulty associated with developing small molecules that modulate GPCRs (Fujioka & Omori 2012). However, some progress has recently been made in developing a small-molecule agonist of RXFP1 (Xiao et al. 2013).

Collectively, the presented data support the clinical development of AT-001 as a new candidate class of anti-hormonal drugs, either as a monotherapy or as a combination therapy with other chemotherapeutic agents and/or anti-hormonal drugs, in particular, those targeting the AR pathway (Fig. 6). AT-001 is unique in that it dually targets two hormonal pathways – androgen and relaxin. Unlike second-generation anti-androgens, anti-relaxin therapies could conceivably be continued once prostate cancer transitions to androgen independence and could be given in combination with aggressive chemotherapeutic agents when the cancer becomes metastatic (stage III and beyond). Therefore, relaxin receptor antagonists offer the potential for patients to continue hormone therapy well beyond the point where prostate cancer becomes insensitive or resistant to anti-androgen therapies. With the majority of anti-hormonal drugs for prostate cancer targeting the AR pathway, an anti-relaxin therapy represents a promising new approach for the clinic.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-14-0088.

Declaration of interest
A Neschadim and J D Silvertown are employees of Armour Therapeutics, Inc. (Toronto, ON, Canada). L B Pritzker and K P H Pritzker are employees of RNA Diagnostics, Inc. (Toronto, ON, Canada). D R Branch, A J S Summerlee, and J Trachtenberg have no conflicts of interest to declare.

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