Effects of treatment with androgen receptor ligands on
microRNA expression of prostate cancer cells

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Abbreviations: microRNA (miRNA), androgen receptor (AR), bicalutamide (BIC), dihydrotestosterone (DHT), mibolerone (MIB), prostate-specific antigen (PSA), androgen responsive elements (AREs), vascular endothelial growth factor (VEGF), epithelial growth factor (EGF)

Abstract

Post-transcriptional regulation by microRNA (miRNA) is an important aspect of androgen receptor (AR) signalling in prostate cancer cells. However, the global profiling of miRNA expression in prostate cancer cells following treatment with AR ligands has not been reported so far. In this study we examined the effect of treatment with two AR agonists (mibolerone (MIB) and dihydrotestosterone (DHT)) and an AR antagonist (bicalutamide (BIC)) on miRNA expression in the human androgen-dependent LNCaP prostate cancer cell line using microarray technology and verification of selected miRNA using quantitative real-time PCR (qRT-PCR). No miRNA were identified as differentially expressed following treatment with the AR antagonist BIC. In contrast, a number of common and compound-specific alterations in miRNA expression were observed following treatment with AR agonists. Unexpectedly it was found that treatment with the AR agonists resulted in the repression of miR-221, a miRNA previously established to be involved with prostate cancer development. This observation indicates that this miRNA may have a more complex role in prostate cancer development than considered previously. Treatment with MIB led to an induction of miR-210 expression, a hypoxia-related miRNA. This miRNA is reported to be involved in cell adaptation to hypoxia and thus induction in conditions of normoxia may be important in driving metabolic changes observed in prostate cancer. Thus examining the effect of AR agonists and antagonists on miRNA expression can provide novel insights into the response of cells to AR ligands and subsequent downstream events.

Introduction

The androgen receptor (AR) is a member of the steroid hormone receptor family that is expressed in various types of tissues. Activation of AR intracellular signalling pathways by natural androgens such as testosterone and dihydroxytestosterone (DHT) is necessary for the development and maintenance of male characteristics in vertebrates (Heinlein & Chang, 2002). The prostate in particular is an organ that requires the action of androgens on the AR in order to develop and function. The dependency of prostate cells on androgen-mediated AR signalling for development and maintenance is also retained in the majority of prostate tumours (Heinlein & Chang, 2004). AR antagonists inhibit the effect of AR, acting by a variety of mechanisms such as competitive binding to the AR and the blocking of androgen synthesis. Given the dependency of prostate cancer cells on AR signalling for growth and survival, AR inhibitors are valuable clinical tools for the treatment of prostate cancer patients (Heinlein & Chang, 2004).

It is being increasingly recognized that post-transcriptional regulation by non-coding miRNA is an important mechanism by which the activated AR modulates the phenotype of prostate cells. In the setting of prostate cancer AR regulated miRNA have been reported to be associated with the development and severity of the disease. Examples include the miR-99/let-7c/miR-125b miRNA cluster involved in the regulation of multiple androgen-induced genes (Sun *et al.*, 2014), the epigenetically repressed miR-31 which is a direct regulator of the AR (Lin *et al.*, 2013), and miR-221 which has been linked to the progression of prostate cancer cells to a state of androgen independence (Sun *et al.* 2009; Sun *et al.*, 2014). Studies aiming to investigate the role of miRNA in AR signalling and prostate cancer have taken a variety of approaches. These include the identification of miRNA that target the AR receptor (Östling *et al.*, 2011), comparison of androgen sensitive and insensitive cell lines (Ottman *et al.*, 2014), comparison of miRNA expression between metastatic and non-metastatic prostate cancer cell lines (Saini *et al.*, 2011), and examination of miRNA expression in prostate tumour samples (Szczyrba *et al.*, 2010).

1 In this study we used a different approach, investigating the effect of direct treatment
2 with two AR agonists and one AR inhibitor on the expression of miRNA in LNCaP
3 cells, an androgen-dependent prostate cancer cell line, using miRNA microarray
4 followed by verification of individual differentially expressed miRNAs using qRT-
5 PCR. This approach has a number of advantages. Firstly, it allows the direct
6 comparison of the effect of treatment with AR agonists and antagonists on the
7 miRNA expression of prostate cancer cell lines. Secondly, it can be used to determine
8 compound-specific effects on miRNA expression. Lastly, it allows the identification
9 of novel AR-regulated miRNA and miRNA implicated in the AR regulatory network.
10 We have used DHT, the most potent endogenous androgenic compound (Luu-The V
11 *et al.*, 2008) and mibolerone (MIB), a potent non-metabolisable synthetic androgen
12 agonist, originally used by veterinarians to prevent oestrus in female dogs
13 (Sokolowski and Geng, 1977). MIB has been used extensively in cell culture assays
14 to study effects of the AR. Additionally we have examined bicalutamide (BIC), also
15 known as Casodex®, a therapeutic non-steroidal androgen antagonist used as part of a
16 regimen in the treatment of androgen-sensitive prostate cancer (Fradet, 2004).
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31 **Materials & Methods**

32 **Cell culture**

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35 All cell culture reagents were supplied by Life Technologies (Paisley, UK) unless
36 otherwise stated, and cells were propagated in Corning® standard tissue culture
37 plastic-ware. Human LNCaP cells were purchased from ATCC at passage number 17
38 and experiments were conducted within 10 passages of the original number. LNCaP
39 were routinely cultured in complete media consisting of phenol red-free minimum
40 essential medium supplemented with 10% v/v foetal bovine serum (FBS), 1% v/v
41 non-essential amino acids, 2mM L-glutamine, and 1% Penicillin/Streptomycin
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55 **Cell treatments**

1 BIC and DHT were purchased from Sigma-Aldrich (Gillingham, UK). MIB was
2 obtained from Perkin Elmer (Waltham, MA, USA). Prior to administration of the
3 treatments LNCaP cells were plated at 50,000 cells/mL in steroid-depleted (stripped)
4 media for 2 days, to reduce the amount of endogenous steroids and growth factors,
5 thus potentially lowering the total testosterone by as much as 80% (Sedelaar and
6 Isaacs, 2009). The actions of exogenous steroids can then be assessed without
7 influence from endogenous steroids. The dextran-coated charcoal stripped FBS
8 (dccFBS) was generated as described previously (Papaioannou *et al.*, 2014) and added
9 to the experimental media at a concentration of 5%.

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22 Cell numbers were assessed using a haemocytometer and viability assessed using
23 trypan blue exclusion or using AlamarBlue® assay (AbD Serotec, 2009).
24 AlamarBlue® indicator dye changes colour and fluoresces in response to the chemical
25 reduction of resazurin to fluorescent resorufin by metabolically competent cells (AbD
26 Serotec, 2009); the amount of reduction is directly proportional to the number of cells
27 present in the sample. Samples were examined at various times after treatment.

28 29 30 31 32 33 34 **Enzyme-linked immunosorbent assay (ELISA) assays**

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38 PSA expression was measured using PSA enzyme-linked immunosorbent assay
39 (ELISA) kit PS067T as per the manufacturer's protocol (Calbiotech, Spring Valley,
40 CA, USA). The amount of PSA was determined by comparison to the standard curve
41 which was run concurrently with each set of samples. The cellular supernatant
42 containing the secreted PSA, diluted with distilled water as required, was used to
43 assess PSA secretion. Samples were examined at various times after treatment.

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51 EGF expression was measured using Human EGF ELISA kit KHG0061 as per the
52 manufacturer's recommendations (Life Technologies). EGF expression was
53 determined by comparison to a standard curve.

Hypoxia culturing conditions

Based on previous reports (Giannakakis *et al.*, 2008) 16 hours of 1% O₂ was used to induce hypoxia in LNCaP. Cells were grown to approximately 70% confluence, the media was refreshed (normal culturing media) and flasks transferred to 1% O₂ (hypoxia) or maintained in control conditions (21% O₂ normoxia). After 16 hours, the normoxic and hypoxic cultures were harvested for RNA and protein extraction.

RNA extraction

To prevent RNA degradation by RNases, all reagents were certified RNase-free and RNaseZap® (Life Technologies) was used to decontaminate surfaces and pipettes. Isolation of RNA from cells was performed using TRIzol® (Life Technologies) following the manufacturer's instructions. RNA pellets were re-suspended in 40µL of Tris/EDTA (TE) buffer pH 8.0 and assessed for concentration and quality. All RNA samples used for subsequent work had 260/280 ratios greater than 2.0 and 260/230 ratios greater than 1.8. Additionally, an RNA integrity number (RIN) was generated for each sample using the RNA Nano reagents and an Agilent Bioanalyser (Agilent Technologies, Santa Clara, CA, USA). All samples used for subsequent work had RIN greater than 7.6.

miRNA microarrays

Profiling of global miRNA expression in LNCaP was performed using the Agilent Human miRNA Microarray kit (V3) and microarray platform as described previously (Koufaris *et al.*, 2012). On each microarray 851 human miRNAs were present, consisting of 680 dominant miRNA and 171 *miRNA.

Microarray pre-processing and normalisation

Processing and normalisation of the microarray data were conducted in the R statistical programming language (R version 13.2; <http://www.r-project.org>), using the "AgiMicroRna" software package, version 2.2, available as part of the BioConductor

Project (<http://www.bioconductor.org>). miRNAs were retained in the analysis if they were declared present in at least 75% of samples within at least one experimental condition (treatment and time) and above the threshold (negative control expression level plus 1.5x standard deviation) in at least 25% of samples within at least one experimental condition. The microarray data were normalised using the robust multi-array average method with quantile normalisation. ComBat was used to reduce batch effects (Johnson *et al.*, 2007).

Microarray analysis

For data collected over multiple time points, time course analysis was conducted in AgiMicroRna using Limma with the method of Benjamini-Hochberg used to control the false discovery rate to 30%

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was used to verify differentially expressed miRNAs identified by microarray. For mature miRNA qRT-PCR reactions, pre-designed Taqman probes were used (Applied Biosystems). The PCR reactions were performed in an ABI Prism 7700 Sequence Detection System. All PCR reactions were run in triplicate to provide technical replicates. Relative quantification was performed using the $2^{-\Delta\Delta CT}$ method and using RNU6B as the reference gene. In addition to the no-RT, a 'no-template control' (no-TC) was also run to ensure the reagents were free of contaminants.

The Applied Biosystems High Capacity cDNA reverse transcription kit with RNase inhibitor (Part number 4374966) with the VEGF TaqMan® Gene Expression Assay (Hs00900055_m1) or TaqMan Ribosomal RNA Control Reagents was employed to measure VEGF and 18S levels, respectively.

Results

Effects of DHT, MIB, and BIC treatments on proliferation and PSA secretion in LNCaP cells

Two established end-points and surrogate markers of AR activation in prostate cells are increased proliferation and PSA secretion. We initially examined the effects of DHT, MIB, and BIC on proliferation (**Suppl.Fig.1A-C**) and PSA secretion (**Suppl.Fig.2A-C**) in LNCaP cells. In addition, the ability of a range of BIC doses to inhibit DHT-induced proliferation (**Suppl.Fig.1D**) and PSA secretion (**Suppl.Fig.2D**) were also examined. As expected DHT and MIB treatments induced dose-dependent increases in LNCaP proliferation and PSA secretion. Whilst BIC treatment alone induced a modest increase in proliferation and PSA secretion, in the presence of DHT it had its reported antagonistic effect. The modest induction by BIC may be due to a mismatch between the availability of co-regulators and the amount required for full antagonism (Hodgson et al, 2007).

Based on these results a single concentration of each compound was selected for subsequent study (BIC 10 μ M – therapeutically relevant concentration, DHT 2nM – physiologically relevant concentration, MIB 100pM – induction of maximal proliferative response). The effects of these doses were examined at multiple time points after treatment initiation (4, 8, 24 and 120 hours) in order to determine temporal changes in cell number, PSA secretion, and miRNA expression. The number of LNCaP cells at each treatment time point was first determined (**Fig.1A**). BIC treatment resulted in a similar trend as the vehicle control, namely a slight increase in cell number over the study period, although at 120 hours of BIC treatment there was a statistically significant decrease in cell number (18% reduction compared to vehicle control; $p<0.05$). In the case of DHT a time-dependent increase in cell number was observed, reaching a statistically significant increase above the time-matched control at 120 hours (122% increase compared to vehicle control; $p<0.01$). Treatment with MIB elicited an even more prominent increase in cell number, reaching statistical significance at 120 hours (190% compared to vehicle, $p<0.001$).

In conjunction with the assessment of cell numbers, PSA secretion was assayed at each time point (**Fig.1B**). In BIC and vehicle treated cells a trend for a steady increase in PSA over time was observed. As expected the two AR agonists caused prominent increases in PSA secretion. There was a 7.3-fold increase and 10.3-fold increase in

DHT-treated cells compared with the control at 24 hours and 120 hours, respectively. Similarly, by 24 hours, MIB had induced an 8-fold increase in PSA and, by 120 hours, PSA was 36.2 times higher than observed in the time-matched control.

Microarray profiling of miRNA expression

Having characterised the phenotypic response of LNCaP cells to the AR ligands (**Fig.1; Suppl.Fig.1-2**), we examined the potential role of miRNA in these responses. Microarray analysis was performed on 3 independent cultures per data point to determine the temporal nature of global miRNA expression profiles in the treated cells. Differentially expressed miRNA (DEM) were identified by performing a time course analysis, implemented in the AgiMicroRna package. This methodology allowed the identification of miRNAs which respond differently over time in each treatment group relative to the control. Treatment with the AR antagonist BIC did not significantly alter the expression of miRNA at any timepoint compared with the time-matched control. In contrast, treatment with the AR agonists MIB and DHT resulted in significant changes in the expression of miRNA (**Tables 1-2**). All the miRNA affected by DHT were found to respond at 24 hours of treatment, with their deregulation being consistent at 120 hours. For MIB, miRNA were only determined to be affected by the treatment at 120 hours. Some of the identified DEM were affected by both AR agonists (miR-221, miR-99a, miR-1246, and miR-1290), while others were affected uniquely by one of the tested compounds (e.g. miR-210 for MIB and miR-494 for DHT).

To validate the reliability of the microarray data qRT-PCR was used to assess the expression levels of five miRNA in the same samples used for microarray analysis. This analysis found a high degree of concordance between the levels of miRNA determined by qRT-PCR and microarrays, verifying the reliability of the microarray data (**Fig.2**).

Expression of miR-221

A defining feature of MIB treatment of LNCaP cells was the significant decrease in the expression of miR-221, also seen in DHT-treated cells, but less pronounced

(Tables 1 and 2). Increased expression of miR-221 has been shown to be involved in prostate cancer development and the progression to AR agonist independence (Gilardi *et al.*, 2007; Sun *et al.*, 2009; Kneitz *et al.*, 2014; Sun *et al.*, 2014). Our findings showing a decrease in expression are consistent with the AR responsiveness of LNCaP cells. Thus understanding this responsiveness may offer insights into the role of miR-221 in the progression of AR agonist independence. To investigate this interaction further we examined how expression of miR-221 relates to exposure to a range of MIB doses (from 1pM to 10nM). As expected MIB treatment resulted in a concentration dependent induction of LNCaP proliferation and PSA secretion (**Fig.3A**). Conversely, MIB treatment resulted in a concentration-dependent decrease in the expression of miR-221, up to 4-fold (**Fig.3B**). In these conditions, both PSA and proliferation were significantly inversely correlated with miR-221 expression ($r=-0.8780$ and -0.9053 , respectively, $p<0.05$).

Expression of miR-210

Among the miRNA displaying significant expression changes in response to MIB treatments was miR-210, a miRNA known to be upregulated by hypoxia and involved in driving cellular adaptations to low oxygen levels (Camps *et al.*, 2008; Chan *et al.*, 2009). Indeed, we verified that in the absence of MIB, miR-210 levels in LNCaP cells were increased following hypoxia (**Fig.4A**). As a positive control it was also demonstrated that this treatment also caused the induction of the prototypical hypoxia responsive gene, the vascular endothelial growth factor (VEGF) (**Fig.4B**).

To examine further the link between AR activation and miR-210 induction we examined the effect of doses of MIB on expression of this miRNA (**Fig.4C**) using qRT-PCR. Induction of miR-210 was observed in response to high doses of MIB (1nM and 10nM), but not as a result to treatment with lower doses. MIB treatment also resulted in increased levels of VEGF (**Fig.4D**), with miR-210 and VEGF displaying significantly correlated increases following MIB treatment (Pearson correlation 0.86, $P<0.05$, **Fig.4E**).

Mabjeesh *et al.*, 2003 reported that treatment of LNCaP cells with AR agonists was able to induce the HIF1 α protein in conditions of normoxia *via* an autocrine loop involving increased epidermal growth factor (EGF) production and secretion. This

was therefore considered a potential mechanism by which MIB could induce miR-210 and *VEGF* in conditions of normoxia. However, no EGF was detected in the cell media in LNCaP cells treated with vehicle or various MIB doses (**Suppl.Fig.3**).

Discussion

In this study we examined the effect of treatment with AR agonists and an AR antagonist on miRNA expression in the androgen-dependent LNCaP prostate cancer cell line. To the best of our knowledge this is the first study to directly investigate the temporal effects of AR agonists and antagonists on global miRNA profiles in prostate cancer cells. One obvious limitation of this approach is that it does not take into account the role of non-parenchymal cells in modulating the prostate response to AR signalling. Nevertheless, although *in vitro* studies can only replicate limited aspects of the *in vivo* response to androgens, valuable insights can still be obtained in regards to the involvement of miRNA to AR signalling and thus potentially to prostate cancer development. Another limitation of this study is that for microarray miRNA profiling a single concentration of each compound was studied, at different phases of the proliferation curve, and therefore in future studies it would be of use to study these compounds at concentrations of similar potency.

An intuitive effect of AR antagonists on miRNA expression in prostate cancer cells would be the reversal of the effects of AR agonists. However, it is also possible that AR antagonists have additional effects on miRNA due to the recruitment of alternative co-regulators, activators and repressors, to the AR or by acting through AR independent pathways. An interesting hypothesis is that miRNA specifically affected by AR antagonists can have important phenotypic consequences, such as being involved in the progression of prostate cancer cells to a state of AR independence. To identify potential subsets of miRNA specifically affected by AR antagonists we examined LNCaP cells, cultured in the absence of AR agonists, treated with BIC. The BIC treatment used here was demonstrated to be able to inhibit AR-agonist induced proliferation of LNCaP cells (**Suppl.Fig.1-2**). Nevertheless, in this study no miRNA were identified as being significantly differentially expressed due to BIC treatment alone. These findings are consistent with the phenotypic effects of BIC being the antagonist of AR agonists, with few, if any, distinct effects on miRNA. However,

1 further investigations with more compounds and in different cell lines are required to
2 rule out the possibility that AR antagonists have effects on prostate miRNA
3 expression that go beyond the reversal of AR agonism.
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7 In contrast to BIC, the AR agonists DHT and MIB had clear effects on the expression
8 of miRNA in the LNCaP cells (**Tables 1-2**). Some of the identified miRNA were
9 differentially expressed in response to treatment with both DHT and MIB, while
10 others were responsive to only one of the tested compounds. It is quite likely that the
11 majority of miRNA affected by both AR agonists are under direct regulation by the
12 AR or are associated with the phenotypic changes induced by the compounds. One of
13 the commonly affected miRNA identified here was miR-99a. This miRNA has been
14 demonstrated previously to be under direct transcriptional regulation of the AR and to
15 be involved in the direct post-transcriptional regulation of a number of AR responsive
16 genes (Sun *et al.*, 2014). This miRNA is also a suppressor of proliferation and PSA in
17 prostate cells (Sun *et al.*, 2011). The repression of this miRNA observed following
18 treatment with both MIB and DHT could contribute to the observed increase in
19 proliferation and PSA secretion of LNCaP cells (**Fig.1**).
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32 An interesting finding was that both DHT and MIB treatment caused a significant
33 downregulation of miR-221, a miRNA strongly implicated in prostate cancer (**Tables**
34 **1-2; Fig.3**). Higher expression of miR-221 has been linked to the progression of
35 prostate cancer cells from androgen-dependency to androgen-independent growth
36 (Sun *et al.*, 2009; Sun *et al.*, 2014), with greater proliferation and aggressiveness of
37 prostate cancer cell lines (Galardi *et al.*, 2007), and with worse prognosis in high-risk
38 prostate cancer patients (Kneitz *et al.*, 2014). However, previous studies had not
39 determined how expression of this miRNA is affected by exposure to AR agonists.
40 Unexpectedly, in this study we have found that treatment with AR agonists caused the
41 reduced expression of oncogenic miR-221 in the LNCaP cell line. Interestingly, the
42 sensitivity of miR-221 to MIB doses appears to be the reverse of the effects of the
43 chemical on cell numbers and PSA secretion (**Fig.3**). This observation indicates a
44 more complex role for miR-221 in prostate cancer development than previously
45 recognised. One possible interpretation of this observation is that AR signalling
46 mediated the repression of miR-221 as a mechanism of maintaining proliferative
47 prostate cells in a state of androgen-dependency. In this case these cells will then
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1 return to a quiescent state more rapidly when the AR signalling is terminated. Further
2 experimental work is required to determine the relevance and importance of the AR-
3 miR-221 interaction *in vivo*.
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7 Another interesting miRNA that was affected by both DHT and MIB was miR-1290.
8 This miRNA has previously been reported to be a circulating prognostic marker for
9 castration-resistant prostate cancer (Huang *et al.*, 2014). This miRNA has been linked
10 to inhibition of the cell cycle and promotion of apoptosis in neuronal and lung
11 carcinoma cells by targeting p27 and BCL2 (Yelamanchili *et al.*, 2014; Kim *et*
12 *al.*2014). Interestingly, this is an evolutionary young, great-ape specific miRNA
13 (Yelamanchili *et al.*, 2014). Such species-specific miRNA repertoire may be
14 important determinants of differential species responses to equivalent xenobiotic
15 exposures such as AR agonists (Koufaris & Gooderham, 2013).
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25 We also determined in this study miRNA responses that were specific to only one of
26 the tested compounds. Such compound-specific effects on miRNA could be due to a
27 variety of technical reasons e.g. different potency of AR activation. It is also known
28 that AR agonists can also have distinct biological effects. For example, in addition to
29 effects on AR, MIB can also activate the progesterone receptor besides the AR, which
30 is not the case for DHT (Cops *et al.*, 2008). The differential effects of AR agonists on
31 miRNA could potentially be important in determining differential effects of
32 compounds on prostate cells or assist in identifying mode of action of such chemicals.
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42 An interesting example of a compound specific miRNA response identified here was
43 the induction of miR-210 following MIB treatment (**Fig.4**). This miRNA is activated
44 by hypoxia and is involved in driving cell adaptations to lack of oxygen (Giannakakis
45 *et al.*, 2008; Chan *et al.*, 2009). In a previous study it has been reported that
46 expression of miR-210 is induced in prostate cancer cell lines exposed to hypoxia
47 (Quero *et al.*, 2010). However, here it was demonstrated that MIB could induce this
48 miRNA in cell culture conditions with adequate oxygen concentrations, suggesting
49 functions for this miRNA beyond its established role in hypoxia. The effect on miR-
50 210 was only observed at high doses of MIB (**Fig.4**). Consequently the lack of
51 response to DHT in this case may reflect the lower potency of the compound to
52 activate the AR. We did not clarify here the mechanism by which AR agonists can
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1 activate miR-210 in conditions of normoxia, but activation of miR-210 *in vivo* could
2 be important in driving the metabolic changes observed in prostate cancer (Vaughan
3 *et al.*, 2013).
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7 Prostate cancer is currently the most commonly diagnosed cancer in males and a
8 prevalent cause of global mortality. Consequently there is considerable interest in
9 delineating the molecular mechanisms implicated in the initiation and progression of
10 the disease. Research into the contribution of miRNA to prostate cancer development
11 holds considerable promise. Firstly, miRNA that are key mediators of AR signalling
12 could be novel therapeutic targets (Deng *et al.*, 2013). Secondly, circulating miRNA
13 could prove to be easily accessible biomarkers for the disease, either in plasma (Lin *et*
14 *al.*, 2014) or in prostate fluid (Selth *et al.*, 2014). This study demonstrates that direct
15 profiling of the effects of AR agonists and antagonists on miRNA expression is a
16 methodological approach that can assist towards clarifying the role of these genes in
17 AR signalling and prostate cancer.
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Figures & Tables

Fig.1 Effects of BIC, DHT, and MIB treatments on proliferation and PSA secretion in LNCaP cells over time (A) Effect of vehicle control and DHT, MIB, and BIC on LNCaP cell numbers with time; (B); Effect of vehicle control and DHT, MIB, and BIC on LNCaP PSA secretion with time. Presented as the mean of three or four independent cultures with S.D. error bars. Statistical analysis was performed by 1-way ANOVA with Dunnett's post hoc test comparing to the vehicle control time point, where *** indicates $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$.

Fig.2 qRT-PCR verification of microarray results for AR agonist treated cells. On the Y-axis the mean hybridization values for the selected miRNA as measured using microarrays are plotted. On the X-axis the mean values for the same miRNA as determined by qRT-PCR are plotted. Pearson's correlation is indicated. Each individual point is the mean value from mibolerone-treated cells at 120 hours (except for miR-494 which is the mean value from DHT-treated cells at 24 hours). qRT-PCR data normalised to RNU6B. Presented as mean of 3 independent cultures.

Fig.3 Effect of MIB on miR-221 expression (A) Effect of MIB on cell numbers and PSA secretion of LNCaP cells ; Presented as mean of at least six independent cultures with S.D. error bars. Statistical analysis performed by 1-way ANOVA with Dunnett's post hoc test, comparing against the vehicle control where *** indicates $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$. (B) Measurement of mature miR-221 levels in same samples by qRT-PCR. Data are mean of 3 independent cultures with S.D error bars. Cells treated for 120 hours.

Fig.4 MIB specific induction of the hypoxia-associated miR-210 (120 hours treatment). (A) Comparison of mature miR-210 levels in LNCaP cells in normoxia and hypoxia (3 independent cultures); (B) Comparison of *VEGF* mRNA levels in LNCaP cells in normoxia and hypoxia (3 independent cultures); (C) Measurement of mature miR-210 levels by qRT-PCR in LNCaP cells treated with range of MIB doses; (3 independent cultures (except VC, N=2) (D) Measurement of *VEGF* mRNA levels by qRT-PCR in LNCaP cells treated with range of MIB doses (3 independent cultures

(except VC, N=2); (E) Pearson correlation between mean miR-210 and *VEGF* levels in same LNCaP samples treated with range of MIB concentrations. All graphs, errors bars indicate S.D

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Tables

Table 1 MIB treatment induced changes in miRNA expression

miRNA	Fold change	FDR pval
miR-221	2.5 DOWN	0.01592
miR-196b	1.5 DOWN	0.15671
miR-1246	1.9 DOWN	0.25493
miR-1290	1.8 DOWN	0.25493
miR-210	1.7 UP	0.25493
miR-182	1.3 DOWN	0.25493
miR-30a	1.8 DOWN	0.28308
miR-99a*	1.2 DOWN	0.28308
miR-720	1.6 UP	0.28308
miR-105	1.1 DOWN	0.28308
miR-99a	1.5 DOWN	0.28308
miR-7	1.8 UP	0.28308

Tabulated miRNA are those found to have a significant temporal change in expression levels following treatment with MIB with a false discovery rate (FDR) less than 0.3 between the examined timepoints (8, 24, and 120 hours of treatment). Values shown are mean fold changes compared to time-matched vehicle control (3 independent cultures per condition).

Table 2 DHT treatment induced changes in miRNA expression

miRNA	Fold change	FDR pval
let-7e	2.1 DOWN	0.27568
miR-16	2.4 DOWN	0.27568
let-7c	1.5 DOWN	0.27568
let-7b	1.3 DOWN	0.27568
miR-494	2.5 UP	0.27568
miR-200b	2.3 UP	0.27568
miR-98	2.0 UP	0.27568
miR-1290	1.7 UP	0.27568
miR-183	2.0 DOWN	0.27568
miR-151-5p	1.9 DOWN	0.27568
miR-23a	2.0 DOWN	0.27568
miR-107	2.2 DOWN	0.27568
miR-23b	2.0 DOWN	0.27568
miR-26b	2.3 DOWN	0.27568
miR-195	2.0 DOWN	0.27568
miR-30c	1.5 DOWN	0.27568
miR-1260	1.7 DOWN	0.27568
miR-100	1.5 DOWN	0.27568
let-7a	1.7 DOWN	0.27568
let-7d	1.5 DOWN	0.27568
miR-1280	2.3 UP	0.27568
miR-99a	1.5 DOWN	0.27568
miR-30b	2.1 DOWN	0.27568
miR-1246	1.6 DOWN	0.27568
miR-125b-2*	1.5 DOWN	0.27568
miR-15b	2.1 DOWN	0.27568
let-7i	1.6 DOWN	0.27568
miR-425	1.7 DOWN	0.27568
miR-24	1.9 DOWN	0.27568
miR-203	1.6 DOWN	0.27568
miR-638	1.4 UP	0.27568
miR-17	2.0 DOWN	0.27568
miR-25	1.5 DOWN	0.27568
miR-148a	2.1 DOWN	0.28423
miR-374b	1.4 DOWN	0.28423
miR-103	2.1 DOWN	0.28423
miR-15a	2.0 DOWN	0.28423
miR-93	1.7 DOWN	0.28423
miR-221	1.5 DOWN	0.28423
miR-200c	1.8 DOWN	0.28423

Tabulated miRNA are those found to have a significant temporal change in expression levels following treatment with MIB with a false discovery rate (FDR) less than 0.3 between the examined timepoints (8, 24, and 120 hours of treatment). Values shown are mean fold changes compared to time-matched vehicle control. (3 independent cultures per condition).

Supplementary Figure Legends

Suppl.Fig.1: Effects of treatments with DHT, MIB, and BIC on LNCaP proliferation.

In panels (A)-(C) the effects of the three tested compounds on LNCaP proliferation as measured by alamarBlue® is shown. In panel (D) the ability of a range of BIC doses to inhibit proliferation induced by 2nM DHT is presented. Shaded area represents 95% confidence interval for determination of Log EC50 (dotted line). Values are mean $\pm$ S.E.M error bars from 4 independent cultures; Vc is vehicle control; -ve is negative control (media); +ve is 2 nM DHT alone.

Suppl.Fig.2: Effects of treatments with DHT, MIB, and BIC on PSA secretion by LNCaP.

In panels (A)-(C) the effects of the three tested compounds on PSA secretion are shown (D) the ability of a range of BIC doses to inhibit PSA secretion induced by 2nM DHT is presented. Values are single determinations,

Suppl.Fig.3: EGF secretion in MIB treated LNCaP

(A) Standard curve of EGF. Data are mean of two replicates per standard; (B) EGF protein expression from MIB-treated LNCaP. Data are mean of seven replicates for samples with S.D. error bars and two replicates for EGF blank (0 pg/mL EGF).

Figure
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Fig.1

Fig.2

Fig.3

Fig.4