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FoxA1 corrupts the antiandrogenic effect of bicalutamide but only weakly attenuates the effect of MDV3100 (Enzalutamide™)

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ABSTRACT

Prostate cancer growth depends on androgens. Synthetic antiandrogens are used in the cancer treatment. However, antiandrogens, such as bicalutamide (BIC), have a mixed agonist/antagonist activity. Here we compare the antiandrogenic capacity of BIC to a new antiandrogen, MDV3100 (MDV) or Enzalutamide™. By reconstitution of a hormone-regulated enhancer in *Xenopus* oocytes we show that both antagonists trigger the androgen receptor (AR) translocation to the nucleus, albeit with a reduced efficiency for MDV. Once in the nucleus, both AR-antagonist complexes can bind sequence specifically to DNA *in vivo*. The forkhead box transcription factor A (FoxA1) is a negative prognostic indicator for prostate cancer disease. FoxA1 expression presets the enhancer chromatin and makes the DNA more accessible for AR binding. In this context the BIC-AR antiandrogenic effect is seriously compromised as demonstrated by a significant chromatin remodeling and induction of a robust MMTV transcription whereas the MDV-AR complex displays a more persistent antagonistic character.

1. Introduction

Androgenic hormones and the androgen receptor (AR), a nuclear receptor family member, are driving the growth of prostate cancer cells. Treatment of prostate cancer usually involves androgen deprivation therapy (Huggins and Hodges, 1972) and the use of AR antagonists (Balk and Knudsen, 2008). These compounds compete with endogenous hormone agonists, testosterone and 5a-dihydrotestosterone (DHT), for binding to AR but do not support the formation of productive AR transcription complexes. This is due to a reduced recruitment of coactivators that are otherwise targeted to the androgen responsive enhancers by the agonist-AR complex (van de Wijngaart et al., 2012). These regimes often lead to cell death or cell cycle arrest and tumor remission. Unfortunately, the remission is usually transient and tumor usually progresses to castration resistance prostate cancer (CRPC) (Balk and Knudsen, 2008). CPRC cells also tend to depend on the AR for growth (Haendler and Cleve, 2012). Experimental studies of the development of resistance to anti-androgen therapy have indicated that an increased level of AR may shift the effect of the today often used antiandrogen, bicalutamide (BIC), into an agonist (Kelly et al., 1997; Chen et al., 2004; Makkonen et al., 2011). However, the resistance that develops during BIC exposure probably involves also other events than merely an increase in AR concentration (Amaral et al., 2012). As an example, hormone resistant prostate cancer cells were recently shown to selectively promote the expression of M-phase cell cycle genes (Wang et al., 2009). One possible route for the development of hormone resistance is the increased expression of other transcription factors (TFs) that may enhance the AR-mediated effect (Sahu et al., 2011 and see below). The mixed agonist/antagonist properties of BIC and other AR antagonists have stimulated the search for a second-generation of antiandrogens with more purely antagonistic properties. One such example is MDV3100 (MDV) (Tran et al., 2009; van de Wijngaart et al., 2012), also dubbed Enzalutamide™. MDV was reported to have several promising properties in comparison to BIC; higher AR affinity, reduced nuclear AR translocation and impaired DNA binding and recruitment of coactivators (Tran et al., 2009).

Recent studies showed other transcription factors (TFs) to be required to assist DNA binding by the nuclear receptors at their respective target enhancers. The occurrence of a DNase I hypersensitive site (DHS) over the receptor binding site prior to hormone

Abbreviations: AR, androgen receptor; ARE(s), androgen response element(s); BIC, bicalutamide; bp, basepair; DBD, DNA binding domain; DMS, dimethylsulphate; DHSs, DNase I hypersensitive sites; DNase I, deoxyribonuclease I; ER, estrogen receptor; FoxA1, forkhead box transcription factor A1; GR, glucocorticoid receptor; LBD, ligand binding domain; LTR, long terminal repeat; MDV, MDV3100/Enzalutamide™; MMTV, mouse mammary tumor virus; MNase, micrococcal nuclease; NF1, nuclear factor 1; Oct1, octamer transcription factor 1; R1881, metyltrienolone; RU486, mifepristone; ss, single stranded; TA, triamcinolone acetonide; TF, transcription factor.

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administration and ligand-induced nuclear translocation argues for that these TFs are involved in the formation of a preset, i.e. more open, chromatin environment at the enhancer. This results in increased access of the nuclear receptor to the chromatinized DNA. An increasing body of evidence argues for that this TF-mediated chromatin presetting is required for the receptor to bind its target DNA (Belikov et al., 2004a,b, 2009; John et al., 2008, 2011; Siersbaek et al., 2011). A common feature of these chromatin-presetting TFs is their tendency to bind constitutively to the DNA sites. Forkhead, GATA and Oct families of TFs fill this criterion and tend to cluster in composite binding sites in the vicinity of ARE (Wang et al., 2007). One of these TFs that was shown to be involved in chromatin presetting, and thereby assist the DNA binding of several steroid receptors is FoxA1 (forkhead box A1). Incidentally, the level of FoxA1 expression in prostate cancer correlates with the tendency of metastatic growth (Gerhardt et al., 2012). Moreover, the FoxA1 gene is often mutated in such tumors (Grasso et al., 2012), and the expression of wild type FoxA1 or these mutant FoxA1 variants caused an increase in tumor proliferation. During embryogenesis, FoxA1 participates in specification of liver- and other gut-specific cell types as well as prostate, lung, and mammary gland cells (Kaestner, 2010). It was dubbed a pioneer TF since FoxA1 can bind to its target site within a nucleosome and open condensed chromatin, thereby facilitating the binding of other TFs nearby (Cirillo et al., 2002; Zaret and Carroll, 2011). FoxA1 acts as a licensing factor for several steroid hormone receptors. It enhances glucocorticoid hormone action in the liver (Rigaud et al., 1991), it is enriched near glucocorticoid response elements in pituitary AtT-20 cells (John et al., 2011), it is required for estrogen receptor (ER)-mediated gene induction in breast cancer (Eeckhoute et al., 2006; Hurtado et al., 2011), for AR action in the prostate (Gao et al., 2003) and in prostate cancer cells (Lupien et al., 2008; Wang et al., 2009) and it is required together with FoxA2 to maintain gender specific AR and ER-mediated gene expression in the liver (Li et al., 2012).

The enhancer and promoter of the mouse mammary tumor virus (MMTV) is a useful model system for studies of hormone regulation by glucocorticoids (Buetti and Diggelmann, 1981) as a well as androgens (Li et al., 2006). Transcription from the MMTV promoter is induced by these hormones via specific DNA binding of the hormone-activated glucocorticoid receptor (GR) or AR to a cluster of receptor response elements (Payvar et al., 1981) (Fig. 4, lower part). The reconstitution of this signal transduction pathway in *Xenopus* oocytes revealed hormone- and receptor-induced translational nucleosome positioning in the MMTV long terminal repeat (LTR) and DNase I hypersensitivity over hormone response elements (Belikov et al., 2000), similar to that found in tissue culture cells (Zaret and Yamamoto, 1984; Richard-Foy and Hager, 1987). Furthermore, FoxA1-binding sites were defined at three separate locations within the MMTV LTR, two double sites at positions −360/−332 and −51/−39, and a single site at position −225 relative to the transcription start site (Holmqvist et al., 2005; Belikov et al., 2009) (Fig. 4, lower part). Binding of FoxA1 to the MMTV LTR rendered a more accessible chromatin structure detectable as a cluster of DHSs. FoxA1 binding also correlated with a robust enhancement of the hormone activated GR-DNA binding and increased transcription from the MMTV promoter (Belikov et al., 2009, 2012). We previously used this system to study the effect of a glucocorticoid antagonist RU486 (mifepristone™). This GR-ligand induces a weak GR-DNA binding, but fails to recruit any chromatin remodeling activity and thus also fails to induce transcription (Belikov et al., 2001, 2004a,b). However, in the presence of FoxA1 the antagonist RU486 is converted into a partial agonist in terms of induction of the MMTV transcription (Belikov et al., 2009). This resides on the capacity of RU486 to induce nuclear translocation of GR and DNA binding. These two effects and the capacity of FoxA1 to open the chromatin structure around the GR-binding sites enables the weakly activating N-terminal domain of GR to recruit coactivators and elicit a transcriptional response as demonstrated with a GR deletion mutant lacking the C-terminal ligand-binding domain (LBD) (Belikov et al., 2009). This mimics the situation in the full length GR-RU486 complex where the activation capacity of the LBD is corrupted by the bound antagonist. Since this finding may be relevant for other steroid hormone receptor antagonists, we decided to analyze this further and to focus on the functionally related AR that is also regulating transcription at the MMTV promoter (Li et al., 2006).

To this end, we exploit the special features of the *Xenopus* oocyte system to compare the anti-androgenic properties of BIC and MDV in the presence or absence of FoxA1. An advantage of the *Xenopus* oocyte system is that protein(s) may be expressed in variable amounts by injection of corresponding *in vitro* transcribed mRNAs (Belikov et al., 2012). The DNA reporter is introduced by intranuclear injection of circular single-stranded (ss) DNA, which in our case yielded approximately 600 million gene copies of the MMTV LTR. Importantly, the injection of ssDNA leads to second-strand DNA synthesis coupled to chromatin assembly (Almouzni and Wolffe, 1993). The so obtained chromatin shares characteristics of the chromatin of stably transfected DNA in tissue culture cells (Li et al., 2006). All injected DNA copies participate in the hormone response after chromatin assembly (Belikov et al., 2000). Because of the high copy number of injected DNA, the specific TF-DNA interactions can be quantified with high precision by DMS *in vivo* footprinting (Belikov et al., 2004a,b, 2012).

Here, we show that the antiandrogenic effect of the BIC-AR complex is corrupted in the presence of FoxA1. This forkhead TF also reduces the AR antagonistic capacity of the MDV-complex, albeit only to a minor extent. We confirm previous findings that the MDV-AR complex is translocated less efficiently to the nuclear compartment than BIC-AR (Tran et al., 2009). However, an increased AR expression results in a correspondingly increased intranuclear uptake of MDV-AR. Importantly, the nuclear MDV-AR complex binds with a similar efficiency to the AREs as the agonist-AR complex, provided that FoxA1 is also present. However, the MDV-AR has a considerably reduced capacity to recruit coactivators, seen as a reduced chromatin remodeling and a reduced MMTV transcription.

2. Materials and methods

2.1. Reagents, plasmids and constructs

AR ligands used were R1881 (Perkin-Elmer Inc, Waltham, MA), MDV3100 (Selleck Chemicals Co. Ltd., Houston, TX) and bicalutamide (Bidragon Pharmservice LLC, Burlingame, CA). The reporter pMMTV:M13 contains the 1.2 kb MMTV LTR fused to the HSV TK gene and its transfer to M13 was described (Belikov et al., 2000), as well as the insertions into RN3P vectors of cDNA coding for mouse FoxA1 (Holmqvist et al., 2005) and the production of mRNAs. The human androgen receptor cDNA was kindly provided by Dr. Jiemin Wong and was described in (Li et al., 2006). The human AR cDNA was cloned between the *BamH1*/*Not I* sites in the vector used for mRNA production that was described (Zernicka-Goetz et al., 1996). The clone was confirmed by DNA sequencing (Supplement 1). The variable poly-Q region harbored 20 Q residues, the variable poly-G region harbored 16 G residues, the total length was 910 amino acid residues. The plasmids were linearized with *Sfi I* and *in vitro* transcribed using the mMESSAGE mMACHINE kit (Ambion) and purified on a spin column (MEGAclear, Ambion); the mRNA stocks were stable for years when saved in small aliquots at −83C. In initial experiments the hAR was expressed from