7-Substituted umbelliferone derivatives as androgen receptor antagonists for the potential treatment of prostate and breast cancer

Sahar Kandil *, Andrew D. Westwell, Christopher McGuigan

School of Pharmacy & Pharmaceutical Sciences, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, Wales, United Kingdom

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A B S T R A C T

The clinically used androgen receptor (AR) antagonists (bicalutamide, flutamide and nilutamide) bind with low affinity to AR and can induce escape mechanisms. Furthermore, under AR gene amplification or mutation conditions they demonstrate agonist activity and fail to inhibit AR, causing relapse into castration resistant prostate cancer (CRPC). Discovery of new scaffolds distinct from the 4-cyano/nitro-3-(trifluoromethyl)phenyl group common to currently used antiandrogens is urgently needed to avoid cross-resistance with these compounds. In this study, a series of twenty-nine 7-substituted umbelliferone derivatives was prepared and their antiproliferative activities were evaluated. The most active compound 7a demonstrated submicromolar inhibitory activity in the human prostate cancer cell line (22Rv1); IC50 = 0.93 μM which represents a 50 fold improvement over the clinical antiandrogen bicalutamide (IC50 = 46 μM) and a more than 30 fold improvement over enzalutamide (IC50 = 32 μM). Interestingly, this compound showed even better activity against the human breast cancer cell line (MCF-7); IC50 = 0.47 μM. Molecular modelling studies provided a plausible theoretical explanation for our findings.

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The androgen receptor (AR) is a member of the steroid nuclear receptor superfamily, which consists of estrogen, progesterone, glucocorticoids, mineralocorticoids and androgen receptors.1 Androgenic steroids have reproductive and anabolic actions in both men and women.2–5 The androgen receptor (AR) is expressed in many cell types and AR signaling has been found to have important roles in modulating tumorigenesis and metastasis in several cancers including prostate, bladder, kidney, lung, breast and liver.6 While supplementing androgens is necessary in the treatment of hypogonadism and may be beneficial in aging individuals to maintain muscle mass, over the last 60–70 years there has been considerable research interest in reducing circulating levels of testosterone and/or blocking the androgen receptor for the clinical management of some diseases such as prostate cancer and polycystic ovarian syndrome. However, more recently, there is also a growing appreciation of the need for selective androgen modulators that would demonstrate tissue-selective agonist or antagonist activity.6,7

AR signalling has an important role in initiation and progression of many hormone-related cancers. Prostate cancer (PC) is one of the leading causes of cancer related death in men. The early stages of PC are treated by surgical excision and radiation therapy. However, the mainstay of advanced stage PC treatment is blockade of the AR signaling pathway by the use of AR antagonists. Initially this androgen ablation treatment is effective, but eventually this treatment strategy fails with the development of a more aggressive form of PC, namely castration resistant prostate cancer (CRPC).7 Currently, the major antiandrogens in clinical use worldwide are bicalutamide, flutamide and nilutamide, Figure 1. However, these compounds bind with low affinity and can induce escape mechanisms.7,8 Furthermore, under AR gene amplification or mutation conditions these compounds demonstrate partial agonist activity and fail to inhibit AR.6 These observations indicate that there is an urgent need to discover a broader chemotype spectrum of AR antagonists distinct from the 4-cyano/nitro-3-(trifluoromethyl)phenyl group in flutamide, nilutamide, bicalutamide, Figure 1, to avoid the cross-resistance with these compounds. A new structural motif without partial agonist activity would be particularly useful.

Prostate cancer and breast cancer share similarities as hormone related cancers with a wide heterogeneity. The AR is involved in both benign and malignant settings in both sexes. Targeting the AR pathway is central to PC therapy. Despite AR expression in breast cancer (BC) was known almost 50 years ago, unlike PC the antiandrogen narrative for BC is still in its infancy.5,9 Recently, there has been increased interest in the role of the AR in BC development and growth, with results indicating AR expression across
all intrinsic subtypes of BC. Targeting the AR axis is an evolving field with novel therapies in development, which may ultimately be applicable to both PC and BC tumour types. Insights into the novel drugs in development for targeting this signaling pathway has been thoroughly reviewed recently by Proverbs-Singh et al.\textsuperscript{9}

X-ray crystallographic studies of AR have elucidated the structures of the AR ligand binding domain (LBD) bound to agonists and of mutant AR bound to antagonists in an agonist like conformation. However, there are no crystal structures of the AR-LBD in an antagonist conformation reported so far.\textsuperscript{10,11} Several studies have used the available structural information, computer modeling and biochemical observations in order to identify new structurally distinct nonsteroidal small molecule competitive AR antagonists.\textsuperscript{12–18} In two independent and recently published studies, a computer aided drug discovery platform was used to identify alternative chemical architectures of AR antagonists. In the first study, the in silico pharmacophore based virtual screening of small molecule libraries led to the identification of six distinct chemotypes, all of which proved to function in vitro as pure antagonists with IC\textsubscript{50} values of approximately 5 \(\mu\text{M}.\)\textsuperscript{14} Of particular interest to us was chemotype A, represented by compound 1, Figure 2, that did not show crossreactivity with the related steroid receptors; glucocorticoid receptor (GR) and progesterone receptor (PR).\textsuperscript{15} In the second study a different strategy was utilised to elucidate a pharmacophore model used in the virtual screening of different commercially available small molecule libraries.\textsuperscript{15} Interestingly, one of the three chemotypes identified in this study i.e. chemotype B, represented by compounds 2 (IC\textsubscript{50} = 3.4 \(\mu\text{M}\)) and 3 (IC\textsubscript{50} = 5.1 - 5.8 \(\mu\text{M}\)), Figure 2, was structurally similar to chemotype A of the first study. Both of these two chemotypes feature a 4-methyl coumarin core fragment and \(\beta\)-keto ether substitution at the 7-position of the coumarin ring. More importantly, neither of these chemotypes show any significant agonistic activity against AR and its mutants (W741C and T877A) and act as pure antagonists.\textsuperscript{14,15}

Noting the value of the findings of these two independent studies, we sought to improve upon these starting point structures by varying the terminal aromatic group of the ketone linkage and exploring the impact of replacing the 4-CH\textsubscript{3} group of the coumarin ring with the more lipophilic 4-CF\textsubscript{3} since the former is assumed to interact with a hydrophobic subpocket within the AR.\textsuperscript{14,15} Moreover, a closely related AR selective modulator (LGD2226) features a CF\textsubscript{3} group in the 4 position of the quinolinone ring that interacts with the same hydrophobic subpocket as confirmed by the X-ray crystal structure (PDB: 2HVC).\textsuperscript{19,20} Generally, introduction of fluorinated substituents into drug candidates can impart a unique variety of properties owing to the special combination of electronegativity, size and lipophilicity features of fluorinated groups. These factors can have a substantial impact on the molecular conformation, which in turn affects the binding affinity to the target protein.\textsuperscript{21–25}

In addition to the substantial role of antiandrogens in the treatment of PC, there has been growing evidence that the androgen signaling pathway can play a critical role in malignant breast tissue.\textsuperscript{9} The AR is the most prevalent sex steroid receptor in situ, invasive, and metastatic breast cancers, occurring in up to 90\% of primary tumours and 75\% of metastases. AR expression has been reported in over 70\% of estrogen receptor ER positive breast cancer and in 45–50\% of patients with ER negative breast cancers.\textsuperscript{26–28} The tumour AR expression level was shown to be inversely associated with the survival of BC patients.\textsuperscript{26} Ablation of the AR in the human BC epithelial cell line, MCF-7, has been shown to suppress cell proliferation, suggesting that AR might have a positive role in promoting BC progression.\textsuperscript{27} Therefore, AR is a highly prevalent target in BC, with potential significance for therapeutic management of both primary and advanced disease.\textsuperscript{9} For example, triple negative breast cancers (TNBC) are characterized by aggressive tumour biology resulting in a poor prognosis. The androgen receptor (AR) is reported to be a newly emerging biomarker in TNBC.\textsuperscript{28,29} Enzalutamide, the second generation AR antagonist, was FDA approved in 2012 for use in prostate cancer. However, recent studies support the initiation of clinical evaluation of enzalutamide for treatment of AR positive breast tumours regardless of ER status, since it

\begin{figure}
\centering
\includegraphics[width=\textwidth]{structures.png}
\caption{Chemical structure of the clinically used nonsteroidal antiandrogens; flutamide, nilutamide, bicalutamide and enzalutamide.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{structures.png}
\caption{Structures of chemotype A (represented by compound 1) and chemotype B (represented by compounds 2 and 3).}
\end{figure}
blocks both androgen- and estrogen-mediated tumour growth.\textsuperscript{28–33} For the above mentioned reasons we decided to screen our compounds against the MCF-7 (human breast cancer) cell line alongside 22Rv1 (human prostate cancer) and interestingly comparable results in both cell lines were observed.

The $\beta$-ketoethers ($7a$–$n$) and ($8a$–$o$) were prepared in good to excellent yields (70–93\%)\textsuperscript{34} by triethylamine-promoted alkylation of 7-hydroxy-2H-1-benzopyran-2-ones ($4$ and $5$) with the appropriate bromoketones ($6a$–$o$) as outlined in Scheme 1.

Compounds ($7a$–$n$) and ($8a$–$o$) were screened for antiproliferative activity using Oncostest's monolayer assay\textsuperscript{35} against the prostate cancer cell line 22Rv1 and the breast cancer cell line MCF-7. Bicalutamide and enzalutamide were used as positive controls. Anticancer activity was assessed after four days of treatment with the compounds using a propidium iodide based monolayer assay.\textsuperscript{35} Potency is expressed as absolute IC$_{50}$ values, calculated by non-linear regression analysis. The twenty-nine compounds were tested at 10 concentrations in half log increments up to 100 µM in triplicate. The results summarised in Table 1 indicated that four compounds ($7a$–$d$) have better antiproliferative activity (IC$_{50}$ = 0.93–20.37 µM) than the positive controls; bicalutamide (IC$_{50}$ = 46.25 µM) and enzalutamide (IC$_{50}$ = 31.76 µM). The 3,5-bis-trifluoromethyl analogue $7a$ proved to be the most potent compound with submicromolar inhibitory activity in prostate cancer (22Rv1; IC$_{50}$ = 0.93 µM), which represents an approximate 50 fold improvement over bicalutamide and more than 30 fold improvement over enzalutamide. Interestingly, this compound showed even better activity against breast cancer (MCF-7; IC$_{50}$ = 0.47 µM).

The second most active compound $7b$, with $m$-methoxy substitution of the terminal phenyl group, displayed a low micromolar activity upon small chemical changes (e.g. 4-CH$_3$ in 4-trifluoromethyl analogues. Considering the surprising drop of activity have the 4-methyl group in the coumarin ring rather than the $\beta$-ketoethers ($7a$–$n$) and ($8a$–$o$). Docking studies suggest that this detrimental effect can be attributed to the steric intolerance of the binding sub-pocket of the aromatase. Generally, activity ridges provide significant opportunities for further SAR study in medicinal chemistry.\textsuperscript{36–38} The homology model for the AR-LBD in the antagonist conformation was constructed using Molecular Operating Environment (MOE) (Chemical Computing Group, Montreal, Canada). It was constructed using the sequence of the hAR in the agonist conformation from the crystal structure of hAR-LBD with dihydrotestosterone (DHT) [ZAMisa, PDB].\textsuperscript{39} To model the antagonistic conformation of hAR-LBD, the progestosterone crystal structure [20VH, PDB] was used as a template since it has sequence identity 54% with the human AR.\textsuperscript{40} Applying the default parameters of MOE homology modeling module and using the AMBER99 force field, a total of 10 homology models were generated. The quality of each model was assessed within MOE, and the best model was chosen for the docking studies. The chemical structures of our compounds were constructed, rendered and minimized with the MMFF94x force field in MOE. Docking simulations were performed using Glide SP in Maestro (Glide, version 9.5, Schrödinger, LLC, New York, NY. http://www.schrodinger.com). The putative docking mode of the most active compounds $7a$ and $7b$ is shown in Figure 4A and B, respectively. Key interactions include two H-bonds between the lactone carbonyl group and both the guanidine group of Arg 752 of helix 5 and the amino group of Gln 711 of helix 3 residues. Another H-bond between the terminal carbonyl group and the side chain OH group of Thr 877, and the H-bond between the methylene group and the side chain carbonyl group of Asn 705 residue of helix 3 were also observed. In addition, hydrophobic interactions were noticed between the 4-methyl coumarin moiety and the surrounding hydrophobic pocket formed of residues; Trp 741, Met 745, Leu 712 and Met 787. The significantly higher activity of compound $7a$ is shown in Figure 4A and B, respectively. On the other hand, there was no or only marginal antagonist activity observed for compounds ($7e$–$n$) and ($8a$–$o$). Interestingly, all the analogues with antiproliferative activity have the 4-methyl group in the coumarin ring rather than the 4-trifluoromethyl analogues. Considering the surprising drop of activity upon small chemical changes (e.g. 4-CH$_3$ in $7a$–$d$ vs. 4-CF$_3$ in $8a$–$d$), we suspect that an ‘activity ridge’ has been identified. Generally, activity ridges provide significant opportunities for further SAR study in medicinal chemistry.\textsuperscript{36–38}

In summary, 7-substituted umbelliferone derivatives have been shown to be potentially useful AR antagonists. Through optimization of the scaffold with regard to the substitution of the terminal

![Scheme 1. Synthesis of $\beta$-ketoethers ($7a$–$n$) and ($8a$–$o$). Reagents and condition: (a) THF, Et$_3$N, rt, 24 h.](image-url)
Table 1
Mean in vitro antiproliferative activity (IC50 μM) of compounds (7a–n) across two human cancer cell lines (MCF-7 and 22Rv1)

<table>
<thead>
<tr>
<th>ID</th>
<th>R1</th>
<th>R2</th>
<th>22Rv1 IC50 (μM)</th>
<th>MCF-7 IC50 (μM)</th>
<th>ID</th>
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Bicalutamide 46.25 — Enzalutamide 31.76 —

Figure 3. Dose response curves of compounds 7a (A), 7b (B), 7c (C) and 7d (D) in the Oncotest monolayer assay of MCF-7 (red) and 22Rv1 (green) cell lines.

Figure 4. Putative binding modes of compounds 7a (A) and 7b (B) inside the antagonistic hAR-LBD showing hydrogen bond interactions with key amino acids: Arg752, Gln711, Thr877 and Asn705.
aryl and the 4-position of the coumarin core ring, significantly potent analogues were identified such as compound 7a, which demonstrated sub-micromolar inhibitory activity in prostate cancer (22Rv1); IC\textsubscript{50} = 0.93 μM (around 50 fold improvement over bicalutamide activity and more than 30 fold improvement over enzalutamide). Interestingly, this compound demonstrated even better activity against MCF-7 (breast cancer); IC\textsubscript{50} = 0.47 μM. Molecular modeling studies cast a light on the SARs observed. These findings provide a basis for further development of umbelliferone derivatives for the potential treatment of human AR related cancers.

Acknowledgement

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Supplementary data

Supplementary data (experimental procedures and spectroscopic characterizations data of the compounds 7a–n and 8a–o) associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.02.088.

References and notes