Androgen glucuronidation: an unexpected target for androgen deprivation therapy with prognosis and diagnostic implications.

Laurent Grosse¹, Sophie Pâquet¹, Patrick Caron¹, Ladan Fazli², Paul S. Rennie², Alain Bélanger³ and Olivier Barbier¹

¹Laboratory of Molecular Pharmacology, CHU-Québec Research Centre and Faculty of Pharmacy, Laval University, Québec, Canada
²Vancouver Prostate Centre at VGH, University of British Columbia, Vancouver, British Columbia, Canada.
³CHU-Québec Research Centre and Faculty of Medicine, Laval University, Québec, Canada

Running title: Ablation therapy improves androgen glucuronidation in PCa

Keywords: Prostate cancer, androgen ablation therapy, glucuronidation, biomarker, drug target.

Financial support: This study was supported by grants from the Canadian Institutes of Health Research (CIHR), the Terry Fox foundation and the Canadian Foundation for Innovation. S. Pâquet is holder of scholarships from CIHR and the Fonds pour la Recherche en Santé du Québec (FRSQ). L. Grosse is supported by a scholarship from the Fonds pour l’Enseignement et la Recherche (FER) from the Faculty of Pharmacy, Laval University (Québec). O. Barbier is holder of salary grants from the CIHR (New Investigator Award #MSH95330) and FRSQ (Junior 2 Scholarship).

Address correspondence to: Olivier Barbier, Ph.D.
Laboratory of Molecular Pharmacology
CHU-Québec Research Centre
2705, boulevard Laurier
Québec (QC) G1V 4G2, Canada
Phone: 418 654 2296
Fax: 418 654 2769
Email: olivier.barbier@crchul.ulaval.ca

Conflict of interests: Authors have nothing to disclose.
ABSTRACT

Androgen deprivation therapy (ADTh) remains a mainstay of prostate cancer treatment but its efficacy is bypassed by mechanisms that are not fully understood. In human prostate cancer cells (CaP cells), androgen glucuronidation catalyzed by the 2-UDP-glucuronosyltransferase (UGT)2B15 and UGT2B17 enzymes is the major androgen inactivation pathway. In this study, we investigated the effect of ADTh on androgen glucuronidation to evaluate its potential clinical utility for CaP prognosis or therapy. UGT2B15 and UGT2B17 expression was evaluated in PCa specimens from untreated or treated patients, and in cell models of PCa exposed to clinically relevant anti-androgens. UGT2B15 and UGT2B17 protein levels in prostate were increased after 5 months of ADTh when compared to specimens from untreated patients. UGT2B15 expression remained elevated for up to 12 months, but UGT2B17 returned to initial levels as soon as after 6 months. Several androgen receptor (AR) antagonists tested caused a dose- and time-dependent stimulation of UGT2B15 and UGT2B17 expression and androgen glucuronidation in CaP cell lines. The role of AR in these regulatory events was confirmed using AR-deficient LNCaP cells, where UGT2B attenuation reduced the anti-proliferative effects of AR pharmacological antagonists. Through this combination of clinical and functional investigations, our work revealed that ADTh stimulates a local androgen metabolism in prostate cells, establishing a foundation to evaluate the potential of UGT2B15 and UGT2B17 as drug targets and/or molecular markers for ADTh responsiveness and maintenance in CaP.
INTRODUCTION

Prostate cancer (PCA) remains the second most frequent type of cancer diagnosed worldwide, and the third leading cause of cancer-related death in men in industrialized countries (1). Prostate cancer is an adenocarcinoma originating from cells of epithelial type, but displays morphologically and genetically very heterogeneous properties (2). Androgens are among the main factors controlling the initiation, maintenance and progression of prostate cancer (3). During carcinogenesis, epithelial cells transform into a malignant phenotype, where the androgen receptor (AR) selectively activates genes positively controlling cell viability to un restraint epithelial cell proliferation (3). For this reason, Androgen Deprivation Therapy (ADTh), also called “medical castration” or “hormone therapy” has become a first-line treatment strategy for advanced PCa (4). ADTh is achieved using gonadotropin releasing hormone (Gn-RH) analogs such as leuprolide, buserelin or goserelin (4), which inhibit testosterone synthesis (2). This medical castration is often combined to anti-androgens such as flutamide, nilutamide or bicalutamide (2). These AR antagonists provide an additional blockade of androgen signalling by preventing AR activation by the locally synthesized androgens. Symptomatic (improvement of quality of life) and/or objective (serum PSA level normalization and measurable tumor response) outcomes to androgen deprivation therapy are observed in approximately 80% of patients (5). However, the duration of these improvements is highly variable, and PCa relapses in the majority of patients evolving to the androgen-independent phenotype within 12 to 18 months of therapy (5). The cancer is then commonly referred to as “castrate resistant prostate cancer” (CRPC), “androgen independent”, or “hormone refractory” (6). CRPC can transiently be treated with alternative ADTh, but the ultimate options for androgen-independent metastatic prostate cancer consists in chemotherapy (5, 7).

The androgen axis continues to play a major role for the progression of CRPCs (8), and a deep understanding of factors affecting androgen inactivation in tumor cells is required. In the prostate, the active hormone dihydrotestosterone (DHT) is extensively metabolized in the prostate to inactive and easily excretable androstane-3α-diol-glucuronide (3α-diol-17G) and androsterone-glucuronide (ADT-3G) derivatives (9, 10). These polar metabolites are formed through a conjugation reaction called glucuronidation, and correspond to the major androgen...
metabolic end-products found in circulation in men (11, 12). In humans, glucuronidation is catalyzed by the 19 functional UDP-glucuronosyltransferase (UGT) enzymes (13). However, in prostate, androgen glucuronidation only involves 2 enzymes: UGT2B15 and UGT2B17 (14). Experimental inhibition of these enzymes significantly improves the pro-proliferative properties of DHT in PCa cells (14), suggesting that they are major determinants for the androgen response. Accordingly, positive associations were reported between PCa risk and a low-activity UGT2B15 allele or a complete UGT2B17 gene deletion (15-19). On the other hand, various studies illustrated the negative control that androgens exert on UGT2B15 and UGT2B17 expression in PCa cells (14, 20, 21). These last observations establish the AR regulatory pathway as a major mechanism for controlling androgen glucuronidation. We therefore sought to test the possibility that ADTh drugs affect UGT2B15 and/or UGT2B17 expression or activity in PCa tumor samples and cell models exposed to anti-androgens.


MATERIALS AND METHODS

Materials

UDP-glucuronic acid and all aglycons were obtained from Sigma (St. Louis, MO) or ICN-Pharmaceuticals Inc. (Québec, Canada). R1881, 3α-Diol, DHT, ADT, and DHT-glucuronide (DHT-G) were purchased from Steraloids (Newport, RI). ADT-3-glucuronide and 3α-Diol-17-glucuronide were provided by the Medicinal Chemistry Division of the “Centre de recherche du CHU-Québec” (21). Cell culture materials, blasticidin and lipofectin were purchased from Invitrogen (Burlington, ON, Canada). Penicillin, streptomycin and IMDM were provided by Wisent (Rocklin, CA), while puromycin, doxycyclin, bicalutamide, nilutamide, flutamide and RPMI were purchased from Sigma. Sybr Green PCR Master mix was purchased from Applied Biosystem (Life Technologies, Carlsbad, CA). Protein assay reagents were obtained from Bio-Rad Laboratories Inc. (Marnes-la-Coquette, France). The anti-calnexin antibody was purchased from Stressgen (Victoria, Canada), the anti-vimentin antibody was from GenScript (Piscataway, NJ), and the anti-UGT2B15 and anti-UGT2B17 antibodies were described previously (24, 31). The secondary antibody against rabbit IgG was purchased from Amersham (Pittsburg, PA). The chemiluminescence kit (ECL) was from Renaissance (Québec, Canada). SiRNA probe for UGT2B15/UGT2B17 (Individual siGENOME duplex D-020195-01) and Non-Target #1 were obtained from Dharmacon (Chicago, IL) as already described (14).

Tissue banks

Tissues used for the microarray construction were from the Vancouver Prostate Centre Tissue Bank (http://www.prostatecentre.com/our-research/core-facilities/biorepository), as described (22, 23). Prostate samples were from 31 benign prostatic hyperplasia (BPH) donors while PCa samples were from 88 advanced patients: 16 from untreated donors, 16 from participants with CRPC, and finally 56 from patients having received ADTh for 1 to 5 months (n=19), 6 to 8 months (n=24) or 9 to 12 months (n=13) (supplementary material SM1). Tissues samples were extracted from the whole tissue after radical prostatectomy, except CRPC samples which were obtained by transurethral resection of the prostate (22, 23). The therapy
consisted in Gn-RH analogs (buserelin, leuprolide or goserelin) and anti-androgens (flutamide, cyprosterone acetate or bicalutamide) used alone or in combination. This protocol was approved by the institutional review boards at the University of British Columbia (Vancouver, BC, Canada), and the “Centre hospitalier universitaire de Québec” (Québec, QC, Canada). Written informed consent was obtained from all participants.

**Tissue microarrays**

Microarrays and immunohistochemical (IHC) stains were obtained as previously described (22, 23), with formalin-fixed and paraffin-embedded 4µm sections of the microarray, using the previously described (24, 31, 40) polyclonal anti-vimentin (positive control, 1:100 dilution), anti-UGT2B15 (1:300 dilution) and anti-UGT2B17 (1:150 dilution) antibodies (SM2-8). The IHC staining (SM2-8) were scored by a pathologist (L. Fazli) for the level of immunoexpression on a scale from 0 to 3, wherein 0 was undetectable, 1 represented a faint or focal questionably present stain, 2 represented a stain of convincing intensity in a minority of cells, and 3 a stain of convincing intensity in a majority of cells.

**Cell culture**

Cell lines were obtained from the American Type Culture Collection (Rockville, MD, Cell authentication through STR profiling), used within the 6 months following reception. LNCaP cells were grown and treated in the 10% FBS-supplemented RPMI 1640 medium. LAPC-4 cells were cultured in IMDM completed with 7.5% FBS and 10nM R1881. Inducible AR-shRNA-expressing-LNCaP cells were cultured in RPMI 1640 supplemented with 10% FBS, puromycin (2.5µg/ml) and blasticidin (1µg/ml), as reported (28). AR deficiency was obtained through a 48H pre-treatment with doxycycline (DOX, 1µg/ml), and the antibiotic was maintained for the complete treatment duration as recommended (28). For RNA isolation, 2.5×10^5 LNCaP or 3.5×10^5 LAPC-4 cells were plated in each well of 12-well plates. For glucuronidation assays and western-blot experiments, 8×10^6 LNCaP cells were plated in 10cm Petri dishes. Cells were then treated for the indicated duration with vehicle (DMSO or ethanol, 0.1%, v/v), bicalutamide (DMSO), nilutamide (ethanol), flutamide (ethanol), R1881 (ethanol) and/or DHT (ethanol) at the indicated
concentrations. Knock-down of UGT2B15 and UGT2B17 expression was obtained using a UGT2B15/UGT2B17 siRNA probe as already reported (14). Following transfection, cells were allowed 18H for recovery, transferred in 96-wells plates and exposed to vehicle (DMSO, 0.1%, v/v) or bicalutamide (10µM) for 72H. Cell proliferation was then ensured using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) and optical density determination (490nm) with a microplate reader Tecan Infinitte M1000 (Männedorf, Switzerland) as recommended by the supplier (Promega).

**Genotyping of the UGT2B17 gene copy number variation (CNV)**

Genomic DNA was isolated from LNCaP and LAPC-4 cells using the ChargeSwitch gDNA Mini Tissue Kit (Life Technologies) and was then used as a template for UGT2B17 deletion genotyping as previously described (23, 26).

**RNA Isolation and quantitative RT-PCR**

Total RNA was isolated according to the Tri-Reagent acid: phenol protocol as specified by the supplier (Molecular Research Center Inc., Cincinnati, OH). The reverse transcription (RT) and quantitative PCR reactions were performed as previously described (23).

**Western-blot analyses and in vitro glucuronidation**

Western-blot analyses and in vitro glucuronidation assays were performed as previously reported (14, 21, 24).

**Statistical analyses**

All data are presented as mean±standard deviation (SD), except for the IHC intensity evaluation where results are presented as mean±standard error to the mean (SEM). Comparisons between experimental groups were performed by a two-tailed Student’s t-test or a Mann-Whitney U test for in vivo assessment of UGT levels using the JMP V7.01 software (SAS Institute, Cary, NC).
RESULTS

**Androgen ablation therapy modulates UGT2B15 and UGT2B17 protein levels in prostate tumors**

According to our previous observation (23), untreated PCa samples presented similar UGT2B15 and UGT2B17 protein levels as in BPH samples (Fig.1, SM2-8). However, when compared to BPH and untreated samples, both UGT proteins were significantly more abundant in tissues from ADTh-treated patients for 1 to 5 months (Fig.1, SM2-5). The highest UGT2B15 expression was observed after 6-8 months of treatment, and remained significantly higher than in untreated samples up to 12 months therapy, even if a tendency to lower again was observed in these last samples (Fig.1A). By contrast, UGT2B17 protein levels were reduced to basal levels (untreated) as soon as after a 6-8 months period of treatment and remained stable for longer exposures (Fig.1B). In patients with CRPCs, both UGT2B15 and UGT2B17 protein levels were not significantly different from in untreated samples. IHC results obtained with the positive control, vimentin (25) indicated that the quantification of UGT2B protein levels was not biased by the tissue preparation (SM2-8).

**Androgen receptor antagonism up-regulates UGT2B15 and UGT2B17 expression and activity in prostate cancer cell models**

*Ex vivo* experiments were performed using PCa cell models and AR antagonists to further investigate the effects and consequences of AR blockade on androgen glucuronidation (Figs.2-4).

First, UGT2B15 and UGT2B17 mRNA and protein levels were quantified in LNCaP cells exposed to increasing doses of bicalutamide (0.1 to 20µM) for 48H. The two UGTs were dose-dependently increased at both mRNA and protein levels (Fig.2). While the amount of UGT2B15 transcripts was statistically increased in the presence of as low as 1µM (Fig.2A), UGT2B17 mRNA accumulation reached the statistical significance only in the presence of 5µM bicalutamide (Fig.2B). In time-course experiments, UGT2B15 and UGT2B17 transcripts were significantly increased as soon as after 12H of treatment, and the maximal induction was reached after a 48H exposure (Fig.2C-D). A similar dose- and time-dependent accumulation
was observed for both UGT proteins in western-blot analyses (Fig.2). In vitro glucuronidation assays confirmed that the bicalutamide-dependent increase in UGT expression results in improved androgen conjugation (Fig.3). Indeed, LNCaP cells exposed to bicalutamide exhibited a dose- and time-dependent improved ability to conjugate DHT (A&D), ADT (B&E) and 3α-Diol (C&F).

To ascertain that results from bicalutamide-treated LNCaP cells were not cell type- or antagonist-specific, additional experiments were performed using the LAPC-4 cell model, and the other AR antagonists, nilutamide and flutamide (Fig.4). In LAPC-4, bicalutamide dose- and time-dependently induced UGT2B15 expression (Fig.4A&B). UGT2B17 transcripts were not detected in these cells. Actually, genotyping analyses revealed that the UGT2B17 gene is absent in LAPC-4 (data not shown), indicating their del/del phenotype for the previously reported UGT2B17 CNV genotype (26). In both LNCaP and LAPC-4 cells, bicalutamide and flutamide were efficient in increasing UGT2B15 mRNA levels (Fig.4C&D). A similar response was also observed for UGT2B17 transcripts in LNCaP cells (Fig.4C). Interestingly, nilutamide exhibited a cell-specific response with a significant induction of UGT2B15 mRNA in LAPC-4 but not in LNCaP cells (Fig.4C&D). Furthermore, not only did nilutamide not improve UGT2B17 expression, but it caused a non significant reduction of these transcripts in LNCaP cells (Fig.4C). This last observation is consistent with the previously reported agonistic effects of nilutamide in front of the mutated AR that is expressed in LNCaP cells (27).

**The androgen receptor mediates the bicalutamide-dependent up-regulation of UGT2B15 and UGT2B17 expression in LNCaP cells**

AR contribution to the bicalutamide-dependent induction of UGT2B15 and UGT2B17 expression was ensured by using LNCaP cells expressing a DOX-inducible anti-AR shRNA (Fig.5) (28). We first confirmed that doxycycline treatment resulted in impaired AR expression and activity (SM9) (28). Subsequently, native (DOX-) and doxycycline-activated cells (DOX+) were cultured in the presence of vehicle, bicalutamide (10μM), or the synthetic (R1881, 1nM) or natural (DHT, 10nM) AR activators (Fig.5).

In native (DOX-) cells, DHT, R1881 and bicalutamide caused the expected down-
(R1881 and DHT, (21)) or up-regulation (bicalutamide) of UGT2B15 (Fig.5A) and UGT2B17 (Fig.5B) mRNA expression.

In AR-deficient (DOX+) cells, doxycycline alone also significantly increased the UGT2B15 (Fig.5A) and UGT2B17 (Fig.5B) transcript levels, thus revealing that inhibition of AR expression has similar consequences as the use of AR antagonists for these UGT gene expression. The addition of R1881 in culture media had no further impact on mRNA levels of the 2 UGTs (Fig.5). Similarly, the negative effect of DHT on UGT2B15 expression was completely lost, since its mRNA levels in vehicle- and DHT-treated DOX+ cells were not significantly different (Fig.5A). By contrast, UGT2B17 mRNA levels were found significantly more abundant in DHT-treated than in vehicle DOX+ cells (Fig.5B). Also surprising was the observation that bicalutamide reduces UGT2B15 and UGT2B17 mRNA levels in cells cultured with doxycycline (Fig.5). This last observation suggests that bicalutamide exerts opposite effects on UGT genes expression in the presence of high (DOX-) or low (DOX+) AR levels. However, when mRNA levels were compared to those quantified in bicalutamide-treated native cells, only the strong reduction in UGT2B17 transcripts remained statistically significant (Fig.5B), indicating that the loss of AR mainly altered the response of this enzyme to bicalutamide.

Nonetheless, the fact that AR knock-down abolishes or reverses the effects of bicalutamide confirms the role played by this receptor in the anti-androgen-induced up-regulation of UGT2B15 and UGT2B17 expression.

Knock-down of UGT2B15 and UGT2B17 reduces the anti-proliferative properties of bicalutamide

We next evaluated whether induction of UGT2B15 and UGT2B17 expression participates to the anti-proliferative properties of bicalutamide (29). For this purpose, LNCaP cells were transfected with a non-target (siRNA control) or the anti-UGT2B15/UGT2B17 siRNA probe (14) (Fig.6A), and then exposed to vehicle (DMSO) or bicalutamide (10µM) for up to 4 days (Fig.6B). As expected (29), bicalutamide caused a significant 24.5% reduction of control cells proliferation after 3 days of exposure. This reduction was further enhanced to 32.5% inhibition after 4 days (Fig.6B). Interestingly, UGT2B-deficient cells were less sensitive to the
antagonist and their proliferation was only 15 and 22.5%-reduced in the presence of the drug after 3 and 4 days, respectively (Fig.6B). Thus, siRNA-mediated knock-down of UGT2B15 and UGT2B17 expression provoked 40% and 32% reductions of the anti-proliferative effects of bicalutamide in LNCaP cells exposed to the drug for 3 and 4 days, respectively.
DISCUSSION

This study evidences a novel and incidental effect by which androgen ablation drugs abolish the ability of carcinogenic androgens to inhibit their own inactivation in prostate cancer cells. This first comprehensive analysis of AR blockade consequences for androgen glucuronidation identifies the 2 androgen-conjugating UGT2B15 and UGT2B17 enzymes as ADTh positive targets, and establishes the major contribution of these effects to the anti-proliferative properties of ADTh drugs.

An interesting observation of the present study is the differential manner in which the 2 UGT genes respond to AR blockade in and ex vivo. These differences are thought to reflect variations in the AR-dependent modulation of the 2 UGT genes. Indeed, the fact that AR agonists/antagonists fail to modulate UGT2B15 expression in receptor-deficient cells, while having inverse effects on UGT2B17 mRNA levels, suggest that alternative regulatory processes drastically modify the AR-dependent modulation of UGT2B17 expression when the androgen receptor is reduced. While thorough investigations are required to fully grasp the mechanisms governing the molecular switch in the UGT2B17 response, it can be envisioned that such UGT-specific processes may be derived from nucleic acid differences in the transcriptional regulatory regions of the UGT2B15 and UGT2B17 genes. Even if the 2 genes share considerable sequence homology (9), previous studies already illustrated that minor differences in their proximal promoters are responsible for their differential response to various regulatory pathways (30, 31). Such changes actually exist within the AR response elements identified in UGT2B15 and UGT2B17 promoters sequences (20), and thus may play a role in their isoform-specific response under low AR levels. Other regulatory pathways may also be involved. For example, the epidermal growth factor (EGF) signalling, which down-regulates UGT2B17 expression without affecting UGT2B15 in LNCaP cells (31), has been identified as a transduction pathway involved in prostate tumor growth (32, 33).

Beyond these mechanistic considerations, the present results are of clinical significance for prostate cancer progression, treatment and diagnosis. In terms of tumor progression, the desensitization of the UGT response to ADTh, as revealed by the similar protein levels detected in untreated tumors and CRPCs, suggests that the loss of UGT induction is involved in tumors
transition to castration resistance. Even if resistant to ablation therapies, CRPC progression remains dependent on the androgen axis (8, 34). Since AR acts as a negative UGT2B15 and UGT2B17 genes regulator (14, 20, 21), the loss of UGT induction detected in vivo may actually reflect an AR reactivation in resistant tumors. On the other hand, as observed with UGT knock-down experiments, the induction of androgen glucuronidation may contribute to the anti-proliferative effects of AR antagonists. It is therefore tempting to speculate that the loss of this induction is likely involved in the phenotypic changes allowing tumor cells to proliferate in the presence of low androgen levels, as observed in CRPCs (8). This idea is supported by the inverse relationship existing between UGT2B15 and UGT2B17 expression and androgen-dependent PCa cell proliferation (14), and by the strong influence that UGT2B15 and UGT2B17 polymorphisms exert on tissue androgen levels (35). The loss of UGT genes sensitivity may allow PCa cells to maintain sufficient DHT levels to activate AR and its regulated genes in CRPCs (36, 37). Based on these observations one can conclude that the UGTs induction in short-term treated samples is an important biochemical component of the initial ADTh benefits (38). Following on that point, it can be envisioned that UGT2B15 and UGT2B17 are actually underexploited therapeutic targets for androgen deprivation therapies. Until now anti-androgenic approaches have been focusing on androgen biosynthesis and activity (38). The present study validates the potential of targeting glucuronidation to reduce active androgen concentrations in PCa cells, thus providing a strong rational for searching pharmacological agents that stimulate androgen glucuronidation in PCa cells. However, such an alternative strategy requires the identification of AR-independent UGT2B15 and UGT2B17 genes inducers.

The present work may also have a significant impact in terms of PCa prognosis and diagnosis. Indeed, the respective changes in UGT2B15 and UGT2B17 protein levels in response to ADTh identify these 2 proteins as potential biomarkers for both evaluating the responsiveness to anti-androgen drug therapies and preventing resistance occurrence. ADTh remains the cornerstone of systemic treatments for locally advanced prostate cancer (37), and biochemical predictors are currently needed to assess whether additional treatments should be initiated (39). Furthermore, CRPC patients have a poor prognosis and account for the majority of PCa deaths (38), thus being able to anticipate ADTh resistance can be helpful in adapting
treatment settings with new effective anti-androgenic agents in order to prevent the development of castrate-resistant tumors (38). A continuous follow-up of UGT2B15 and/or UGT2B17 intra-prostatic protein levels from the time of diagnosis constitutes a potential approach to evaluate therapy responsiveness, and measuring UGT2B17 levels may also be helpful to anticipate the loss of optimal ADTh effects in UGT2B17-positive patients, and then initiate alternative therapeutics, even before the resistance occurrence.

The above discussed diagnostic and therapeutic opportunities are mainly based on results from the tissue microarray study, while largely supported by results from functional investigations. However, sample heterogeneity is a major problem in tissue array studies, especially in PCa, where patients receive varied drugs or combination of drugs, and the tumor areas are small and often surrounded by normal cells (39). Therefore, the role of UGT2B15 and UGT2B17 as drug targets and/or molecular biomarkers remains to be validated through large and accurate clinical settings.

Nevertheless, the combination of clinical and functional approaches used for the current investigation demonstrates that ADTh drugs stimulate the local androgen metabolism in prostate cells, and identifies UGT2B15 and UGT2B17 as potential anti-cancer drug targets and/or biomarkers for androgen ablation responsiveness and maintenance.
ACKNOWLEDGMENTS

We wish to thank Dr. Virginie Bocher for critical reading of the manuscript, and Dr. Éric Lévesque for helpful discussion.
REFERENCES


Figure 1. Androgen ablation therapy modulates UGT2B15 and UGT2B17 protein levels in prostate tumors.

UGT2B15 (A) and UGT2B17 (B) protein levels were quantified in prostate tumors using immunohistochemical analyses of a tissue microarray with specific antibodies as illustrated in SM2-8. Tumor samples were from 31 BPH donors or 88 advanced prostate cancer patients (SM9), who were either untreated (n=16), or treated with androgen deprivation therapy (ADTh) for 1 to 5 months (1-5; n=19), 6 to 8 months (6-8; n=24) and 9 to 12 months (9-12; n=13) or with castrate resistant tumors (CRPC, n=16).

The staining intensity was visually scored on a scale of 0 to 3 (see materials and methods), and values represent the mean±SEM of the scores obtained for each experimental group (expressed relatively to untreated samples). Statistically significant differences between experimental groups are indicated by asterisks (Mann-Whitney test: *: p<0.05; **: p<0.01; ***: p<0.001; n.s: not significant).

Figure 2. Bicalutamide dose- and time-dependently increases UGT2B15 (A&C) and UGT2B17 (B&D) expression in LNCaP cells.

(A&B) LNCaP cells were treated with DMSO (vehicle, V) or increasing bicalutamide concentrations for 48H.

(C&D) LNCaP cells were treated with DMSO (vehicle) or 10µM bicalutamide for the indicated duration.

UGT2B15 (A&C) and UGT2B17 (B&D) mRNA levels (upper panels) were quantified from total RNA through quantitative RT-PCR analyses, and normalized with the housekeeping RNA 36B4. Values (mean±SD) are expressed relatively to control (vehicle, V) set at 1. Statistically significant differences between control and treated cells are indicated by asterisks (Student $t$ test: *: p<0.05; **: p<0.01; ***: p<0.001; n.s: not significant).

UGT2B15 (A&C) and UGT2B17 (B&D) protein levels (lower panels) were visualized in cell homogenates (30µg) through immunoblotting using the anti-UGT2B15 (1/1500 dilution) (A&C) and anti-UGT2B17 (1/2000 dilution) (B&D) antibodies. The same membranes were
subsequently hybridized with an anti-calnexin antibody (1/5000 dilution) to ensure the equal loading of each lane.

**Figure 3. Bicalutamide dose- and time-dependently stimulates androgen glucuronidation in LNCaP cells.**

(A-C) LNCaP cells were treated with DMSO (vehicle, V) or increasing bicalutamide concentrations for 48H.

(D-F) LNCaP cells were treated with DMSO (vehicle) or 10µM bicalutamide for the indicated duration.

Cell homogenates (70 to 210µg) were used for *in vitro* glucuronidation assays performed for 1H in the presence of 100µM of the active androgen dihydrotestosterone (DHT, A&D), or its reduced metabolites androsterone (ADT, B&E) and androstane-3α,17β-diol (3α-diol, C&F). The formation of glucuronide derivatives was measured through LC/ESI-MS/MS analyses. Statistically significant differences between control and treated cells are indicated by asterisks (Student *t* test: *: p<0.05; **: p<0.01; ***: p<0.001).

**Figure 4. The AR antagonists bicalutamide (A-D), nilutamide (C&D) and flutamide (C&D) differentially modulate UGT2B15 (A-D) and UGT2B17 (C) mRNA levels in LNCaP and LAPC-4 cells.**

(A) LAPC-4 cells were treated with DMSO (vehicle) or increasing bicalutamide concentrations for 48H.

(B) LAPC-4 cells were treated with DMSO (vehicle) or 10µM bicalutamide for the indicated duration.

(C&D) LNCaP (C) and LAPC-4 (D) cells were treated with vehicle, bicalutamide (10µM), nilutamide (10µM) or flutamide (20µM) for 48H.

UGT2B15 (A-D) and UGT2B17 (C) mRNA levels were quantified from total RNA through quantitative RT-PCR analyses, and normalized with the housekeeping RNA 36B4. Values (mean±SD) are expressed relatively to control (vehicle) set at 1. Statistically significant differences between control and treated cells are indicated by asterisks (Student *t* test: *: p<0.05; **: p<0.01; ***: p<0.001).
Figure 5. ShRNA-mediated knock-down of the androgen receptor modifies the ability of its agonists and antagonists to modulate UGT2B15 (A) and UGT2B17 (B) mRNA expression in LNCaP cells.

LNCaP cells stably expressing an inducible anti-AR shRNA were pre-treated (DOX+) or not (DOX-) with doxycycline (1µg/ml) for 48H, and then cultured in media enriched with DMSO or ethanol (vehicle), R1881 (1nM), DHT (10nM) or bicalutamide (10µM) for an additional 48H. The antibiotic was maintained at the same concentration for the complete treatment duration.

UGT2B15 (A) and UGT2B17 (B) mRNA levels were quantified from total RNA through quantitative RT-PCR analyses, and normalized with the housekeeping RNA 36B4. Values (mean±SD) are expressed relatively to vehicle-treated DOX- cells set at 1.

Statistically significant differences between DOX- and DOX+ (white bars versus grey bars) cells cultured with or without AR agonists/antagonists are indicated by asterisks (Student t test: **: p<0.01; ***: p<0.001; n.s: not significant). The statistical significance of the differences in mRNA levels between vehicle versus R1881-, DHT- or bicalutamide-treated DOX– cells (white bars; †††: ***: p<0.001) and between vehicle versus DHT- or bicalutamide-treated DOX+ cells (grey bars #: p<0.05; ###: p<0.001) were also determined using the parametric Student t test.

Figure 6. UGT2B15- and UGT2B17-deficient LNCaP cells are less sensitive to the anti-proliferative effects of bicalutamide.

(A&B) LNCaP cells were transiently transfected with a non-target (control) or an anti-UGT2B15/UGT2B17 siRNA probe (UGT deficient) using lipofectin for 6H, allowed 18H for recovery and then exposed to vehicle (DMSO) or bicalutamide (10µM) for up to 2, 3 or 4 days (D).

(A) UGT2B15 and UGT2B17 mRNA levels were quantified from total RNA through quantitative RT-PCR analyses, and normalized with the housekeeping RNA 36B4. Values (mean±SD) are expressed relatively to control (non-target siRNA probe) set at 1.

(B) Cell viability was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation
Assay kit. Proliferation rate values (mean±SD) are expressed as number of cells per well. Percentages of reduction in cell proliferation were determined. Statistically significant differences between siRNA control and siRNA UGT2B15/B17-transfected cells are indicated by asterisks (Student t test: *: p<0.05; **: p<0.01; ***: p<0.001; n.s: not significant).
Figure 2: Bar graphs showing the relative UGT2B15 and UGT2B17 mRNA levels after treatment with different concentrations of Bicalutamide and over time.

A) UGT2B15
- Bicalutamide (μM): V, 0.1, 1, 5, 10, 20
- Relative mRNA levels:
  - Vehicle: V
  - Bicalutamide: 0.1 μM, 1 μM, 5 μM, 10 μM, 20 μM

B) UGT2B17
- Bicalutamide (μM): V, 0.1, 1, 5, 10, 20
- Relative mRNA levels:
  - Vehicle: V
  - Bicalutamide: 0.1 μM, 1 μM, 5 μM, 10 μM, 20 μM

C) UGT2B15
- Time (Hours): 6, 12, 24, 48, 72
- Relative mRNA levels:
  - Vehicle: 6, 12, 24, 48, 72
  - Bicalutamide: 6, 12, 24, 48, 72

D) UGT2B17
- Time (Hours): 6, 12, 24, 48, 72
- Relative mRNA levels:
  - Vehicle: 6, 12, 24, 48, 72
  - Bicalutamide: 6, 12, 24, 48, 72

**Note:** n.s. indicates not significant, *p < 0.05, **p < 0.01, ***p < 0.001.
A) LAPC-4

![Graph showing relative UGT2B15 mRNA level vs. Bicalutamide (µM).](image)

B) LAPC-4

![Graph showing relative UGT2B15 mRNA level vs. Time (Hours).](image)

C) LNCaP

![Graph showing relative mRNA level vs. treatment.](image)

D) LAPC-4

![Graph showing relative UGT2B15 mRNA level vs. treatment.](image)

Figure 4
Figure 5
Figure 6
Androgen glucuronidation: an unexpected target for androgen deprivation therapy with prognosis and diagnostic implications.

Laurent Grosse, Sophie Paquet, Patrick Caron, et al.

Cancer Res Published OnlineFirst October 11, 2013.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-1462

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2013/10/11/0008-5472.CAN-13-1462.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.