Valproic acid induces neuroendocrine differentiation and UGT2B7 up-regulation in human prostate carcinoma cell line

Alessandra Valentini, Michela Biancolella, Francesca Amati, Paolo Gravina, Roberto Miano, Giovanni Chillemi, Alessio Farcomeni, Susana Bueno, Giuseppe Vespasiani, Alessandro Desideri, Giorgio Federici, Giuseppe Novelli and Sergio Bernardini.

AV, PG, GF, SB Dept. of Internal Medicine-PTV University of Rome “Tor Vergata”,
MB, FA, GN Dept. of Biopathology and Diagnostic Imaging University of Rome “Tor Vergata”,
RM, GV Dept. of Urology University of Rome “Tor Vergata”,
GC, AF, SB CASPUR- Rome,
AD Dept. of Biology University of Rome “Tor Vergata”,
GF Santa Lucia Foundation-IRCSS, Rome,
SB Bambino Gesù Children’s Hospital-IRCSS, Rome-Italy.
Running Title: Acquisition of Neurondocrine Characteristics and up-regulation of UGT2B7 in prostate cancer cells after VPA treatment

Corresponding Author:
Alessandra Valentini, PhD
Dept. of Internal Medicine PTV-Università di Roma Tor Vergata
Via Montpellier 81 00133 Roma - Italy
Phone +39 0620902259 Fax +39 0620902537
E-mail: ales_valentini@yahoo.it

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Abbreviations: CaP, prostate cancer; NE, neuroendocrine; VPA, valproic acid; HDAC, histone deacetylase; UGT, UDP-glucuronosyltransferases; PSA, prostate-specific antigen; NSE, neuron-specific enolase; AR, androgen receptor
ABSTRACT:

Prostate cancer originates as an androgen-dependent hyperproliferation of the epithelial cells of the gland and it evolves in an androgen-independent, highly aggressive cancer for which no successful therapy is available to date. Neuroendocrine (NE) differentiation plays an important role in the progression of prostate cancer to an androgen independent state with profound impact on CaP therapies. Actually new approaches on treating advanced prostate cancer are focused on modulators of epigenetic transcriptional regulation. New class of antitumoral agents is emerging: histone deacetylase (HDAC) inhibitors are interesting for their ability to arrest cell growth, to induce cell differentiation, and in some cases, to induce apoptosis of cancer cells. We studied the effect of Valproic acid (VPA), an inhibitor of HDAC, in the human prostate androgen-dependent cancer cell line LNCaP. We observed that VPA promotes neuroendocrine-like differentiation associated with an increase in the expression of Neuron Specific Enolase, a decrease in PSA, and a down-regulation of AR protein, suggesting a modulation in the responsiveness to androgen therapy. Furthermore, selective gene expression profiling using a low-density microarray showed that VPA was able to modulate the expression of different androgen metabolism genes. We observed a down regulation of androgen receptor coregulator (ARA24) and prostate-specific antigen, while an up-regulation of some of the UDP-glucuronosyltransferases (UGT2B11 and UGT2B7) implicated in catabolism of dihydrotestosterone (DHT) was detected. Even though UGT2B7 has only about one tenth to one hundredth the activity of UGTs 2B15 and 2B17 towards active androgens and we did not found any modulation in gene expression of these enzymes it can be hypothesised that VPA might enhance DHT catabolism in this in vitro model and induces NE differentiation. Our data seem to be concerning CaP treatment with VPA.
Recently modulators of epigenetic transcriptional regulation have been suggested to be useful for treating advanced prostate cancer. Indeed it is well known that acetylation and deacetylation of histones play an important role in the epigenetic regulation of gene expression (Roth et al., 1996; Minucci et al., 2006). There are at least two classes of enzymes involved in determining the acetylation state of histones, histone acetyl transferases and histone deacetylases (HDAC). Altered states of these enzymes have been associated with various malignancies (Blaheta et al., 2005; Angelucci et al., 2006). News data provide evidence that prostate cancer may particularly benefit from Valproic Acid (VPA) treatment. In fact, some authors demonstrated that VPA in vitro is able to induce apoptosis in LNCaP prostate cancer cell line (Angelucci et al., 2006; Thelen et al., 2004) inducing a dramatic decrease of PSA mRNA expression (Thelen et al., 2004). Moreover recent studies demonstrate that chronic VPA treatment results in statistically significant reduction of tumor xenograft growth in vivo, independently of androgen regulation (Xia et al., 2006).

Androgens play a central role in promoting the development and progression of prostate cancer, and androgen ablation through the androgen receptor (AR) has been the cornerstone of treatments for advanced prostate cancer. In this context, the identification of pharmacological targets and the investigation of the mechanisms underlying the regulation of AR function is of critical importance for the design and development of new therapies (Chen et al., 2005). Moreover, it has been reported that after long-term antiandrogen therapy, tumor cell populations become enriched for neuroendocrine (NE) cells and that NE differentiation is associated with progression of CaP toward an androgen-independent state, a condition for which there is currently no successful therapy (Abrahamasson et al., 1989). In fact, neuroendocrine differentiation (NED) has been implicated in prostate cancer progression and hormone-therapy failure since neuroendocrine cells are non-proliferating and then avoid apoptotic cell death (Cox et al., 1999).
The progression of CaP from androgen-responsive to an androgen-unresponsive state remains one of the major obstacles in the treatment of this disease. Therefore, VPA might represent an effective therapy of androgen-unresponsive cancer (Angelucci et al., 2006).

In this study, we evaluated the expression profile of genes involved in the biosynthesis and metabolism of androgens in LNCaP cells after VPA treatment. At this purpose, we developed a low-density oligo-array containing 103 genes able to delineate the effects of VPA treatment on androgen signalling pathway. Using this approach, we were able to provide additional findings to understand the cellular and molecular effects of VPA in androgen responsive CaP cells in vitro.
Materials and Methods

**Chemicals and cells.** Human prostate cancer cell line (LNCaP) was grown in RPMI-1640 (Gibco) supplemented with 10% of fetal bovine serum (GIBCO), 5% of L-Glutamine (GIBCO) and antibiotics, under standard conditions (37°C temperature, 5% CO₂ in a humidified atmosphere). Valproic Acid (Sigma, St.Louis, MO, USA) dissolved in sterile water were administered to the cells after 24 hour after seeding, at the final concentration of 1 mM and 5 mM.

**Prostate-specific antigen (PSA) and neuron-specific enolase (NSE) assay.** The Elecsys total PSA immunoassay (Roche) was used to quantify the PSA in medium of cells treated or not with valproic acid. The neuron-specific enolase (NSE) immunoassay (Roche) was used to measure the quantity of NSE in medium of cells treated or not with VPA. The electrochemiluminescence immunoassays “ECLIA” were used on the MODULAR ANALYTICS (Elecsys module – Roche). The results of PSA and NSE levels are related to the cell number.

**Western blotting.** Total cell lysates were obtained resuspending the cells in buffer containing 1% Triton, 0.1% SDS, 2 mM CaCl₂, 100 µg/ml phenylmethyl sulfonyl fluoride. Protein content was determined using the Protein Assay Kit 2 (Bio-Rad Laboratory, Hercules, CA, USA). 30 µg of proteins were electrophoresed in 10% SDS-polyacrylamide gel and then electrotransferred to nitrocellulose membrane (Amersham biosciences, Piscataway, NJ, USA), which was then blocked overnight with 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween-20 (TBS-T) containing 5% non fat dry milk. The membrane was then incubated with 1µg/ml of primary antibody in TBS-T [AR (clone G122-434, BD Pharmingen) or UGT2B7 (BD Gentest)] and with specific horseradish peroxid
ase-conjugated secondary antibodies in TBS-T. Protein bands were visualized using a chemiluminescent detection system (Amersham biosciences, Piscataway, NJ, USA).

**Microarray design and printing.** We developed a low density home made oligo-array composed of 103 genes selected on the basis of their proved or potential role in prostate cancerogenesis related to androgen signalling ("AndroChip"). They have been subdivided in different classes, according to their molecular function or to the biological process in which they are active; genes regulating the androgen metabolism within the prostate, androgen receptor (AR) and genes that bind to the AR-complex (such as ARA70), genes whose expression is androgen-regulated (ARGs), genes involved in transcriptional regulation, and in signalling pathway.

A complete list of genes present onto the “AndroChip” is available as supplementary data.

The oligonucleotide set (MWG Biotech, Ebersberg, Germany) was printed in triplicate onto UltraGAPS glass slides (Corning, Schiphol-rijk, The Netherlands) using TheRoboArrayer™ (Microgrid Compact Plus, BioRobotics). Printed slides were dried overnight and cross-linked with UV light at 600 mJ using a Strata-linker 2400 (Stratagene, Glenville, VA) and stored in a desiccator at room temperature. Before hybridization, each slide was incubated in a prehybridization buffer (5XSSC, 0.1% SDS, and 0.1 mg/ml BSA) at 42°C for 45–60 min.

**RNA extraction, labeling, hybridization.** Total RNA was isolated from LNCaP cells untreated and valproic acid (VPA) treated cells for 48 hour using TRIZOL standard protocol (Invitrogen). A small aliquot of RNA was then used for quantification and quality control using respectively a spectrophotometer (Nanodrop™, Wilmington, Delaware, USA) and an agarose gel electrophoresis.

Synthesis of the labelled first strand cDNA was conducted using the Superscript Indirect
cDNA labeling system (Invitrogen Corporation) with starting material of 10 µg of total RNA. The amino-allyl labeled dNTP mix was added to the reaction to generate amino-allyl labelled second strand cDNA. Following the hydrolysis reaction, single-stranded cDNA probes were purified using a Purification Module (Invitrogen Corporation). Probe mixtures where then evaporated in a vacuum centrifuge, and the cDNA pellet resuspended in 3 µl of water. The dye coupling reactions were performed by mixing the cDNA samples with AlexaFluor Dyes 555 or 647 and were incubated overnight in the dark. The reactions were purified with a Purification Module (Invitrogen Corporation) to remove the unincorporated/quenched dyes. After the purification, samples were combined for hybridization. The labelled cDNAs were co-hybridized to microarrays in duplicate. Slides were scanned on the GenePix 4000B Microarray Scanner (Axon instruments, Union City) at the optimal wavelength for the Alexa555 (F532) and Alexa647 (F635) (Invitrogen Corporation) using lasers.

**Image Analysis and Processing Bionformatics.** The acquired images were analysed with Genepix Pro 5.0 software (Axon instruments, Union City). Oligonucleotide spots were automatically segmented, local backgrounds (B635 and B532) were subtracted, and total intensities (F635 and F532) as well as the fluorescence ratios of the two dyes were calculated for each spot. The spots were rejected when they exhibited poor hybridisation signals or when they were saturated (F635 median = 65534 or F532 = 65534). Genes with signal to background ratio below two were flagged, together with genes strongly disagreeing by fold change: if two out of three replicates were overexpressed, while the third had a fold change below the cut off -2, the gene was flagged; and similarly for under expressed genes.

Data has been normalized with a separate global normalization for each slide. This permits a better stabilization of the MA-plots than a multi-slide or intensity dependent normalizations. Genes were selected through T-testing, and we corrected for the multiplicity by controlling the False
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Discovery eXceedance (FDX) of (Van der Laan et. al. 2004), in order to avoid an explosion of the number of false rejections. FDX control was given by the GAUGE procedure, and guarantees that the proportion of erroneously selected genes is below 10% with a probability at least 95%. Finally, among the significant genes we considered only the ones with a fold change at least equal to +/-1.5.

Reverse Transcription and Quantitative Real-Time RT-PCR. cDNA synthesis was performed from 1µg of total RNA for each samples, by using AMV Reverse Transcriptase and as primers oligo-p(dT) (1st Strand cDNA synthesis kit for RT-PCR (AMV)-Roche Applied Science).

Real-Time quantitative RT-PCR was carried out using the Mx3000P™ (Strategene®) which detects the signal from the fluorogenic SYBR green® during PCR. The volume of each reaction was 25 µl containing 5 µl of the cDNA sample, 5 pmol each primer UGT2B7 [5’-AGA ATT TCA TCA TGC AAC AG-3’-GTT ATG TCA CCA AAT ATT G-], ARA24 [5’-CCA CCA GAA GTT GTC ATG GAC-3’-ACA AGG GAT GAG TTC ACT TGC-], UGT2B11 [5’-TTC CAT TC TTT TTG ATCC CAA TGA TG-3’-TAG GTA TGA GGA AGG AGG GAA AAT C-], UGT2B15 [5’-GTG TTG GGA ATA TTA TGA CTA CAG TAA C-3’-TCA GCC AGT AGC TCA CCA CAG GG-], UGT2B17 [5’-TGT TGG GAA TAT TCT GAC TAT AAT C-3’-TCA GCC AGT AGC TCA CCA CAG GG-] and for β2-Microglobulin [5’-GAG TAT GCC TGC CGT GTG- 3’-AAT CCA AAT GCG GCA TCT -] 12,5µl of SybrGreen Universal PCR Master Mix®, (PE Applied Biosystems). The specificity of the primers used for UGTs was confirmed by BLAST program considering the Query coverage of 100%. The thermal cycling conditions comprised 10 min at 95°C. Thermal cycling consisted of 44 cycles at 95°C for 30 s, 55°C for 1 min and 72°C for 30 s. Each assay include: a standard curve (from 1µg to 125ng of cDNA) in duplicate, a no-template control (water), 250 ng of calibrator cDNA in triplicate (from DU145 cells). All samples with a coefficient of variation (CV) higher than 10 % were rested.
QReal-Time PCR Validation Experiment. The amount of target, normalized to the endogenous reference (β2-microglobulin) and relative to the calibrator, was performed by $2^{-\Delta\Delta CT}$ method (Livak et al., 2001). The relative expression was calculated with the “delta-delta Ct method” ($\Delta\Delta Ct=\Delta Ct_{sample}-\Delta Ct_{calibrator}$). The Ct (threshold cycle) values were normalised against the endogenous reference gene β2-microglobulin ($\Delta Ct_{target\ gene}-\Delta Ct_{β2\ -}\text{microglobulin}$) and were compared with the calibrator. The ΔΔCt validation required equal efficiencies of target genes and reference amplification. Therefore, standard curve assays were obtained for target gene and reference by cDNA sample diluted at two fold intervals. For each dilution, cycle threshold were plotted against the Log value of the input cDNA concentration. Quantitative PCR efficiencies (E) were calculated from the slopes, where one cycle in the exponential phase yielded the efficiency: $E=10^{\frac{-1}{\text{slope}}}$. 

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VPA induced PSA decrease and Neuron-endocrine differentiation in LNCaP. We analyzed the effect on PSA secretion after VPA treatment (1 mM and 5 mM) at different time of exposure. Figure 1a shows that the effect of VPA on PSA secretion was dose-dependent starting from 24 h of exposure. A reduction of PSA secretion of about 50% was detected after 24 h of treatment with 1mM VPA as well as after 48 and 72 hour. At the concentration of 5 mM VPA, stronger reduction in PSA production was observed (Fig. 1a).

After 48 h of treatment with VPA 5 mM we observed a dose-dependent increase of neuron-specific enolase (NSE), indicating that VPA is able to induce an early neuron-endocrine differentiation in LNCaP. In addition after 72 h of VPA exposure the secretion of NSE increased even in cells treated with 1 mM VPA. The NSE increase is consistent with morphological changed observed in VPA treated cells (Fig. 1b), indeed fewer attached cells which showed long dendritic processes remained after 72 hour of VPA treatment (lower panel) compared to untreated cells (upper panel).

Down regulation of Androgen Receptor protein expression induced by VPA. Since valproic acid is expected to modulate androgen receptor (AR) activity with downstream consequences as suggested by PSA decrease (Thelen et al., 2004). We investigated the effect of VPA treatment on androgen receptor (AR) expression in LNCaP cells by Western blot analysis. Figure 2 shows that the down-regulation of AR protein is VPA dose-dependent. After 48 h of exposure to valproic acid was evident a decrease of AR protein expression with a complete down-regulation of AR protein expression after 72 h of exposure for both the doses employed of 1 and 5 mM.

Gene expression profile of LNCaP cells treated with VPA. To monitor changes in gene
expression we performed microarray experiments in LNCaP cells treated with 5 mM VPA after 48 h. Experiments were done in duplicate using reverted labelled RNA. After data normalisation, we considered only genes showing a fold change (FC) at least equal to +/− 1.5. A total of 15 differentially expressed genes were detected (Table 1). Of these, 10 were underexpressed and 2 (UGT2B7 and UGT2B11) were overexpressed.

Microarray results were confirmed by QReal-Time PCR for both UGT2B7 and UGT2B11 genes (Fig. 3a). In addition, as shown in figure 3b the UGT2B7 protein up-regulation was evaluated. Using $\Delta \Delta CT$ gene expression quantification, we observed that the up-regulations of UGT2B7 and UGT2B11 genes were VPA dose-dependent. In fact, as shown in figure 3 at the concentration of 1 mM of VPA we observed a 2-fold up-regulation of UGT2B7 gene expression, whereas at 5 mM, a 7-fold higher increase was detected. Analogously, UGT2B11 was up-regulated 2.2-fold after 1 mM VPA exposure and 5-fold after 5 mM VPA. ARA 24 was found 2-fold down-regulated after 1mM VPA and 5,2-fold after 5 mM VPA exposure. Moreover we analyzed the expression of UGT2B15 and UGT2B17, implicated in DHT glucuronidation, and we did not found any modulation induced by VPA treatment. Relative expression level of gene in VPA treated vs untreated cells, reported as $2^{\Delta \Delta CT}$, was 1.0 for both genes.
Discussion

We report that VPA promotes neuroendocrine-like differentiation of androgen-dependent LNCaP prostate cancer cells. This differentiation is associated with an increase in the secretion of Neuron Specific Enolase (NSE), a decrease in PSA and a down-regulation of AR protein, suggesting a role of VPA in the mechanism of androgen responsiveness of prostate cancer. Being AR expression influenced by post-translational and epigenetic modifications, it could be of interest to better understand the role of VPA in modulating this protein. Inhibition of cell proliferation and of AR protein expression have been observed in prostate cancer cells after exposure to chemopreventive compounds, such as vitamin E, resveratrol and VPA as well (Angelucci et al., 2006; Culig et al., 2005). Since elevated NSE levels in prostate carcinomas during androgen ablation therapy correlates with hormone-refractory growth and poor prognosis (Hvamstad et al., 2003), the ability of the HDAC inhibitor VPA to induce NE differentiation in LNCaP-derived prostatic carcinoma cell lines should be elucidated by studying genes involved in homeostasis of prostate cancer cells.

Low-density microarray, as our custom “Androchip”, is a reliable technique through which, in a single assay, the quantitative expression of each gene in response to a change in the cellular state can be measured simultaneously. Gene profiling by “Androchip” of LNCaP treated with valproic acid showed that genes differentially expressed are mostly involved in steroids metabolism. In particular, we observed a down-regulation of androgen receptor co-regulator (ARA24), of farnesyl pyrophosphate synthase which is a cholesterol-repressible protein (FDPS), of Isopentenyl-diphosphate δ-isomerase (IDI1), of prostate androgen-regulated transcript (PART1), of prostate-specific antigen (PSA) and upregulation of UDP-glucuronosyltransferases (UGT2B11 and UGT2B7) involved in the catabolism of dihydrotestosterone (DHT).
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It should be pointed out that ARA24 is a co-factor implicated in the enhancement of AR transactivation, in AR nuclear translocation and in chromatin organization. The AR co-activator effect of ARA24 is not only at the physiological concentration of DHT but also at low DHT level ($10^{-10}$ M) usually found in patients undergoing androgen ablation therapy (Hsiao et al., 1999). Moreover ARA24 is significantly higher in prostate tumour tissue compared with non neoplastic prostate tissue (Li et al., 2002). In vitro studies have shown that altered expression of AR co-regulators may significantly modify transcriptional activity of AR, suggesting that these co-regulators could also contribute to the progression of prostate cancer. AR co-activators, showing histone acetyltransferase activity, interact with one or more domains of the AR, thus leading to remodelling the chromatin structure through the acetylation of histones (Culig et al., 2005). Not only expression, but also functional interactions of co-regulatory proteins, may be altered in the late stage of prostate cancer. This mechanism might be active with other co-factors in prostate cancer thus enhancing expression of AR target genes in late tumour stages. In cellular context, AR over-expression is clearly associated with malignant phenotype.

Furthermore among VPA up-regulated genes we have found UDP-glucuronosyltransferases. Valproic acid is a specific substrate for glucuronidation through UGT2B7 (Ethell et al., 2003; Hum et al., 1999), in addition UGT2B7 catalyzes the conjugation of the 17β-hydroxy position of DHT, testosterone and 3α-Diol, and glucuronidate androsterone (ADT) (Turgeon et al., 2001). UGT2B7 has only about one tenth to one hundredth the activity of UGT2B15 and UGT2B17 towards these active androgens. Although it has been demonstrate that human prostate and LNCaP cells express both UGT2B15 and UGT2B17, which are two widely distributed UGT isoforms implicated in steroid glucuronidation (Lévesque et al., 1998), we did not found any modulation in gene expression of these two enzymes in LNCaP cells treated with VPA (data not shown). Thus it could be hypothesised that
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VPA might enhance DHT catabolism in this *in vitro* model.

Glucuronidation is catalyzed by UDP-glucuronosyltransferases (UGTs), which transfer the polar moiety from UDP-glucuronic acid to a wide variety of endogenous compounds, including steroid hormones. UGT2B7 has been demonstrated to glucuronidate estrogens, catechol estrogens, and androstane-3α,17β-diol more efficiently than any other human UGTB isoform (Turgeon et al., 2001).

Moreover major pathways of valproate metabolism include glucuronidation by UGTs, mitochondrial β oxidation and a minor cytochrome P450-dependent oxidation pathway and desaturation (Baille et al., 1995). In androgen sensitive tissues, such as skin and breast, UGT2B7 probably contributes to the conjugation of phase 1 DHT metabolites, particularly 3α-Diol and ADT (Bélanger et al., 2003). So far it is known that UGT2B11 is involved in xenobiotic metabolism (Jinawath et al., 2006) and recently it has been demonstrated that treatment of the prostate cancer cells LNCaP with natural and synthetic androgens, IL-1α, or epidermal growth factor (EGF) differently modulates the glucuronidation of androgen and bioactive lipid metabolites. Indeed, glucuronidation of 5α-androstane-3α,17β-diol and 13-hydroxyoctadecadienoic acid was drastically reduced, whereas 12-hydroxyeicosatetraenoic acid conjugation by UGT2B11 was increased after androgen treatment. In LNCaP cells UGT2B11 mRNAs are detected and are regulated by androgens in a similar manner as in human prostate epithelial cells (Chouinard et al., 2006). Recently Bélanger has postulated that UGTs activities may be implicated as a mean to control androgen levels in target tissues (Bélanger 2003).

However, being VPA a short chain branched fatty acid and hence a substrate of UGT2B7, we can not exclude a competitive effect of VPA in steroid catabolism (Ethell et al., 2003). Thus, the action of VPA has to be further investigated before considering it for therapeutic protocols in prostate cancer.
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References


Lévesque É, Beaulieu M, Guillemette C, Hum DW, Bélanger A (1998) Effect of Interleukins on UGT2B15 and UGT2B17 steroid uridine diphosphate-glucuronosyltransferase expression and
activity in the LNCaP cell line, *Endocrinology* **139**:2375-2381.


Footnote section

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Legends

Figure 1. A: PSA and NSE analysis expressed as ng/ml of protein in culture medium of LNCaP cells after different time of exposure to valproic acid and results are related to the cells number for each point. The electrochemiluminescence immunoassays “ECLIA” were used on the MODULAR ANALYTICS (Elecsys module – Roche). U untreated cells, I 1 mM VPA, 5 5 mM VPA. Results are expressed as mean ± standard deviation (SD) for n=3 independent experiments. B: Fewer attached cells which showed long dendritic processes (arrows) remained after 72 hour of VPA treatment (lower panel) compared to untreated cells (upper panel).

Figure 2. Representative western blotting illustrating the expression of AR protein in LNCaP cells after different time of exposure to VPA. β-actin has been used as a control for sample loading.

Figure 3. A: UGT2B7(■), UGT2B11 (□) gene expressions increase and ARA 24 (□) decrease in the LNCaP cells exposed to VPA for 48 h. Target mRNA levels were evaluated by quantitative RT-PCR and standardized to β2-microglobulin mRNA levels (ΔΔCT method). Expression levels in the control group were designated as 1.0, and relative expression levels in the VPA 1 mM (upper) and VPA 5 mM (lower) LNCaP treated cells are shown. Data are represented as the mean ± SD from 3 independent experiments. B: Representative western blotting illustrating the expression of UGT2B7 protein in LNCaP cells after 48h of exposure to VPA. β-actin has been used as a control for sample loading.
Table 1. Genes differentially expressed in LNCaP treated with valproic acid

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<td>ARA24</td>
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<td>NM_001083 phosphodiesterase 5A, cGMP-specific</td>
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<td>PSA</td>
<td>NM_001648 kallikrein 3, (prostate specific antigen)</td>
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<td><strong>Up-regulated genes</strong></td>
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Figure 2

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<td>5mM</td>
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AR
β-actin
Figure 3

A

![Graph showing relative expression vs untreated cells for different concentrations of VPA (VPA 5mM, VPA 1mM).](image)

B

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