Primary hepatocytes as an useful bioassay to characterize metabolism and bioactivity of illicit steroids in cattle

Primary hepatocytes as an useful bioassay to characterize metabolism and bioactivity of illicit steroids in cattle

Toxicology In Vitro 26(2012):1224-1232

http://dx.doi.org/10.1016/j.tiv.2012.06.003

PRIMARY HEPATOCYTES AS AN USEFUL BIOASSAY TO CHARACTERIZE METABOLISM AND BIOACTIVITY OF ILLICIT STEROIDS IN CATTLE

Mery Giantin[a], Guglielmo Gallina[a], Sara Pegolo[a], Rosa Maria Lopparelli[a], Clara Sandron[a], Vanessa Zancanella[a], Carlo Nebbia[c], Donata Favretto[b], Francesca Capolongo[a], Clara Montesissa, Mauro Dacasto[a]*

[a] *Dipartimento di Biomedicina Comparata e Alimentazione, Università di Padova, viale dell'Università 16, I-35020 Agripolis Legnaro (Padova), Italia;*
[b] *Dipartimento di Medicina Molecolare, Università di Padova, via Falloppio 50, I-35121 Padova, Italy;*
[c] *Dipartimento di Patologia animale, Università di Torino, via Leonardo da Vinci 44, I-10095 Grugliasco (Torino), Italy.*

---

* Corresponding author. Address: Dipartimento di Biomedicina Comparata e Alimentazione, area di Farmacologia e Tossicologia, viale dell'Università 16, I-35020 Agripolis Legnaro (Padova) – Italy. Tel.: +39.049.827.2935; Fax: +390.049.827.2973.

E-mail addresses: mery.giantin@unipd.it (M. Giantin); guglielmo.gallina@libero.it (G. Gallina); sara.pegolo@unipd.it (S. Pegolo); rosa.lopparelli@unipd.it (R.M. Lopparelli); clara_128@hotmail.com (C. Sandron); vanessa.zancanella@unipd.it (V. Zancanella); carlo.nebbia@unito.it (C. Nebbia); donata.favretto@unipd.it (D. Favretto); francesca.capolongo@unipd.it (F. Capolongo); clara.montesissa@unipd.it (C. Montesissa); mauro.dacasto@unipd.it (M. Dacasto).

Abbreviations

β-actin, ACTB;

analysis of variance, ANOVA;

androgen receptor, AR;

androsta-1,4-diene-3,17-dione, boldione or ADD;

androsta-l ,4-diene-17β-01-3-one, 17β-boldenone or BOLD;

4-androsten-2β, 17β-diol-3-one, 2βOH-TST;

4-androsten-3,17-dione, androstenedione or AND;

4–androsten-6β, 17β-diol-3-one, 6βOH-TST;

4-androsten-16β, 17β-diol-3-one, 16βOH-TST;

arbitrary units, a.u.;

constitutive androstan receptor, CAR;

cytochromes P450, CYPs;

dehydroepiandrosterone, DHEA;

drug metabolizing enzymes, DMEs;

ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, EGTA;

foetal calf serum, FCS;

glutathione *S*-transferase A1, GSTA1-*like*;

Hanks' balanced salts solutions, HBSS;

hepatocyte nuclear factor 4-alpha, HNF4α;

high-performance liquid chromatography, HPLC;

17β-hydroxysteroid dehydrogenase type 2, 17β-HSD2;

lactate dehydrogenase, LDH;

liquid chromatography-high resolution mass spectrometry, LC-HRMS;

nuclear receptors, NRs;

phosphate buffer solution, PBS;

peroxisome proliferator-activated receptor-alpha, PPARα;

pregnane X receptor, PXR;

quantitative real time polymerase chain reaction, qPCR;

retinoic X receptor alpha, RXRα;

standard deviation, S.D.;

sulfotransferase 2A1, SULT2A1;

testosterone, TST;

uridin diphosphate glucuronosyltransferase 2B17-*like*, UGT2B17-*like*;

William's E medium, WEm.

Abstract

Cattle hepatocytes have already been used in veterinary *in vitro* toxicology, but their usefulness as a multi-parametric screening bioassay has never been investigated so far. In this study, cattle hepatocytes were incubated with illicit steroids/prohormones (boldenone, BOLD; its precursor boldione, ADD; dehydroepiandrosterone, DHEA; an association of ADD:BOLD), to characterize their transcriptional effects on drug metabolizing enzymes (DMEs) and related nuclear receptors (NRs), on cytochrome P450 3A (CYP3A) apoprotein and catalytic activity as well as to determine ADD and BOLD metabolite profiling.

DHEa-exposed cells showed an up-regulation (higher than 2.5 fold-changes) of three out of six NRs, CYP2B22 and CYP2C87; likewise, ADD:BOLD increased CYP4A11 mRNA levels. In contrast, a reduction of CYP1A1 and CYP2E1 mRNAs (lower than 2.5^{-1} fold-changes) was noticed in ADD- and DHEA-incubated cells. No effect was noticed on CYP3A gene and protein expression, though an inhibition of 6β-, 2β and 16β- hydroxylation of testosterone (higher than 60% of control cells) was observed in ADD- and BOLD-exposed cells. Finally, 17α-BOLD was the main metabolite extracted from hepatocyte media incubated with ADD and BOLD, but several mono-hydroxylated BOLD and ADD derivatives were detected, too.

Collectively, cattle hepatocytes can represent a complementary screening bioassay, useful to characterize growth promoters metabolite profiling and their effects upon DMEs expression, regulation and function.

Key words

cattle hepatocytes, bioassay, illicit steroids, prohormones, drug metabolism

1. Introduction

Steroids are illicitly used in cattle fattening to improve growth rate and reduce breeding costs, although their use has been banned for over twenty years in Europe. Such a phenomenon represents a major public concern for the risk of potentially harmful drug–drug interactions and the presence of uncontrolled drug residues in edible tissues (Nebbia et al., 2011). Illicit steroids more commonly used in cattle fattening are either natural compounds (i.e., 17β-oestradiol, progesterone and testosterone, TST) or synthetic derivatives like medroxyprogesterone 17-acetate, 19-nortestosterone, and trenbolone (Noppe et al., 2008). Moreover, breeders sometimes make use of certain prohormones like dehydroepiandrosterone (DHEA) and androsta-1,4-diene-3,17-dione or (boldione, ADD), whose exogenous origin cannot be easily determined (Draisci et al., 2007). Steroids misuse monitoring usually consist in the detection of the parental compound nor its derivatives in urine samples, but these analytical assays are often unsuccessful. This may be due to the use of outdated and insensitive analytical methods, the use of compounds of unknown chemical structure, the ever changing illicit schedules and a general lack of information about the kinetics of growth promoters in cattle (Nebbia et al., 2011; Vincenti et al., 2009). To challenge the black-market trade in hormones, genomic and proteomic approaches have been recently used to unveil tissue-specific biomarkers of steroid abuse (Giantin et al., 2010; Lopparelli et al., 2010; Lopparelli et al., 2011; Mooney et al., 2009; Nebbia et al., 2011; Rijk et al., 2010). Furthermore, bioassay-based screening tools have also been developed, validated and

shown to be complementary to official analytical methods (Bovee et al., 2009; Divari et al., 2010; Rijk et al., 2008; Willemsen et al., 2002). Clearly, these indirect molecular biomarkers cannot replace official analytical methods; rather, they might be successfully used in the screening phase to "filter" thousands of randomly collected samples, thereby improving the overall efficacy of controls. These bio-molecular tools are highly sensitive, easy and cost-effective; in contrast, they cannot either establish the identity of steroids/prohormones nor provide information about their biotransformation pathways, which can result in the formation of inactive metabolites or more active derivatives (Wang et al., 2010).

Several liver-derived *in-vitro* models are currently used in biomedical research for metabolic and/or toxicological applications. These ones range from simple enzyme preparations (i.e., S9 fractions, microsomes, Supersomes™) up to whole cell systems (i.e., primary cell cultures, transformed cell lines, tissue slices). Cultured hepatocytes are recognized as the closest model to the whole liver in the *in vivo* situation: in fact, they retain most of differentiated liver-specific functions, drug metabolizing enzyme (DMEs) capacity and drug transporters, although for a limited period of time (Gómez-Lechon et al., 2010; Li, 2007; Wang et al., 2010). Therefore, hepatocyte primary cultures represent a valuable model to study drug metabolism, drug-drug interactions and drug toxicity (Li, 2007).

In vitro liver models (including primary hepatocytes) have already been successfully used in cattle to study the metabolism and bioactivity of illicit steroids and prohormones (Clouet et al., 1998; Merlanti et al., 2007; Rijk et al., 2008; Van

Puymbroeck et al., 1998; Wang et al., 2010); nonetheless, nobody has still investigated the usefulness of cattle hepatocytes as a multiparametric screening assay, helpful to characterize illicit steroids/prohormones metabolite profiling as well as to investigate their effects on DMEs expression and regulation. In the present study cattle hepatocytes were at first incubated with some illicit steroids/prohormones; then, their transcriptional and post-transcriptional effects upon DMEs and related transcription factors were measured by using quantitative real time RT-PCR (qPCR), immunoblotting and high-performance liquid chromatographic assays; finally, their derivatives were identified by liquid chromatographic-high resolution mass spectrometry (LC-HRMS).

2. Materials and methods

2.1. Products and solutions

Chemicals and commercial kits used in the study, together with companies from whom they were obtained are hereby listed. Glucose (AppliChem, Milano, Italy); plastic cell-ware (Becton Dickinson, Milano, Italy); testosterone, 4-Androsten-2β, 17β-diol-3-one (2βOH-TST), 4-androsten-16β, 17β-diol-3-one (16βOH-TST), 4–androsten-6β, 17β-diol-3-one (6βOH-TST), 4-androsten-3,17-dione (androstenedione, AND), androsta-l ,4-diene-17β-01-3-one (17β-boldenone, BOLD) and ADD (Steraloids, Newport, RI, USA); 6α-, 6β-, 16α- and 16β-hydroxy BOLD (Alltech, State College, PA, USA); acetonitrile, methanol and dichloromethane (Mallinckrodt-Baker, Milano, Italy); sheep anti-mouse peroxidase-conjugated IgG (GE Healthcare, Chalfont St. Giles,

Buckinghamshire, UK); agarose, oligonucleotide primers and TRIzol® reagent (Invitrogen, Paris, France); High Capacity cDNA Reverse Transcription Kit and Power SYBR® Green PCR Master Mix (Applied Biosystems, Courtaboeuf, France); SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Milano, Italy); cell culture medium and supplements and all other reagents (Sigma Aldrich, Milano, Italy). The anti-rat CYP3A1/2 monoclonal antibody was a kind gift from H.V. Gelboin (National Institutes of Health, Bethesda, MD, USA). Purified deionised water was obtained through a MilliQ Synthesis water purification system (Millipore SpA, Vimodrone, Milano, Italy).

2.2. Cell culture and incubation with steroids and prohormones

2.2.1. Isolation of cattle hepatocytes

The liver caudate lobe of two healthy heifers (15-18 months old, about 400 kg) was collected at the slaughterhouse and immediately after the bleeding step. The lobe or a part of it (about 100-150 g) was rinsed, by inserting into major veins a cannula connected to a 60 mL disposable syringe, with 300-400 mL of ice-cold Eurocollins buffer (15 mM KH_2PO_4, 42 mM K_2HPO_4, 15 mM KCl, 1 mM $NaHCO_3$ and 0.2 M glucose, pH 7.4) supplemented with 1 mM ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA) to remove blood and deplete calcium. To favour buffer diffusion, the tissue was gently kneaded during all the washing step. Then, the

lobe was plunged in a beaker containing fresh (4°C) buffer, kept on ice and immediately transported to the laboratory.

Hepatocytes were isolated according to the method of Van't Klooster et al. (1992) and Kuilman et al. (1998), with few modifications. Briefly, the lobe was placed on a Buchner funnel and submitted to a retrograde three-steps perfusion by using a peristaltic pump (Minipulse 3, Gilson, Milano, Italy) and four cannulas. Buffers were sterile, adjusted to pH 7.4 and saturated, at 37°C, with carbogen (O_2/CO_2, 95:5, v/v). The lobe was at first perfused (100 $mL \cdot min^{-1}$) with 1 L of buffer I (142 mM NaCl, 0.5 mM KCl, 9.2 mM HEPES and 0.5 mM EGTA); then, with 1 L of buffer II (buffer I without EGTA); finally, with about 65.000 I.U. type IV collagenase (from rat tail) diluted in 9.2 mM HEPES, 66.7 mM NaCl, 6.7 mM KCl and 4.74 mM $CaCl_2$. This latter solution was recycled through the lobe for 17-20 min.

Then, the lobe was transferred under a laminar flow cabinet; the glissonian capsule was torn off and non-perfused tissue trimmed. Cells were dispersed in a buffer (pH 7.65) containing 9.3 mM HEPES, 9.91 $mg \cdot L^{-1}$ modified Hanks' balanced salts solution (HBSS) and 3% bovine serum albumin; afterwards, the cell suspension was gently shaken (10 min, 37°C) in a water bath under O_2/CO_2 (95:5, v/v), sieved through a nylon mesh (100 μm) and centrifuged (80x*g*, 5 min). Cells were washed for three times with William's E medium (WEm). The final pellet was weighed to estimate an appropriate amount of culture medium to be added (6 mL of medium per g of hepatocytes); finally, the total number of isolated hepatocytes and the percentage of viable cells were determined by using the trypan blue dye exclusion test.

2.2.2. Cell culture

The cell suspension was diluted to a final density of 10^6 cells·mL^{-1} with WEm containing 10% foetal calf serum (FCS), 1.67 mM glutamine, 26.6 μM $NaHCO_3$, 50 μg·mL^{-1} gentamicin sulphate, 1 μM hydrocortisone 21-hemisuccinate and 1 μM insulin (pH 7.4). Six mL of the cell suspension (for a total of $6 \cdot 10^6$ cells) were seeded onto 9 cm plastic Petri dishes coated with type I collagen from rat tail (150 μL of a 1 mg·mL^{-1} collagen solution). Petri dishes had been previously re-hydrated at first with 4 mL HBSS and, then, with 4 mL WEm. Cells were incubated (Infrabator® F.lli Galli, Vetrotecnica, Padova, Italy) in a humidified atmosphere (O_2/CO_2 95:5) at 37°C and left to attach for 4 hours. The medium was then replaced with the same WEm cited above without FCS.

2.2.3. Incubation with steroids and prohormones

Twenty-four hours after seeding, cell culture medium was replaced with WEm containing 100 μM ADD, 100 μM BOLD, 10:90 μM ADD:BOLD and 100 μM DHEA (final concentrations). These concentrations were chosen according to previous *in vitro* drug metabolism studies (Clouet et al., 1998; Van Puymbroeck et al., 1998; Merlanti et al., 2007; Rijk et al., 2008; Wang et al., 2010; Nebbia et al., personal data). Steroids and prohormones were dissolved in methanol, whose concentration in cell cultures never exceeded 1%. Petri dishes containing only methanol were prepared, too. Incubations were performed at 37°C for a maximum of 24 hours. At scheduled time-points (0, 3, 6

and 24 hours: see Figure 1), cells and/or medium aliquots were respectively scraped off or withdrawn for the execution of the bio-molecular and analytical investigations hereby reported.

2.3. Cytotoxicity

The cytotoxic effect of illicit steroids and prohormones was estimated, after 24 hours of incubation, by measuring the release of lactate dehydrogenase (LDH) in cell medium, according to Bergmeyer et al. (1963). The enzyme activity in culture media was referred to the total LDH activity, that is the activity measured in cell medium and in cytosolic subcellular fractions.

2.4. Effects of steroids and prohormones on DMEs and related transcription factors mRNAs

After 6 hours of incubation, the effects of aforementioned steroids/prohormones on mRNA levels of the androgen receptor (AR), major phase I and phase II DMEs as well as of nuclear receptors (NRs) involved in their regulation (e.g., the constitutive androstan receptor, CAR; the hepatocyte nuclear factor 4-alpha, HNF4α; the peroxisome proliferator-activated receptor-alpha, PPARα; the pregnane X receptor, PXR; and the retinoic X receptor alpha, RXRα), were measured by using a qPCR approach.

2.4.1. Cell harvesting

The medium of two Petri dishes was aspirated and 2 mL of ice-cold 0.1 mM phosphate buffer solution (PBS, pH 7.4) were added to each dish; monolayers were then scraped off. Cell suspensions obtained from each Petri dish were centrifuged (1500x*g,* 5 min at 4°C); the buffer discarded and the remaining pellet added with 200 µL of RNAlater® solution. Tubes were then stored at -20°C until use.

2.4.2. Total RNA extraction and reverse transcription

Total RNA was isolated by using the TRIzol® reagent and according to the manufacturer's instructions, with minor modifications. In short, 1 mL TRIzol® was added to the cellular pellet after RNAlater® solution aspiration, and the tube was then immediately vortexed. Samples were purified with a standard phenol-chloroform extraction and washed twice with ethanol 70%. Finally, the RNA pellet was dissolved in an appropriate volume of DEPC water.

Total RNA concentration and quality were checked by using a Nanodrop ND-1000 spectrophotometer (Labtech France, Paris, France). The RNA quality was estimated by the 260/280 and 260/230 nm absorbance ratios and confirmed by denaturing agarose gel electrophoresis.

The reverse transcription was performed as previously reported (Giantin et al., 2008), by using the High Capacity cDNA Reverse Transcription kit and according to the manufacturer's instructions.

2.4.3. Quantitative Real Time RT-PCR

Eighteen candidate genes, gathered from previously published studies and including the internal control gene β-actin (ACTB), were considered in the present study: *Bos taurus* cytochromes P450 (CYPs) 1A1, 1A2, 2B22, 2C87, 2E1, 3A28 and 4A11; the 17β-hydroxysteroid dehydrogenase type 2 (17βHSD2); the glutathione *S*-transferase A1-*like* (GSTA1-*like*), the uridin diphosphate glucuronosyltransferase 2B17-*like* (UGT2B17-*like*) and the sulfotransferase 2A1 (SULT2A1); the androgen receptor and NRs CAR, HNF4α, PPARα, PXR and RXRα (Toffolatti et al., 2006; Giantin et al., 2008; Cantiello et al., 2009; Giantin et al., 2010; Lopparelli et al., 2010; Lopparelli et al., 2011).

For each target gene the GenBank accession number, primer oligonucleotide sequences used for qPCR and the respective amplicon size are listed in Table 1. Each qPCR assay was validated according to previously published criteria (Lopparelli et al., 2011). The qPCR amplification was performed with 5 ng of cDNA in a final volume of 20 μL by using the Power SYBR® Green PCR Master Mix in an ABI-Prism 7000 thermal cycler (Applied Biosystems, Foster City, CA) under standard PCR conditions. The ΔΔCt method (Livak and Schmittgen, 2001) was used to analyse the obtained results.

2.5. Effects of steroids and prohormones on CYP3A apoprotein and catalytic activity

The post-translational effects of illicit steroids and prohormones on CYP3A were measured, on microsomal subcellular fractions isolated after 24 hours of

incubation, by using an immunoblotting technique (CYP3A apoprotein amount) and a TST hydroxylase activity assay (CYP3A-dependent catalytic activity).

2.5.1. Cells harvesting

After 24 hours of incubation, 150 µL of cell medium were collected from three Petri dishes, gathered together and stored at 4°C for LDH leakage cytotoxicity test. The remaining medium was aspirated and 4 mL of ice-cold 0.1 mM PBS (pH 7.4) was added; then, monolayers were removed by scraping. After a further 4 mL PBS addition, cell suspensions obtained from three Petri dishes were put together and centrifuged (250x*g,* 3 min at 4°C), the buffer discarded and the remaining pellet added with other 2 mL of PBS. Tubes were finally stored at -80°C until use.

2.5.2. Preparation of subcellular fractions

Tubes containing cell suspensions were thawed on ice and sonicated (four times, 20 s each) with a Microson™ XL Ultrasonic Cell Disruptor (Misonic Inc., Farmingdale, NY, USA). Then, the suspension was homogenized (four to six passes) in a 15 mL Wheaton Tenbroeck Tissue Grinder (Vetrotecnica, Padova, Italy). Subcellular fractions were prepared by differential centrifugation as reported by Daujat et al. (1987). Aliquots of the supernatants (cytosolic fractions) were frozen at -80 °C for further biochemical assays (LDH leakage cytotoxicity test). Pellets (microsomal fractions) were resuspended in 500 uL PBS 0.1M (pH 7.4) containing glycerol 20% (v/v) and stored as above. The protein content was determined by Lowry's method (Lowry et al.,1951).

2.5.3. Cytochrome P450 3A28 immunoblotting

The CYP3A immunoblotting was executed according to Cantiello et al. (2009). Briefly, microsomal proteins (30 μg) were separated by a 10% sodium dodecyl sulphate polyacrilamide gel electrophoresis and, then, transferred to nitrocellulose membranes. Afterwards, membranes were firstly incubated with an anti-rat CYP3A1/2 monoclonal antibody (final dilution of 1:500) and, then, with a peroxidase-conjugated sheep anti-mouse IgG (final dilution of 1:5000). The CYP3A apoprotein was detected by a chemiluminescent kit.

2.5.4. Cytochrome P450 3A28 catalytic activity

The cytochrome P450 3A28-dependent hydroxylation of TST at the 2β-, 6β- and 16β-position was measured by high-performance liquid chromatography (HPLC) technique according to Pegolo et al. (2010) and using 250 μM TST as the final substrate concentration.

2.6. Boldione and BOLD metabolite profiling

The time-dependent metabolism of ADD, BOLD and ADD:BOLD was investigated by LC-HRMS on medium aliquots withdrawn at 0, 3, 6 and 24 hours of incubation, quickly frozen and stored at -80°C until analysis.

2.6.1. Deconjugation and metabolite extraction

Before hydrolysis, l mL of hepatocyte medium was thawed and added with 1mL of acetate buffer (pH 5.0). Therefore, the sample was incubated (3 hours at 37°C) with β-glucuronidase/arylsulfatase from *Helix pomatia*. After cooling, 50 µL of the internal standard 17α-TST (25 µM stock solution) were added and the liquid was extracted twice with 10 mL ethylacetate. After vortexing for 10 min, organic phases were collected and evaporated to dryness at 37°C under air flow. The residue was dissolved in 1 mL of mobile phase A/B (50:50, v/v); (A) 5 mM ammomium acetate pH 5.0/formic acid (100:0.1 v/v) and (B) acetonitrile/formic acid (100:0.1 v/v) and diluted 1:5 with the same solvents prior to analysis.

2.6.2. Liquid chromatographic-high resolution mass spectrometry analysis

The LC-HRMS system consisted of a Thermo Electron model LTQ-FT Orbitrap hybrid MS system equipped with a Surveyor HPLC system (Thermo Electron, San Jose, CA, USA).

The chromatographic separation was achieved with an Atlantis T3 (50 mm x 2,1 mm, 3 µm i.d.) column (Waters, Elstree, UK). The elution was performed at a constant flow rate of 150 µl·min^{-1}. The mobile phase was programmed as follows: initial conditions 10% B, linear gradient to 90% B in 15 min, 90% B hold from 15 to 18 min. The re-equilibration time was 4 min. The injection volume was 5 µL. The hybrid MS system was equipped with an electrospray ionization (ESI) probe operating in the positive ion mode: spray voltage -4500 V, sheath gas (N_2) 40 arbitrary units (a.u.),

auxiliary gas (N_2) 5 a.u., capillary temperature 275°C, capillary voltage 37 V, tube lens voltage 130 V.

The acquisition was made in full scan at high resolution (60,000-150,000 FWHM) for accurate mass measurements, scanning from *m/z* 200 to *m/z* 400. For supplemental ion structure investigations, multiple mass spectrometry (MS^2 or MS^3) experiments were realized in the LTQ trap by collisionally induced dissociations of selected $[M+H]^+$ species, by using a supplementary RF voltage and helium as a target gas. The normalized collision energy was varied in the range 15-35 %. Product ions were transferred to the Orbitrap part of the instrument for an accurate mass measurement at high resolution. Data were processed using XCalibur v.2.0 software (Thermo Electron, San Jose, CA, USA).

2.7. Data analysis

Data were expressed as arithmetic means ± standard deviation (S.D., Prism 4 for Windows, GraphPad Software Inc., La Jolla, CA, USA) of two independent cultures, each one performed in duplicate. More in detail, LDH leakage data were expressed as a percentage of cytotoxicity, while candidate genes relative quantification values as fold-change (a.u.) of the ΔΔCt mean value of respective control cells, to whom an arbitrary value of 1.00 was assigned. Likewise, immunoblotting data (a.u.) and catalytic activity ($nmoles \cdot min^{-1} \cdot mg\ protein^{-1}$) were expressed as a percent of control cells, to whom an arbitrary value of 100.00 was given. Finally, the amounts of produced metabolites were expressed as $nmoles \cdot mL^{-1}$. As two cultures did not allow the execution of classical

statistical tools, a threshold value was defined to interpret the obtained data. In *–omic* investigations, a cutoff value of 2 fold-change is usually established as a rule (Giantin et al., 2010); in the present study, a higher value (e.g., 2.5-fold change) was taken into consideration. This means that only data $\geq (2.5)^{+1}$ or $\leq (2.5)^{-1}$ (e.g., $\geq$ 2.5 fold-changes or $\geq$ 250% and $\leq$ 0.4 fold-changes or $\leq$ 40% of CTRL value, respectively) were considered of importance.

3. Results

3.1. Hepatocytes isolation and viability

The present isolation protocol yielded an average of 459.76 ± 119.59·10^6 cells (3.68 ± 0.96·10^6 cells·g^{-1} of liver), and the mean cell viability was 88.0 ± 4.23%. Hepatocytes attached to coated Petri dishes and began to spread within 4 hours after seeding, and formed monolayers within 24 hours. No morphological changes or cell detachment were ever noticed in vehicle- or xenobiotic-exposed cultures. The LDH leakage percentage, after 24 hours of incubation, was 15.03 ± 7.65%, 14.34 ± 9.80%, 14.79 ± 7.26%, 15.32 ± 7.83% and 22.47 ± 12.83% for vehicle-, ADD-, BOLD-, ADD:BOLD- and DHEA-exposed cells, respectively.

3.2. Effects of steroids and prohormones on DMEs and related NR mRNAs

Drug metabolizing enzyme mRNAs were differentially modulated depending on the illicit steroid/prohormone to whom cells were exposed. A common trend of up-

regulation was noticed, with few exceptions (see Table 2). An increase above 2.5 fold-change of CYP2C87, CYP2B22, and CYP4A11 mRNA levels was observed in DHEA- and ADD:BOLD-exposed cells, respectively; in contrast, DHEA and ADD down-regulated (fold-changes lower than 0.4 fold-changes) CYP2E1 and CYP1A1 gene expression, respectively.

As regards target NRs, fold-changes greater than 2.5 were just noticed for AR, CAR and PXR mRNAs in DHEA-exposed cells (see Figure 2).

3.3. Effects of steroids and prohormones on CYP3A mRNA, apoprotein and catalytic activity

Cattle CYP3A mRNA expression and apoprotein amount were never affected by steroids/prohormones here used. In contrast, a common inhibitory trend in the pattern of TST hydroxylation (lower than 40% of CTRL) was observed in cells exposed to ADD, BOLD or ADD:BOLD (in this last case limited to the hydroxylation at 2β- and 16β-position: see Fig.3).

3.4. Time-dependent metabolism of ADD and BOLD

The LC-HRMS investigations confirmed a time-dependent increase of ADD, 17β-BOLD and 17α-BOLD amounts in the medium of exposed cells (see Table 3).

Other polar derivatives (see Table 4), corresponding to mono-hydroxylated BOLD were noticed; four of them, e.g. 6β-OH,17β-BOLD, 6β-OH,17α-BOLD, 16α-OH,17α-BOLD and 16β-OH,17α-BOLD, were particularly abundant. Some mono-hydroxylated

ADD derivatives were also detected, albeit in minor abundance; among these ones there were 16α-OH-ADD, 16β-OH-ADD and two 6-0H-ADD epimers. The presence of some di-hydroxylated derivatives was also demonstrated. Finally, a new metabolite eluting after 24 hours of incubation at Rt 8,96 was identified.

4. Discussion

Steroids misuse in cattle fattening is still a major concern within the European Union (Willemsen et al., 2002; Mooney et al., 2009; Nebbia et al., 2011). Whenever natural steroid esters and/or prohormones such as ADD and DHEA are used, their illicit use is hard to prove; unknown metabolites are likely to be generated, or the parental compound nor its derivatives concentrations might not exceed fluctuating endogenous levels, thereby preventing to unveil their exogenous origin (Draisci et al., 2007; Rijk et al., 2008). A further crucial issue is represented by the fact that recent complementary bio-molecular tools cannot provide information about illicit steroid/prohormone drug metabolism (Wang et al., 2010). As a consequence, *in vitro* liver models might be considered important tools to monitor the abuse of anabolic steroids/prohormones in cattle fattening (Van Puymbroeck et al., 1998; Wang et al., 2010).

Hepatocyte primary cultures represent the best liver *in vitro* model for toxicokinetic, toxicodynamic and toxicogenomic investigations (Li, 2007; Gómez-Lechon et al., 2010; Guguen-Guillouzo and Guillouzo, 2010). Cattle hepatocytes have already been used in the screening of steroids and prohormones misuse (Clouet et al.,

1998), but their usefulness as a multiparametric screening bioassay has not been investigated so far. This represented the key objective of the present study.

Drug metabolizing enzymes play a central role in the metabolism of xenobiotics and endogenous relevant compounds, including steroids and DHEA. Many important advances have been made in the molecular mechanisms governing DMEs expression, regulation and biological activity, and NRs have been shown to play a role in the transcriptional regulation of DME genes coding for proteins involved in steroid/DHEA drug metabolism (Ripp et al., 2002; You, 2004; Xu et al., 2005; Kohalmy et al., 2007; He et al., 2010). Collectively, data here obtained indicate that DHEA and ADD (albeit to a lower extent) affect target gene expression profiles. Dehydroepiandrosterone up-regulated AR, CAR and RXRα above the defined threshold value (2.5 fold-changes), and such a finding is consistent with previous studies in which supraphysiological concentrations of DHEA (i.e., 50-100 μM) were shown to activate these NRs, although this molecule is considered as a weak PXR agonist in humans (Fujita et al., 2002; Ripp et al., 2002; Köhalmy et al., 2007; Poczatková et al., 2007).

Nuclear receptors transactivation by DHEA is a prerequisite for transcriptional activation of CYP2B, 2C, 3A and 4A (Peters et al., 1996; Köhalmy et al., 2007; Poczatková et al., 2007). In cattle hepatocytes, DHEA upregulated CYP2B22 and 2C87 mRNAs, and the increased (about 3-fold) CYP2C87 gene expression substantiates previous data obtained in beef cattle administered with high DHEA amounts *in vivo* (Rijk et al., 2010). Similarly, an increase of CYP4A11 mRNA was noticed in cells given ADD and BOLD in combination; no data are actually available for cattle, but the

few comparative ones are suggestive of a positive relationship between androgens and CYP4A gene expression (Singh et al., 2007). On the contrary, ADD and DHEA prompted on a down-regulation of CYP1A1 and CYP2E1, respectively; data referring to CYP1A1 would confirm previous in vivo post-transcriptional data obtained either in cattle administered with ADD:BOLD (Merlanti et al., 2006) then in rat liver microsomes incubated with different steroid μM concentrations (Klinger et al., 2002). The cytochrome P450 2E1 is neither considered a DHEA molecular target nor involved in its metabolism, but this gene has been shown to be deeply down-regulated in cattle administered with two illicit schedules containing dexamethasone (Giantin et al., 2010).

Among conjugative enzymes involved in steroid conjugation (You, 2004), increasing amounts of GSTA1-*like* and SULT2A1-*like* mRNAs were only found in DHEA-exposed cells; these results agree with previously published ones, for which a substrate-based mechanism of induction, occurring through the activation of abovementioned NRs, has been hypothesized (Echchgadda et al., 2004a; Echchgadda et al., 2004b; El-Sayed, 2011).

The cytochrome P450 3A4 is the major DME in the adult human liver, and most drugs currently used in clinics are metabolized by this CYP isoform. This same CYP is responsible for the metabolism of endogenous steroids like TST, DHEA, androstenedione and cortisol (Nakamura et al., 2002; Ripp et al., 2002; Zhou, 2008). Some growth promoters (including also ADD, BOLD and DHEA) have been shown to affect to a various extent (induction, inhibition, no effect) cattle CYP3A expression and function *in vivo* (Capolongo et al., 2006; Merlanti et al., 2006; Cantiello et al., 2008;

Cantiello et al., 2009). Illicit steroids/prohormones here used never affected CYP3A mRNA and apoprotein amounts; in contrast, a general inhibition of a CYP3A-dependent catalytic activity (TST hydroxylation at positions 6β-, 2β, and 16β-), greater than the expected cutoff value (below 40% of CTRL) was observed. Many xenobiotics (including steroids and their modulators) behave as substrates, inhibitors and inducers of human CYP3A4 (Zhou, 2008); likewise, endogenous steroids (and especially androgens) inhibit human CYP3A-mediated drug metabolism *in vitro* (Nakamura et al., 2002). Such a phenomenon occurs mostly through two post-transcriptional mechanisms: a reversible inhibition and a mechanism-based inactivation. The former refers to competition of two substrates for CYP3A4, while the second one lies in the formation of a stable intermediate-metabolite complex, with an irreversible loss of catalytic function and reduced clearance of other CYP3A substrates (Polasek and Miners, 2006; Zhou, 2008). Differences in the interaction between the steroid and the CYP3A active site have also been considered to explain the steroid inhibitory effects on CYP3A4-dependent TST hydroxylation (Torimoto et al., 2007). Taken together, these evidences would be helpful to justify the inhibitory effect of ADD, BOLD and DHEA on CYP3A-dependent TST hydroxylation here observed.

In vitro liver models have already been successfully used to investigate anabolics and prohormones biotransformation in cattle (Clouet et al., 1998; Merlanti et al., 2007; Rijk et al., 2008; Van Puymbroeck et al., 1998; Wang et al., 2010); in the present study a LC-HRMS with a LTQ-FT Orbitrap hybrid MS system was used, for the first time, to characterize the metabolite profiling of anabolic steroids in cattle hepatocytes. After 24

hours of incubation, 17α-BOLD was the chief derivative extracted from hepatocyte media incubated with ADD and BOLD; however, several mono-hydroxylated BOLD and ADD derivatives (including 6β-OH,17β-BOLD, 6β-OH,17α-BOLD, 16α-OH,17α-BOLD and 16α-OH-ADD, 16β-OH-ADD, respectively) were detected, too. Among these ones, 6β-OH,17β-BOLD had already been identified *in vitro* by using bovine liver microsomes (Merlanti et al., 2007) and proposed as an urinary marker of BOLD illicit use (Blokland et al., 2007). On the other hand, the new metabolite found after 24 hours of incubation and eluting at Rt 8.96 might be probably related to the one described for TST by Nagata et al. (1986), whose structure entails a double bond in C_6-C_7, as a consequence of H_2O loss after 6β-hydroxylation. Present data prove that LC-HRMS and multiple mass spectrometry experiments, executed by using cattle hepatocytes, are suitable for the structural identification of unknown steroid metabolites.

In conclusion, cattle primary hepatocytes were used to study the effects of illicit steroids and prohormones (ADD, BOLD, DHEA) upon DMEs expression, regulation and function as well as to characterize their pattern of biotransformation in terms of produced derivatives. Collectively, data obtained confirm that cattle primary hepatocytes are a helpful complementary tool for the screening of illicit steroids/prohormones as well as, in a wider scenario, of potentially harmful xenobiotics. As most pharmaco-toxicological available data refer to hepatocytes isolated from human and model species or to established cell lines, this whole-cell *in vitro* system might be used, in perspective, to investigate more in depth the possible species-differences in DMEs expression, regulation and function. Additionally, this *in vitro*

model might reduce the number of experimental animals, because liver tissue can be easily obtained at the slaughterhouse. In this respect, a major concern is still represented by the time from the exanguination step and the hepatic lobe obtainment (about 20-30 min), that may affect isolated cell viability and, then, their usefulness for these studies.

Acknowledgements

This work was supported by grants from the Regione del Veneto, Italy (Valutazione del profilo genomico e proteomico in tessuti di bovini da carne trattati con composti ad attività anabolizzante: DGR 3655 29.11.05 and DGR 4313 30.12.05) to C.M. and M.D. and Dipartimento di Sanità pubblica, Patologia comparata ed Igiene Veterinaria (Setting up and validation of a protocol for the obtainment of cattle hepatocyte primary cultures to be used in drug metabolism studies, GIANPRGR09) to M.G.

Authors would like to thank Drs. C. Baruzzi and A. Dalla Pria for their help during cell culturing.

References

Bergmeyer, H.U., Bernt, E., Hess, B., 1963. Lactic dehydrogenase, in: Bergmeyer, H.U. (Ed.), Methods of Enzymatic Analysis. Academic Press, New York, pp. 736-741.

Blockland, M.H., Van Rossum, H.J., Sterk, S.S., Van Ginkel, L.A., Stephany, R.W., 2007. Development of a method which discriminate between endogenous and exogenous β-boldenone. Analytica Chimica Acta, 586, 147-153.

Bovee, T.F.H., Bor, G., Heskamp, H.H., Lasaroms, J.J.P., Sanders, M.B., Nielen, M.W.F., 2009. Validation and application of a yeast bioassay for screening androgenic activity in calf urine and feed. Analytica Chimica Acta, 637, 225-234.

Cantiello, M., Carletti, M., Dacasto, M., Martin, P.G., Pineau, T., Capolongo, F., Gardini, G., Nebbia, C., 2008. Cytochrome P450 inhibition profile in liver of veal calves administered a combination of 17beta-estradiol, clenbuterol, and dexamethasone for growth-promoting purposes. Food and Chemical Toxicology, 46, 2849-2855.

Cantiello, M., Giantin, M., Carletti, M., Lopparelli, R.M., Capolongo, F., Lasserre, F., Bollo, E., Nebbia, C., Martin, P.G., Pineau, T., Dacasto, M., 2009. Effects of dexamethasone, administered at growth promoting purposes, upon the hepatic cytochrome P450 3A expression in the veal calf. Biochemical Pharmacology, 77, 451-463.

Capolongo, F., De Bona, C., Giantin, M., Merlanti, R., Cantiello, M., Nebbia, C., Montesissa, C., Dacasto, M., 2006. Effects of illicit drug treatments upon the

cattle liver cytochrome P4503A (CYP3A) dependent hydroxylation of testosterone and protein expression. *Proceedings of the 16th International Symposium on Microsomes and Drug Oxidations*, Budapest, September 3rd-7th, 159.

Clouet, A-S., Le Bizec, B., Boerlen, F., Monteau, F., André, F., 1998. Calf primary hepatocyte culture as a tool for anabolic steroid metabolism studies. Analyst, 123, 2489-2492.

Daujat, M., Pichard, L., Dalet, C., Larroque, C., Bonfils, C., Pompon, D., Li, D., Guzelian, P.S, Maurel, P., 1987. Expression of five forms of microsomal cytochrome p450 in primary cultures of rabbit hepatocytes treated with various class of inducers. Biochemical Pharmacology, 36, 3597-3606.

Divari, S., De Maria, R., Cannizzo, F.T., Spada, F., Mulasso, C., Bovee, T.F.H., Capra, P., Leporati, M., Biolatti, B., 2010. A RIKILT yeast estrogen biomassa (REA) for estrogen residue detection in urine of calve experimentally treated with 17β-estradiol. Food Additives and Contaminants, 27, 19-28.

Draisci, R., Merlanti, R., Ferretti, G., Fantozzi, L., Ferranti, C., Capolongo, F., Segato, S., Montesissa, C., 2007. Excretion profile of boldenone in urine of veal calves fed two different milk replacers. Analytica Chimica Acta, 586, 171–176.

Fujita, A., Furutama, D., Tanaka, T., Sakai, R., Koyama, A., Hanafusa, T., Mitsuhashi, T., Ohsawa, N., 2002. In vivo activation of the costitutive androstane receptor beta (CARbeta) by treatment with deydroepiandrosterone (DHEA) or DHEA sulphate (DHEA-S). FEBS Letters, 532, 373-378.

Giantin, M., Carletti, M., Capolongo, F., Pegolo, S., Lopparelli, R.M., Gusson, F., Nebbia, C., Cantiello, M., Martin, P., Pineau, T., Dacasto, M., 2008. Effect of breed upon cytochromes P450 and phase II enzyme expression in cattle liver. Drug Metabolism and Disposition, 36, 885-893.

Giantin, M., Lopparelli, R.M., Zancanella, V., Martin, P.G., Polizzi, A., Gallina, G., Gottardo, F., Montesissa, C., Ravarotto, L., Pineau, T., Dacasto, M., 2010. Effects of illicit dexamethasone upon hepatic drug metabolizing enzymes and related transcription factors mRNAs and their potential use as biomarkers in cattle. Journal of Agricultural Food and Chemistry, 58, 1342–1349.

Gómez-Lechon, M.J., Lahoz, A., Gombau, L., Castell, J.V., Donato, M.T., 2010. *In vitro* evaluation of potential hepatotoxicity induced by drugs. Current Pharmaceutical Design, 16, 1963-1977.

Guguen-Guillouzo, C., and Guillouzo, A., 2010. General review on in vitro hepatocyte models and their applications. Methods in Molecular Biology, 640, 1-40.

He, J., Cheng, Q., Xie, W., 2010. Minireview: nuclear receptor-controlled steroid hormone synthesis and metabolism. Molecular Endocrinology, 24, 11-21.

Klinger, W., Lupp, A., Karge, E., Baumbach, H., Eichhorn, F., Feix, A., Füldner, F., Gernhardt, S., Knels, L., Kost, B., Mertens, G., Werner, F., Oettel, M., Römer, W., Schwarz, S., Elger, W., Schneider, B., 2002. Estradiol, testosterone, dehydroepiandrosterone and androstenedione: novel derivatives and enantiomers. Interactions with rat liver microsomal cytochrome P450 and antioxidant/radical scavenger activities in vitro. Toxicology Letters, 128, 129-144.

Köhalmy, K., Tamási, V., Kóbori, L., Sárváry, E., Pascussi, J-M., Porrogi, P., Rozman, D., Prough, R.A., Meyer, U.A., Monostory, K., 2007. Dehydroepiandrosterone induces human CYP2B6 through the constitutive androstan receptor. Drug Metabolism and Disposition, 35, 1495-1501.

Kuilman, M.E.M., Maas, R.F.M., Judah, D.J., Fink-Gremmels, J., 1998. Bovine hepatic metabolism of aflatoxin B_1. Journal of Agricultural and Food Chemistry, 46, 2707-2713.

Li, A.P., 2007. Human hepatocytes: isolation, cryopreservation and applications in drug development. Chemico-Biological Interactions, 168, 16-29.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-ΔΔC(T)) method. Methods, 25, 402-408.

Lopparelli, R.M., Zancanella, V., Giantin, M., Ravarotto, L., Cozzi, G., Montesissa, C., Dacasto, M., 2010. Constitutive expression of drug metabolizing enzymes and related transcription factors in cattle testis and their modulation by illicit steroids. Xenobiotica, 40, 670–680.

Lopparelli, R.M., Zancanella, V., Ginatin, M., Ravarotto, L., Pozza, G., Montesissa, C., Dacasto, M., 2011. Steroidogenic enzyme gene expression profiles in the testis of cattle treated with illicit growth promoters. Steroids, 76, 508-516.

Lowry, O., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193, 265-275.

Merlanti, R., Carletti, M., De Bona, C., Capolongo, F., Nebbia, C., Poppi, L., Dacasto, M., Montesissa, C., 2006. Influence of diets containing animal or plant sterols and

a boldenone/boldione combination on liver drug metabolism in veal calves. Journal of Veterinary Pharmacology and Therapeutics, 29 (Spl.1), 126-127.

Merlanti, R., Gallina, G., Capolongo, F., Contiero, L., Biancotto, G., Dacasto, M., Montesissa, C., 2007. An in vitro study of 17beta-boldenone and boldione using cattle liver and kidney subcellular fractions. Analytica Chimica Acta, 586, 177-183.

Mooney, M.H., Le Bizec, B., Elliott, C.T., 2009. Combining biomarker screening and mass-spectrometric analysis to detect hormone abuse in cattle. Trends in Analytical Chemistry, 28, 665-675.

Nagata, K., Liberato, D.J., Gillette, J.R., Sasame, H.A., 1986. An unusual metabolite of testosterone, 17 beta-hydroxy-4,6-androstadiene-3-one. Drug Metabolism and Disposition, 14, 559-565.

Nakamura, H., Nakasa, H., Ishii, I., Ariyoshi, N., Igarashi, T., Ohmori, S., Kitada, M., 2002. Effects of endogenous steroids on CYP3A4-mediated drug metabolism by human liver microsomes. Drug Metabolism and Disposition, 30, 534-540.

Nebbia, C., Urbani, A., Carletti, M., Gardini, G., Balbo, A., Betarelli, D., Girolami, F., 2011. Strategies for tracing the exposure of meat cattle to illegal growth promoters. The Veterinary Journal, 189, 34-42.

Noppe, H., Le Bizec, B., Verheyden, K., De Brabander, H.F., 2008. Novel analytical methods for the determination of steroid hormones in edible matrices. Analytica Chimica Acta, 611, 1-16.

Pegolo, S., Giantin. M., Dacasto, M., Montesissa, C., Capolongo, F., 2010. Testosterone hydroxylation in bovine liver: enzyme and inhibition study. Xenobiotica, 40, 255-261.

Peters, J.M., Zhou, Y-C., Ram, P.A., Lee, S.S.T., Gonzalez, F.J., Waxman, D.J., 1996. Peroxisome proliferator-activated receptor α required for gene induction by dehydroepiandrosterone-3β-sulfate. Molecular Pharmacology, 50, 67-74.

Poczatková, H., Bogdanovaá, K., Uherková, L., Cervenková, K., Riegrová, D., Rypka, M., Veselŷ, J., 2007. Dehydroepiandrosterone effects on the mRNA levels of peroxisome proliferator-activated receptors and their coactivators in human hepatoma HepG2 cells. General Physiology and Biophysics, 26, 268-274.

Polasek, T.M., Miners, J.O., 2006. Quantitative prediction of macrolide drug-drug interaction potential from in vitro studies using testosterone as the human cytochrome P4503A substrate. European Journal of Clinical Pharmacology, 62, 203-208.

Rijk, J.C.W., Bovee, T.F.H., Groot, M.J., Peijnenburg, A.A.C.M., Nielen, M.W.F., 2008. Evidence of the indirect hormonal activity of prohormones using liver S9 metabolic bioactivation and an androgen bioassay. Analytical and Bioanalytical Chemistry, 392, 417-425.

Rijk, J.C., Peijnenburg, A.A., Hendriksen, P.J., Van Hende, J.M., Groot, M.J., Nielen, M.W., 2010. Feasibility of a liver transcriptomics approach to assess bovine treatment with the prohormone dehydroepiandrosterone (DHEA). BMC Veterinary Research, 6, 44.

Ripp, S.L., Fitzpatrick, J.L., Peters, J.M., Prough, R.A., 2002. Induction of CYP3A expression by dehydroepiandrosterone: involvement of the pregnane X receptor. Drug Metabolism and Disposition, 30, 570-575.

Singh, H., Cheng, J., Deng, H., Kemp, R., Ishizuka, T., Nasjletti, A., Schartzmann, M.L., 2007. Vascular cytochrome P450 4A expression and 20-hydroxyeicosatetranoic acid synthesis contribute to endothelial dysfunction in androgen-induced hypertension. Hypertension, 50, 123-129.

Toffolatti, L., Rosa Gastaldo, L., Patarnello, T., Romualdi, C., Merlanti, R., Montesissa, C., Poppi, L., Castagnaro, M., Bargelloni, L., 2006. Expression analysis of androgen-responsive genes in the prostate of veal calves treated with anabolic hormones. Domestic Animal Endocrinology, 30, 38–55.

Torimoto, N., Ishii, I., Hata, M., Kobayashi, Y., Nakamura, H., Ariyoshi, N., Kitada, M., 2007. Theoretical calculation of triazolam hydroxylation and endogenous steroid inhibition in the active site of CYP3A4. Biochemica et Biophysica Acta, 1774, 223-232.

Van Puymbroeck, M., Kuilman, M.E.M., Maas, R.F.M., Witkamp, R.F., Leyssens, L., Vanderzande, D., Gelan, J., Raus, J., 1998. *In vitro* liver models are important tools to monitor the abuse of anabolic steroids in cattle. Analyst, 123, 2453-2456.

van't Klooster, G.A.E., Woutersen-van Nijnanten, F.M.A., Klein, W.R., Blaauboer, B.J., Noordhoek, J., van Miert, A.S.J.P.A.M., 1992. Effects of various medium formulations and attachment substrata on the performance of cultured ruminant hepatocytes in biotransformation studies. Xenobiotica, 22, 523-534.

Vincenti, M., Girolami, F., Capra, P., Pazzi, M., Carletti, M., Gardini, G., Nebbia, C., 2009. Study of dexamethasone urinary excretion profile in cattle by LC-MS/MS: comparison between therapeutic and growth-promoting administration. Journal of Agricultural Food and Chemistry, 524, 1299-1306.

Wang, S., Rijk, J.C.W., Riethoff-Poortman, J.H., Van Kuijk, S., Peijnenburg, A.A.C.M., Bovee, T.H.F., 2010. Bovine liver slices combined with an androgen transcriptional activation assay: an in-vitro model to study the metabolism and bioactivity of steroids. Analytical and Bioanalytical Chemistry, 397, 631-641.

Willemsen, P., Scippo, M-L., Maghuin-Rogister, G., Martial, J.A., Muller, M., 2002. Use of specific bioluminescent cell lines for the detection of steroid hormones (ant)agonists in meta producing animals. Analytica Chimica Acta, 473, 119-126.

Xu, C., Li, C.Y., Kong, A.N., 2005. Induction of phase I, II and III drug metabolism/tran sport by xenobiotics. Archives of Pharmaceutical Research, 28, 249-268.

You, L., 2004. Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolizing enzymes. Chemico-Biological Interactions, 147, 233-246.

Zhou, S-F., 2008. Drugs behave as substrates, inhibitors and inducers of human cytochrome P450 3A4. Current Drug Metabolism, 9, 310-322.

Figure captions

Fig. 1. Exposure protocol and sample collection. Cattle hepatocyte primary cultures were incubated up to 24 hours with ADD, BOLD, ADD:BOLD and DHEA.
Cytotoxicity and post-translational effects on CYP3A were evaluated after 24 hours of incubation. Pre-transcriptional effects on DMEs and NRs were measured after 6 hours of incubation. Finally (except for DHEA), ADD, BOLD and ADD:BOLD metabolite profiling was investigated on medium samples collected after 0, 3, 6 and 24 hours of incubation. For further details see Materials and methods section.

Fig. 2. Effects of illicit steroids and prohormones on NR mRNA relative abundances (a.u.) in cattle hepatocyte primary cultures incubated for 6 hours with ADD, BOLD, DHEA and ADD:BOLD. Data (arithmetic means ± S.D.) are expressed as –fold change (normalized to $\Delta\Delta C_t$ mean value of respective CTRL cells). Data marked with a black arrow represent those exceeding the selected cutoff value ($\geq (2.5)^{+1}$ or $\leq (2.5)^{-1}$ fold-changes) and, therefore, considered of value. The cytochrome P450 3A28 data are reported in Figure 3. For further details see Section 2.

Fig. 3. Effects of illicit steroids and prohormones on CYP3A mRNA relative abundances (a.u.), apoprotein amount (a.u.) and catalytic activity (nmoles·min^{-1}·mg protein^{-1}). Cells were incubated for 6 or 24 hours with ADD, BOLD, DHEA and ADD:BOLD. Immunoblotting data and catalytic activity are expressed as a percent of

control cells, while CYP3A mRNA data are expressed as –fold change (normalized to $\Delta\Delta C_t$ mean value of respective CTRL cells). Data marked with a black arrow represent those exceeding the selected cutoff value (≥ 250% or ≤ 40% of CTRL value) and, therefore, considered of value. For further details see Section 2.

Table 1. GenBank accession numbers, oligonucleotide sequences used for qPCR and amplicon size.

Gene acronym	GenBank ID	Primers sequences (5'→3')	Amplicon size (bp)
AR	XM_001253942	F: CGGTCCTTCACCAATGTCAAC R: ATGCGGTACTCATTGAAAACCA	71
CAR	NM_001079768	F: GAAGGACATGATCCTATCGACAGA R: CGTCGCTGGGCCTGTCT	63
HNF4α	NM_001015557	F: CGACAACGAGTACGCCTGC R: CCCCTTGGCATCTGGGTC	58
PPARα	NM_001034036	F: GGATGTCCCATAACGCGATT R: CACACGTAAGGATTTCTGCCTTC	81
PXR	NM_001103226	F: TGAAGGCCTACATCGAGTTCAAC R: GGCCATGATCTTCAGGAACAA	68
RXRα	XM_881943	F: GCCTCAATGGTGTCCTCAAAG R: AGCTGTACACCCCGTAGTGCTT	120
CYP1A1	XM_588298	F: GACCTGAATCAGAGGTTCTACGTCT R: CCGGATGTGACCCTTCTCAA	81
CYP1A2	NM_001099364	F: ACCATGACCCGAAGCTGTG R: CAATGGTGGTGCCATCAGAC	78
CYP2B22	NM_001075173	F: GCGGACCTCATCCCCATT R: GTGCCCTTGGGAAGGATGT	80
CYP2C87	XM_612374	F: TCCCTGGACATGAACAACCC R: TTGTGCTTTTCCTGTTCCATCTT	71
CYP2E1	NM_174530	F: ACCCGGAGGTTGAAGAGAAAC R: GCCCAATCACCCTGTCAATTT	51
CYP3A	NM_174531	F: GCCAGAGCCCGAGGAGTT	77

		R: GCAGGTAGACGTAAGGATTTATGCT	
CYP4A11	NM_001077908	F: AACTCGGTTTGCGCCAAGT R: CCCGATGCAGTTCCTGGAT	78
17βHSD2	NM_001075726	F: CTTACTTTTCTGGCCAAGAGTTGTTA R: CAAACCCAGAATCACAACCTGTT’	75
GSTA1-*like*	NM_001078149	F: TTCCCTCTGCTAAAGGCCCTA R: CTTCCTCTGGCTGCCAGG	84
SULT2A1-*like*	NM_001046353	F: GAAGGCCCACGTCTCATCAG R: GATCACCTTGGCCTTGGATTT	81
UGT2B17-*like*	NM_001075724	F: GCAAAGCCCCTACCTAAGGAATTA R: AGTAAACACCACGACTCCATCTTTT	72
ACTB	NM_173979	F: GTCATCACCATCGGCAATGAG R: AATGCCGCAGGATTCCATG	84

Table 2. Effects of illicit steroids and prohormones on CYPs, 17β-HSD2 and conjugative DMEs mRNA relative abundances (a.u.) in cattle hepatocyte primary cultures. Cells were incubated for 6 hours with 100 μM ADD, BOLD, DHEA and an association ADD:BOLD 10:90 μM (final concentrations). Cells were then harvested, total RNA extracted, reverse-transcribed and finally submitted to qRT-PCR investigations. Data (arithmetic means ± S.D.) are expressed as –fold change (normalized to $\Delta\Delta C_t$ mean value of respective CTRL cells). Data against a grey background represent those exceeding the selected cutoff value ($\geq (2.5)^{+1}$ or $\leq (2.5)^{-1}$ fold-changes) and, therefore, considered of value. For further details see Section 2.

Gene acronym	**fold-change (a.u.) ±S.D.**				
	CTRL	**ADD**	**BOLD**	**ADD:BOLD**	**DHEA**
CYP1A1	1.00 ± 0.21	0.37 ± 0.20	0.86 ± 0.47	0.73 ± 0.14	0.47 ± 0.04
CYP1A2	1.00 ± 0.16	0.96 ± 0.55	1.22 ± 0.63	0.94 ± 0.20	0.71 ± 0.16
CYP2B22	1.00 ± 0.14	1.77 ± 0.22	1.74 ± 0.74	1.42 ± 0.37	2.60 ± 0.89
CYP2C87	1.00 ± 0.20	1.11 ± 0.30	1.31 ± 0.45	1.33 ± 0.42	2.95 ± 0.13
CYP2E1	1.00 ± 0.13	1.01 ± 0.34	1.27 ± 0.53	1.10 ± 0.42	0.32 ± 0.04
CYP4A11	1.00 ± 0.16	1.09 ± 0.41	1.44 ± 1.06	2.58 ± 2.45	2.07 ± 0.94
17β-HSD2	1.00 ± 0.28	1.32 ± 0.14	1.27 ± 0.06	1.44 ± 0.21	1.98 ± 0.65
GSTA1-*like*	1.00 ± 0.16	1.34 ± 0.21	1.35 ± 0.22	1.23 ± 0.40	1.81 ± 0.09
SULT2A1-*like*	1.00 ± 0.23	1.30 ± 0.25	1.29 ± 0.23	1.08 ± 0.34	1.93 ± 0.14
UGT2B17-*like*	1.00 ± 0.27	1.18 ± 0.13	1.40 ± 0.32	1.24 ± 0.44	0.61 ± 0.01

Table 3. Metabolites produced by cattle primary hepatocytes following the incubation with 100 μM ADD, BOLD, and an association ADD:BOLD 10:90 μM (final concentrations). Medium aliquots were withdrawn at scheduled time-points (0, 3, 6 and 24 hours), metabolites were deconjugated, extracted with ethyl acetate and finally submitted to LC-HRMS investigations. Data (arithmetic means ± S.D.) are expressed as nmoles·mL^{-1}. For further details see Section 2.

	ADD	**17β-BOLD**	**17α-BOLD**	**6β-OH, 17β-BOLD**	**6β-OH, 17α-BOLD**	**16α-OH, 17β-BOLD**	**16β-OH 17α-BOLD**
ADD							
T_0	67.4 ± 0.8	1.2 ± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.
T_3	32.4 ± 7.7	3.0 ± 0.6	1.6 ± 0.8	2.2 ± 0.7	1.4 ± 0.3	n.d.	0.6 ± 0.1
T_6	25.3 ± 5.8	3.4 ± 0.7	3.2 ± 0.5	3.4 ± 0.8	1.9 ± 0.4	0.4 ± 0.1	0.7 ± 0.1
T_{24}	n.d.	n.d.	5.2 ± 1.1	2.5 ± 0.2	2.2 ± 0.2	0.9 ± 0.1	0.8 ± 0.1
BOLD							
T_0	n.d.	68.5 ± 6.6	1.1 ± 0.1	n.d.	n.d.	n.d.	n.d.
T_3	17.0 ± 3.7	6.9 ± 3.8	1.8 ± 0.9	4.3 ± 3.4	0.9 ± 0.4	n.d.	2.3 ± 0.3
T_6	15.0 ± 0.9	9.2 ± 0.4	2.5 ± 0.1	5.3 ± 0.2	1.4 ± 0.1	n.d.	3.7 ± 0.3
T_{24}	n.d.	6.4 ± 0.1	5.1 ± 0.1	3.0 ± 0.1	2.1 ± 0.1	0.7 ± 0.1	2.3 ± 0.1
ADD:BOLD							
T_0	8.7 ± 1.8	50.8 ± 13.6	1.1 ± 0.1	n.d.	n.d.	n.d.	n.d.
T_3	20.0 ± 3.0	8.0 ± 3.7	1.8 ± 0.1	5.9 ± 0.7	1.2 ± 0.1	0.3 ± 0.1	3.4 ± 0.3
T_6	12.1 ± 2.7	6.9 ± 1.3	2.7 ± 0.1	4.6 ± 0.6	1.5 ± 0.1	0.4 ± 0.1	3.2 ± 0.1
T_{24}	n.d.	5.4 ± 0.9	3.6 ± 0.8	2.5 ± 0.1	1.7 ± 0.1	0.8 ± 0.1	3.8 ± 0.7

n.d.: not detectable.

Table 4. Metabolites identification. Metabolites identified in hepatocyte primary cultures media following the incubation (up to 24 hours) with 100 μM ADD, BOLD, and an association ADD:BOLD 10:90 μM (final concentrations).

	retention time	**accurate mass of MH^+**	**elemental composition**
17β-BOLD	9.21	287.20056	$C_{19}H_{27}O_2$
17-α BOLD	10.06	287.20056	$C_{19}H_{27}O_2$
ADD	9.84	285.18491	$C_{19}H_{25}O_2$
6-OH ADD	6.15, 6.61	301.17982	$C_{19}H_{25}O_3$
16-OH ADD	6.97, 7.05	301.17982	$C_{19}H_{25}O_3$
Monohydroxylated ADD	7.22, 7.47	301.17982	$C_{19}H_{25}O_3$
Monohydroxylated BOLD	5.75, 7.14	303.19547	$C_{19}H_{27}O_3$
6β-OH,17β-BOLD	6.09	303.19547	$C_{19}H_{27}O_3$
6β-OH,17α-BOLD	6.63	303.19547	$C_{19}H_{27}O_3$
16β-OH,17α-BOLD	7.03	303.19547	$C_{19}H_{27}O_3$
16α-OH,17α-BOLD	7.90	303.19547	$C_{19}H_{27}O_3$

Figure 1

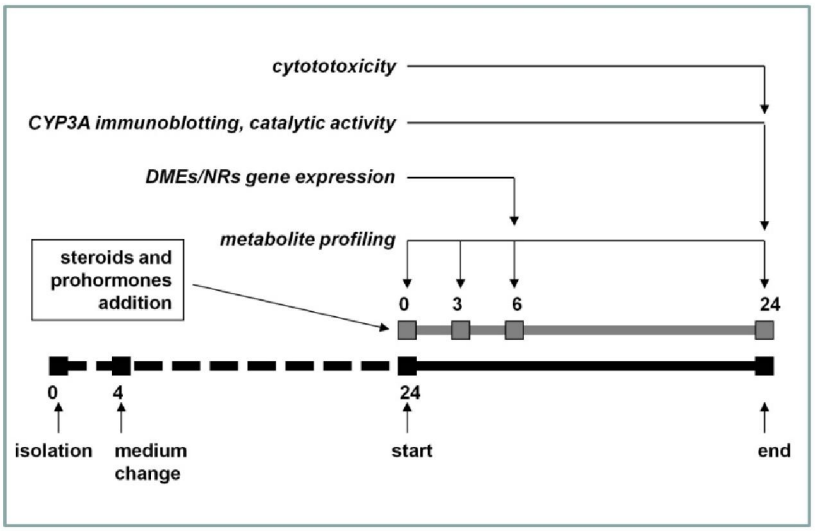

cytototoxicity
CYP3A immunoblotting, catalytic activity
DMEs/NRs gene expression
metabolite profiling
steroids and prohormones addition
0
3
6
24
0
4
24
isolation
medium change
start
end

Figure 2

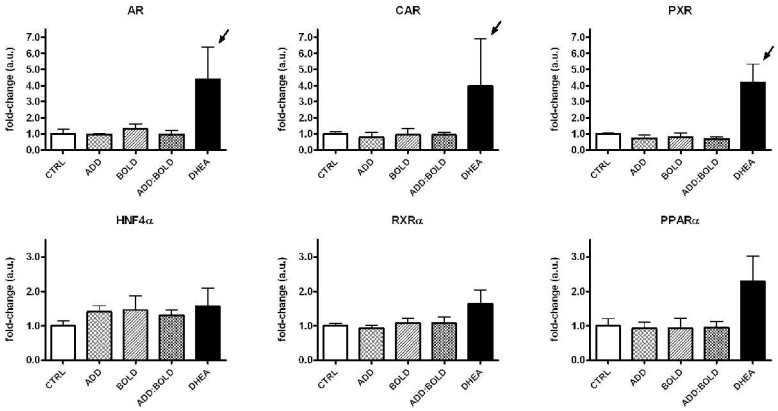
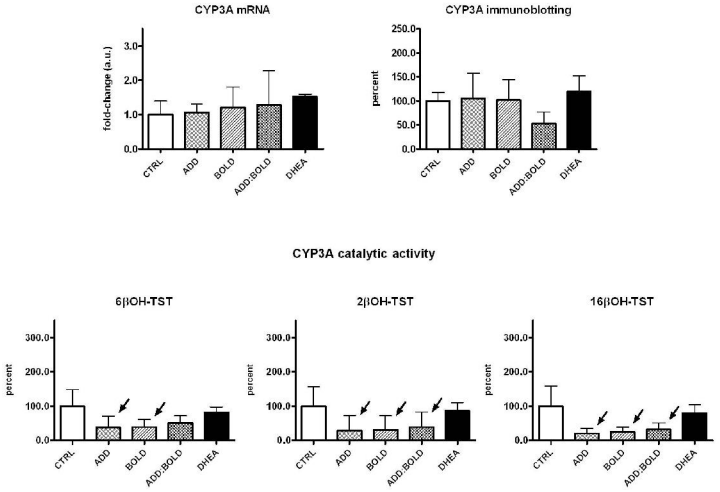

AR
CAR
PXR
HNF4α
RXRα
PPARα
fold-change (a.u.)
CTRL
ADD
BOLD
ADD:BOLD
DHEA

Figure 3


CYP3A mRNA
fold-change (a.u.)
3.0
2.0
1.0
0.0
CTRL
ADD
BOLD
ADD:BOLD
DHEA
CYP3A immunoblotting
percent
250.0
200.0
150.0
100.0
50.0
0.0
CYP3A catalytic activity
6βOH-TST
2βOH-TST
16βOH-TST
300.0
200.0
100.0