Expression and localisation of rat aldo-keto reductases and induction of the 1B13 and 1D2 isoforms by phenolic antioxidants


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Expression, localisation and induction of rat AKRs

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Text pages: 19
Tables: 2
Figures: 5
References: 34
Abstract words: 203
Introduction words: 389
Discussion words: 1474

Abstract

The aldo-keto reductase (AKR) phase I drug metabolism enzyme superfamily is implicated in detoxification or bioactivation of a wide variety of carbonyl-bearing compounds. In this study, we have used antibodies raised against purified recombinant rat AKR isoforms 1A3, 1B4, 1C9, 1D2 and 7A1 to characterise the expression profile of these superfamily members in the rat and define their localisation by immunohistochemistry. Western blotting showed that AKR1A3, AKR1B4, and AKR1C9 are ubiquitously expressed, whereas AKR1D2 and AKR7A1 are present in liver, adrenal gland and kidney, with the latter also present in testis, spleen and stomach. Immunohistochemical analysis of the kidney demonstrated the localisation of AKR1A3 in proximal convoluted tubules, AKR1B4 in the Loop of Henle and AKR1C9 in the pars recta S3 segment of proximal tubules. We also report localisation of AKR1B4 in the adrenal gland (parenchymal cells of the zona reticularis) and testis (sertoli cells and late spermatids), of AKR1D2 in the liver (hepatocyte nuclei), and of AKR7A1 in the pancreatic duct and bronchiolar epithelium. Previous studies have shown that expression of AKR7A1 is induced in response to dietary administration of the phenolic antioxidants butylated hydroxyanisole and ethoxyquin. Here we identify AKR1B13 and AKR1D2 as further inducible members of the rat AKR superfamily.
Aldo-keto reductases (AKRs) metabolise a diverse range of compounds by catalysing NAD(P)(H)-dependent reduction or oxidation reactions (Jin and Penning, 2007). Usually, an aldehyde or ketone moiety is converted to the corresponding alcohol. AKR substrates include sugars, steroids, amino acids, pesticides, neurotransmitters, substituted benzenes, polycyclic aromatic hydrocarbons, chemotherapeutics and lipid aldehydes. In the case of xenobiotics, metabolism by AKRs often prepares the substrate for phase II conjugation by sulfotransferases or UDP-glucuronosyl transferases, leading ultimately to excretion of the product. Certain AKR isoforms may have detrimental effects because they convert pro-carcinogenic polycyclic aromatic trans-dihydrodiols of cigarette smoke to o-quinones, leading to the generation of reactive oxygen species (Park et al., 2008). In the case of steroid hormones, AKRs can modulate the bioavailability of pro-proliferative nuclear receptor ligands by ketosteroid reduction and thereby may contribute to the development of hormone-dependent cancers (Bauman et al., 2004; Penning and Byrns, 2009).

To date, a total of seventeen rat AKR isoforms have been identified, comprising members of the AKR1A (aldehyde reductase), AKR1B (aldose reductase), AKR1C (hydroxysteroid dehydrogenase), AKR1D (Δ4-3-ketosteroid-5β-reductase), AKR6A (shaker channel β-subunit) and AKR7A (aflatoxin B1 aldehyde reductase) subfamilies (Table 1). All of AKR proteins share a common fold, the (αβ)8-barrel, and while the AKR1 family members are monomeric, the AKR7 family is tetrameric and the AKR6 family appear as subunits of large multiprotein complexes. The latter (comprising...
AKR6A2, AKR6A12 and AKR6A13) are expressed in brain, where they effect rapid inactivation of voltage-gated potassium channels (Rettig et al., 1994). As the AKR6 family are not thought to serve a detoxification function, they will not be discussed further in this report.

Amongst the rat AKR1 family members, those in the 1A, 1B and 1C subfamilies have been best characterised, with several studies defining their catalytic properties and tissue distribution. By contrast, relatively little is known about the 1D2 isoform, a ketosteroid 5β-reductase which is capable of reducing double bonds. In the present paper the tissue-specific profile of expression of rat AKR isoenzymes 1A3, 1B4, 1C9, 1D2 and 7A1 has been examined. We also discuss novel immunohistochemical data concerning their localisation. We sought to determine whether AKR7A1 is alone amongst the rat superfamily members in its induction by butylated hydroxyanisole (BHA) and ethoxyquin (EQ), and herein the first experimental evidence that the AKR1B13 and AKR1D2 isoforms are also subject to this form of regulation is presented.
Materials and Methods

Chemicals

All chemicals were of analytical grade and were purchased from Sigma-Aldrich Company Ltd. (Poole, UK), unless stated otherwise. Complete EDTA-free tablets of protease inhibitors were obtained from Roche Diagnostics Ltd. (Lewes, UK). Rabbit polyclonal antisera were previously generated against his-tagged recombinant aldo-keto reductases 1A3, 1B4, 1C9 and 1D2 (Kelly et al., 2000). Antibodies against AKR7A1 purified from rat liver have been described previously (Hayes et al., 1993). All pre-designed TaqMan oligonucleotide assays (NQO1: Rn00566528_m1, AKR1B13: Rn00756509_g1, AKR1D2: Rn00594102_m1, AKR7A1: Rn00566256_m1) and TaqMan Master Mix were supplied by Applied Biosystems (Foster City, CA).

Animals

All tissue profiling and immunohistochemical analyses were performed on tissues from untreated HanWistar rats (AstraZeneca; Alderley Park, UK). For induction experiments, Western blotting and RT-PCR analyses were performed on tissue from Fischer 344 rats (Harlan Laboratories Inc; Bicester, UK). The health of these animals was monitored daily. Prior to dietary intervention, rats were fed the RM1 diet (Special Diet Services; Witham, UK) for one week. The synthetic antioxidants BHA and EQ were dissolved in peanut oil and added to the RM1 diet at a concentration of 0.75% (w/w) and 0.5% (v/w), respectively, with an equal volume of peanut oil alone serving as a vehicle control. The rats were fed for five days on a diet containing BHA, EQ, or vehicle control before being culled. Tissues were excised and washed immediately in...
PBS, before they were placed in 20 ml universal tubes, snap-frozen in liquid nitrogen and stored at -80C until analysed.

**Isolation of protein and immunoblotting**

Tissues that had been stored at -80C were immersed in liquid nitrogen and pulverized to a fine powder using a pestle and mortar. Portions of the pulverized tissue (100 – 300 mg) were placed in an ice-cold Eppendorf microfuge tube and homogenised in ice-cold HEPES buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1mM DTT) with complete EDTA-free protease inhibitors (Roche; Basel, Switzerland, 1 tablet / 10 ml) using an Ultra-Turrax T8 rotor-stater homogeniser (IKA-Werke; Staufen, Germany, 90 sec at 90% power). Homogenised samples were centrifuged at 16,000 g and 4C for 10 min to remove insoluble debris. Supernatants were collected and centrifuged at 100,000 g and 4C for 80 min to remove the microsomal fraction for sample preparation. Western blotting was carried out as described previously (Ellis et al., 1996).

**Preparation of tissues for immunohistochemistry**

Tissue sections for immunohistochemical analysis (3 mm thick) were fixed in 10% (v/v) neutral buffered formalin at 20C for a minimum of 24 hr and embedded in paraffin. Serial 3 mm sections were cut for each tissue and mounted on Colourfrost Plus slides (Thermo Shandon; Waltham, MA). For immunogold labelling and analysis, tissue sections (3 mm thick) were fixed in Karnovsky's primary fixative (1% (w/v) paraformaldehyde, 3% (v/v) glutaraldehyde in 70 mM sodium cacodylate buffer, pH 7.3) for a minimum of 24 hr at 4C and embedded in LR White acrylic
resin. Semi-thin sections (0.5 μm) were cut and mounted on Colourfrost Plus slides (Thermo Shandon).

**Immunohistochemistry**

Slides were stained using the DAKO EnVision+ System (Ely; Cambridge, UK), with 3,3’-diaminobenzidine (DAB) as the chromogen (Sabattini et al., 1998). Briefly, mounted tissue sections were dewaxed and rehydrated by sequential placement in aqueous solutions containing decreasing amounts of ethanol (95-70%). Antigen retrieval was achieved by microwave heating to 110°C for 5 min followed by 5 min cooling in a citrate or EDTA buffer or by proteolytic digestion using DAKO Pronase solution. Subsequent steps were performed using a Labvision Autostainer. Endogenous peroxide activity was quenched with an aqueous solution of 3% (v/v) hydrogen peroxide and nonspecific antibody binding was blocked by incubating sections with DAKO Protein Block Serum-Free solution or by incubation in 100 μl of a 5% (w/v) BSA solution in 20 mM Tris-buffered saline, pH 7.4, containing 0.2% (v/v) Tween (TBST) for 20 min at 20°C. Sections were incubated in appropriate dilutions of primary antibody for 1 hr at 20°C. Dilutions and wash steps were carried out using TBST. Incubation in the absence of primary antibody served as negative controls. After primary antibody incubation, sections were washed in TBST for 5 min at 20°C and incubated with DAKO EnVision+ System-labeled polymer (peroxidase-labeled polymer conjugated to goat anti-rabbit or goat anti-mouse IgG) for 30 min at 20°C. Sections were washed in TBST for 5 min at 20°C. Peroxidase activity was developed with a buffered DAB chromogen solution (DAKO). Slides were removed from the autostainer and tissue counterstained in Carazzi’s hematoxylin for exactly one minute before being dehydrated, cleared, and mounted.
**Immunogold labelling**

Immunostaining of semi-thin sections was carried out using an indirect immunocolloidal gold / silver detection method. Briefly, the sections were equilibrated with TBST for 5 min at 20°C, then incubated with rabbit anti-AKR1B4 primary antibody or pre-immune serum (1:100) diluted with TBST for 1 hr at 20°C. Negative control sections were incubated in TBST for 1 hr at 20°C. Following primary application, sections were incubated for 1 hr at 20°C in an anti-rabbit IgG 10 nm colloidal gold conjugate diluted 1:10 using TBST (BB International; Cardiff, UK). Sections were then enhanced using a silver enhancing kit (BB International; Cardiff, UK). Counterstaining was carried out for 1 min with 0.5 % Toluidine Blue.

**Isolation of RNA and TaqMan quantitative Real-Time PCR**

Portions of frozen and pulverized liver (10 – 30 mg) were placed in an ice-cold Eppendorf microfuge tube and homogenised in 600 µl ice-cold RLT containing 1% (v/v) β-mercaptoethanol using an Ultra-Turrax T8 rotor-stater homogeniser (IKA-Werke, 60 sec at 90% power). Homogenised samples were centrifuged at 16,000 g and 4°C for 10 min to remove debris and the supernatants were loaded onto QIAshredder spin columns for isolation of RNA using the RNeasy Mini Kit (QIAGEN; Crawley, UK), including on-column DNA digestion step. RNA was reverse-transcribed to cDNA using the Omniscript Reverse Transcription Kit (QIAGEN). TaqMan reactions were performed on the Prism Model 7700 Sequence Detector (Perkin Elmer/Applied Biosystems) and fold changes calculated using the comparative CT method.
Statistics

Statistical significance for TaqMan RT-PCR values were calculated on Prism 4 software (Graphpad, CA) using the unpaired Student’s *t* test, where “*” denotes $P = 0.05-0.01$, “**” denotes $P = 0.01-0.001$, and “***” denotes $P = <0.001$. 
Results

Expression profile of aldo-keto reductases

Rabbit polyclonal antibodies raised against AKRs 1A3, 1B4, 1C9, 1D2 and 7A1 were used to profile the distribution of these isoforms across rat tissues by Western blot (Figure 1). Reductases 1A3, 1B4 and 1C9 were found to be ubiquitously expressed, with 1A3 and 1B4 exhibiting uniform levels of expression across all fifteen tissues examined (Figure 1). The level of AKR1A3 was slightly higher in ileum, spleen and kidney, and was lower in duodenum. The AKR1B4 isoform was enriched in the adrenal gland, with the lowest level of expression apparent in liver; this is in agreement with the findings of others (Zeindl-Eberhart et al., 1997). Our polyclonal anti-AKR1C9 serum detected two distinct protein bands in the kidney. This is probably due to its cross-reaction with other AKR1C isoforms such as 1C15, 1C16 or 1C17 (Qin and Cheng, 1994).

The AKR1D2 and AKR7A1 proteins presented a more tissue-specific expression profile than did AKR1A3, AKR1B4 and AKR1C9. Immunoblotting showed AKR1D2 was strongly enriched in liver, with moderate expression in adrenal gland and a low level of expression in kidney. AKR7A1 was expressed at a high level in kidney, with moderate expression in liver and stomach, and lower levels in testes, adrenal gland, and spleen. These observations complement a previous study in which AKR7A1 expression was detected in liver, kidney, testes and small intestine (Grant et al., 2001).

Localisation of rat AKR isoenzymes
In the adrenal gland, immunohistochemical (IHC) staining for AKR1B4 was mainly cortical, although there was some medullary expression (Figure 2A). The most intense staining of the adrenal gland was found in parenchymal cells of the zona reticularis, immediately adjacent to the medulla (Figure 2A). AKR1B4 was also expressed in the testis with the most concentrated immunoreactive staining observed in Sertoli cells and late spermatids of the seminiferous tubule germinal epithelium (Figure 2B). In agreement with the findings of others (Terubayashi et al., 1989), IHC staining for AKR1B4 in the kidney was observed exclusively in the medulla (data not shown), with a signal detected in the ascending thin limb of the Loop of Henle (LoH) in the inner medulla, and the ascending thick limb of the LoH in the inner stripe of the outer medulla (data not shown). We also used the same anti-AKR1B4 serum for immunogold labelling of the antigen in semi-thin kidney sections (Figure 2C and D). Localisation of staining to the ascending thin limb of the LoH (arrows) was observed throughout the inner medulla. No staining was detected following incubation of tissue with pre-immune serum in place of anti-AKR1B4 serum (Figure 2E).

In the kidney, IHC staining for AKR1C9 localised exclusively to the pars recta S3 segment of the proximal tubule (data not shown). AKR1D2 protein was expressed at substantially higher levels in the liver than in any other tissue (Figure 1). Immunohistochemical staining of this isoform localised to hepatocytes uniformly throughout the organ (Figure 2F). Within these cells, the immunoreactive signal detected by our antiserum was stronger in nuclei than in cytoplasm.

AKR7A1 is subject to both developmental and sex-specific regulation of expression (Grant et al., 2001). Immunohistochemical analysis has shown previously that
AKR7A1 is expressed in collecting ducts of the kidney, small intestinal villi, testes and skeletal muscle nerve bundles (Grant et al., 2001). Our results are in agreement with these observations. In addition, we found AKR7A1 staining localised to enterocytes and goblet cells of the mucosal villi in duodenum and ileum, with more intense signal towards the base of the mucosa (data not shown). Staining was also apparent in parietal cells of the gastric mucosa. In the kidney, AKR7A1 localised to the inner medullary collecting ducts (IMCDs), but also to the descending thick limb of the LoH in the outer medulla, and to the proximal convoluted tubules of the cortex (data not shown). Western blotting showed that AKR7A1 was not expressed in homogenised pancreas at a level comparable to that of tissues such as the liver or stomach (Figure 1), but IHC demonstrated localisation of this isoform to pancreatic duct epithelium (Figure 2G). AKR7A1 was also observed in mucosal cells of the bronchiolar epithelium (Figure 2H).

**Induction of AKR by antioxidants**

The role of antioxidants in induction of AKR expression in the rat was investigated as, to date, only the 7A1 protein has been shown to be induced by BHA and EQ (Hayes et al., 1993; McLellan et al., 1994). Male Fischer 344 rats (n = 12) were divided into three groups and placed on different diets. In the first group (n = 4), rats were fed the vehicle control (peanut oil only) powdered RM1 diet. In the second group (n = 4), this diet was supplemented with 0.75% (w/w) BHA dissolved in peanut oil. In the third group (n = 4), the diet was supplemented with 0.5% (v/w) EQ dissolved in peanut oil. Following 5 days on this diet, rats were culled and livers removed for analysis. Cytosolic protein samples were prepared as described in Materials and Methods. Western blotting of these samples indicated that AKR1D2
and 7A1 were induced by both antioxidant treatments, whereas AKR1A3, 1B4 and 1C9 were not (Figure 3). A Western blot of the inducible antioxidant defence enzyme, NAD(P)H quinone oxidoreductase 1 (NQO1) was included as a positive control for the action of these compounds.

Total RNA was isolated from samples of each of the 12 rat livers and TaqMan RT-PCR analysis was carried out on AKR1D2 and AKR7A1 mRNA transcript levels, with an assay for NQO1 again included as a positive control for induction. As we have recently identified the human AKR1B10 as inducible in a manner dependent on nuclear factor-erythroid 2-related factor 2 (NRF2) (MacLeod et al., 2009), we also performed RT-PCR analysis on AKR1B13, the rat orthologue of this gene (83% and 81% identity at the coding DNA and protein levels, respectively). Data from the four animals in each treatment group were combined and fold change calculated relative to the control group. AKR1B13 mRNA was induced approximately 3.2- and 3.8-fold in response to dietary supplementation of BHA and EQ, respectively. AKR1D2 mRNA showed a more modest, though significant, level of induction at approximately 1.5-fold in response to BHA and 2.2-fold in response to EQ. As expected, AKR7A1 mRNA was upregulated following treatment with both BHA and EQ, by approximately 6- and 24-fold, respectively (Figure 4).
Discussion

In comparison with other supergene families of drug metabolising enzymes, relatively little is known about the expression of aldo-keto reductases. As the rat is frequently used to study drug metabolism, we investigated the distribution and regulation of AKRs in this species.

Over the past 20 years, the rat aldehyde reductase AKR1A3 and aldose reductase AKR1B4 proteins have been the subject of numerous investigations. Some of these studies have shown AKR1A3 and AKR1B4 to be ubiquitously expressed (Terubayashi et al., 1989; Jung et al., 2002) and our western blotting data support such conclusions. With the exception of localisation of AKR1B4 to kidney IMCDs, which we could not detect in ultrathin sections (Figure 2C and D), our IHC data is also in agreement with previously published findings (Terubayashi et al., 1989; Jung et al., 2002).

The rat AKR1C subfamily comprises AKR1C8 (ovarian 20α-hydroxysteroid dehydrogenase), AKR1C9 (liver 3α-hydroxysteroid dehydrogenase), AKR1C15, AKR1C16, AKR1C17 and AKR1C24 (Table 1). The best studied of these, AKR1C9 is broadly expressed and shows sexual dimorphism (Hou et al., 1994; Azzarello et al., 2008). The present study agrees with this observation.

Herein we have found that the expression of AKR1D2 is restricted to the liver and adrenal gland, with a low level of expression also apparent in the kidney. In the liver, AKR1D2 catalyses 5β-reduction of the C4-C5 double bond of bile acid (BA).
intermediates bearing the $\Delta^4$-3-one structure (Berseus, 1967). BAs activate the farnesoid X receptor (FXR), transcriptional consequences of which ultimately lead to the inhibition of their synthesis and promotion of their excretion (Makishima et al., 1999). In addition, FXR regulates cholesterol and triglyceride metabolism, inhibits gluconeogenesis, and promotes glycogen storage and insulin signalling (Modica and Moschetta, 2006; Zhang and Edwards, 2008). Our IHC analysis of AKR1D2 localisation in hepatocytes suggests that it is primarily found in nuclei (Figure 2F), a location that may permit the enzyme to influence FXR activity. As well as for FXR, BAs act as ligands for the G-protein-coupled receptor, TGR5, and activate mitogen-activated protein kinase signalling (Houten et al., 2006). Our data suggest that, as it is absent from most other tissues, AKR1D2 will not exert a global effect on FXR, TGR5 and MAP kinase signalling. Due to its activities towards steroid hormones (Okuda and Okuda, 1984) it is likely that, in the adrenal gland, AKR1D2 may be involved in their clearance.

The founding member of the seventh aldo-keto reductase family, AKR7A1, reduces a dialdehyde metabolite of aflatoxin B$_1$ (Hayes et al., 2005). It is expressed in rat liver, kidney (collecting ducts), small intestine (villi), and testis, and is strongly induced in the liver by the cancer chemopreventive agents BHA and EQ (Hayes et al., 1993; McLellan et al., 1994; Grant et al., 2001). Herein, we have identified pancreatic duct and bronchiolar epithelia as novel sites of AKR7A1 localisation (Figure 2G and H). Interestingly, in the bronchiolar mucosum, the reductase is not present in every cell, but is only apparent in certain cells.
It has been recognised for many years that AKR7A1 can be induced in rat liver by the cancer chemopreventive agents BHA, EQ and oltipraz (Hayes et al., 1993; Ellis et al., 1996; Primiano et al., 1996), and is up-regulated in liver bearing pre-neoplastic nodules (Hayes et al., 1993). Evidence suggests that induction of AKR7A1 is mediated by the redox-sensitive transcription factor NRF2 through one or more antioxidant response elements (AREs, core element 5′-TGACGNNNGC-3′) in its upstream regulatory region (Ellis et al., 2003). Recently we have created a luciferase reporter plasmid that contains ~4 kb of the AKR7A1 gene promoter, and shown that transcription from this reporter construct is up-regulated several fold when it is co-transfected into the rat liver cell line RL-34 cells with an expression construct for NRF2 (unpublished results). These data, together with the finding that NRF2 is regulated primarily by protein stabilisation, suggest that BHA and EQ induce AKR7A1 through inhibiting the degradation of NRF2. Indeed, BHA is metabolised to tert-butylhydroquinone, an electrophile that has been shown to stabilize NRF2 (Nguyen et al., 2003).

In the present study, we have shown for the first time that AKR1B13 is induced in the liver of adult rats upon feeding of a diet containing BHA or EQ. Interestingly, this AKR has been shown to be expressed in embryonic liver and adult hepatoma, but not in healthy adult liver (Zeindl-Eberhart et al., 1997). Moreover, it is inducible in response to hydrogen peroxide and 1,4-naphthoquinone (Endo et al., 2009). There are four putative ARE enhancers within the 10 kb region upstream of the AKR1B13 translational start site (Table 2). One of these, the core sequence of which begins at -3597 on the antisense strand, is highly homologous to a functional ARE within the promoter of glutathione S-transferase pi 1 (GSTP1), a gene that is regulated by NRF2.
and serves as a biomarker for pre-neoplastic foci in rat liver (Hayes et al., 2005) (Figure 5A). Furthermore, AKR1B13 and GSTP1 both possess an additional putative ARE proximal to the translational start site which may act synergistically with the distal cis-element (Suzuki et al., 2005). As we have shown AKR1B13 to be inducible in response to synthetic antioxidants, it is likely that this gene is regulated by NRF2 in a manner similar to GSTP1 (Figure 5B).

Our investigation of hepatic AKR expression in the rat has provided evidence that AKR1D2 is modestly inducible by BHA and EQ. In this case, there are eight putative elements which match the core ARE consensus sequence in the 10 kb upstream of the AKR1D2 translational start site (Table 2), but none of these are flanked by A or G nucleotides, a feature of previously identified AREs (Nioi et al., 2003). Weak association of NRF2 with one or more of these “non-ideal” AREs may therefore explain the smaller magnitude of induction of AKR1D2 observed in response to BHA or EQ compared to that of other enzymes, such as AKR7A1, NQO1 or GSTA2. Of the five candidate AREs we have identified, the sequence beginning at -5494 is the most promising as there are three half-ARE sequences, TGAG, TGAG and TGAT, between -5453 and -5473 on the opposing strand that might contribute to enhancer activity (Figure 5C). The closest of these to the putative ARE is also part of a TGATTCTA palindrome (from -5470 to -5476), and therefore a potential Activator Protein 1 (AP1) binding site. As the accumulation of BAs intermediates in the liver may result in increased lipid peroxidation, oxidative stress, and ultimately hepatic injury (Perez and Briz, 2009), upregulation of AKR1D2 by NRF2 in response to this stress may constitute a mechanism by which normal BA metabolism is maintained.
We have recently found that, in human HaCaT keratinocytes (MacLeod et al., 2009) and HepG2 hepatoma cells (unpublished results), the AKR1B10, AKR1C1, AKR1C2 and AKR1C3 genes are highly inducible in an NRF2-dependent fashion. However, no study has been produced to date showing induction of the human AKR1D1 gene, or the human AKR7A2 and AKR7A3 genes. In the present study we have shown that AKR1D2 protein (Figure 3) and mRNA for both AKR1D2 and AKR1B13 (Figure 4) are increased in response to dietary administration of the antioxidants BHA and EQ. As for AKR7A1, characterisation of functional ARE(s) in the flanking regulatory regions of AKR1B13 and AKR1D2 are subjects for future investigation. We have found no evidence, however, for the induction of either AKR1B4 or AKR1C9. Although the latter is inducible in response to estrogens and prostaglandin E2 (Hou et al., 1994; McCarthy et al., 2007), our findings are in complete agreement with previous observations of the rat liver H4IIE cell line, where this isoform was found to be unresponsive to several inducers of phase I and II drug-metabolising enzymes, including BHA and EQ (Buller et al., 1989).

The data described herein suggest that significant differences exist in the regulation of rat and human AKR genes by inducing agents. Whilst parallels may be drawn between rat AKR1B13 and human AKR1B10 genes, the rat and human AKR1C, AKR1D and AKR7 gene families seem to be regulated by distinct mechanisms. Due to the availability of animals at the two locations where experiments were carried out, our localisation and induction analyses were carried out in different rat strains; HanWistar and Fischer 344, respectively. However, we do not expect significant or confounding disparities in AKR localisation or regulation to occur between these strains.
The rat is frequently used as an experimental model to study drug metabolism. In the present investigation, we have shown marked variation in the distribution of individual AKRs and demonstrated that certain isoforms are highly inducible in the liver of this animal. Importantly, the pattern of AKR induction in rat liver appears to differ from that observed in humans. These observations must be considered when conclusions about drug metabolism and the response to xenobiotics are drawn based on data from the rat as a model organism.


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Footnotes

This work was funded by Cancer Research-UK (C4909/A5942) and the Association for International Cancer Research (05-154). A.K.M. was supported by a Biotechnology and Biological Sciences Research Council (UK) Collaborative Awards in Science and Engineering PhD studentship with AstraZeneca.
Figure 1 Tissue profile of rat AKR expression

Western blots for AKR isoforms in homogenised rat tissue samples. A coomassie-stained gel is presented as a control for equal protein loading.

Figure 2 AKR Localisation

IHC staining of AKR1B4 in adrenal gland (A) and testis (B). Immunogold staining of AKR1B4 in kidney inner medulla (C, D), with AKR1B4 pre-immune serum control stain (E). Ascending thin limb of the Loop of Henle is identified with arrows. IHC staining of AKR1D2 in liver (F), and of AKR7A1 in pancreas (G) and lung (H).

Figure 3 Induction of AKR1D2 and AKR7A1 proteins in liver

Western blotting was performed for AKR isoforms 1A3, 1B4, 1C9, 1D2 and 7A1. An NQO1 blot is included as a positive control for induction by antioxidants, with a GAPDH blot included as a loading control. Data are representative of three separate experiments.

Figure 4 Induction of AKR1B13, AKR1D2 and AKR7A1 mRNA in liver

TaqMan RT-PCR was carried out to measure the levels of mRNA for NQO1, AKR1B13, AKR1D2 and AKR7A1. Reactions were performed in triplicate and all 12 readouts from each treatment group averaged then normalised against the control group. Statistical significance was calculated for each antioxidant-treated group relative to mean ± standard error of the control group. Transcript levels are presented...
in Relative Transcription Units (RTU) and data are representative of three separate experiments.

**Figure 5 Candidate ARE enhancers in the AKR1B13 and AKR1D2 promoter regions**

(A) and (B) Candidate AREs at position -3597 and -104 on the antisense strand of the AKR1B13 promoter was aligned with the functional and putative AREs of rat GSTP1, respectively (boxed regions). TRE-like sequences of the GSTP1 promoter are also labelled. Nucleotide numbering refers to the AKR1B13 promoter sequence. (C) Candidate ARE at position -5494 on the sense strand of the AKR1D2 promoter is depicted (boxed region) alongside putative AP1 and half-ARE sequences.
Table 1 Rat aldo-keto reductases

Known rat aldo-keto reductase isoforms are presented alongside accession number. The 1B10 isoform is shown in brackets as it is yet to be given a correct systematic designation.

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Table 2 Candidate ARE enhancers in the AKR1B13 and AKR1D2 promoter regions

The 10 kb DNA sequence upstream of the AKR1B13 and AKR1D2 translational start sites were searched for candidate AREs using the consensus sequence 5’-TGA(C/G)NNNGC-3’. Nucleotide location of the first consensus matched base (T) relative to the start codon is presented.

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Figure 3

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Figure 4

**NQO1**

- **RTU**
  - Control: 
  - BHA: 
  - EQ: 

- **AKR1B13**
  - **RTU**
  - Control: 
  - BHA: 
  - EQ: 

- **AKR1D2**
  - **RTU**
  - Control: 
  - BHA: 
  - EQ: 

- **AKR7A1**
  - **RTU**
  - Control: 
  - BHA: 
  - EQ: 

Significance levels: 
- *****p < 0.001**
- ****p < 0.01**
- **p < 0.05**
Figure 5

A

\[ \text{AKR1B13 (-3597): } 5' - \text{TTTTTCTTTGTCAGCA} \quad \text{TGATTCAGC} \quad \text{AGTTTAACCG-3'} \]

\[ \text{GSTP1 (GPE1, -2430): } 5' - \text{CAAAAGTAGTCAGTCACTA} \quad \text{TGATTCAGC} \quad \text{AACAAACCC-3'} \]

\[ \text{TRE-like} \quad \text{TRE-like} \]

B

\[ \text{AKR1B13 (-104): } 5' - \text{ACTGTG} \quad \text{TGAGCAAGC} \quad \text{AGCCAC-3'} \]

\[ \text{GSTP1 (-61): } 5' - \text{CTGTGT} \quad \text{TGACTCAGC} \quad \text{ATCCGG-3'} \]

C

\[ \text{AKR1D2 (-5494): } 5' - \text{ATCT} \quad \text{TGAGGCAGC} \quad \text{TCCCAACATTTGAATCAATGATACTCATGCCTCAAAC-3'} \]

\[ \text{AP1} \quad 1/2 \text{ ARE} \quad 1/2 \text{ ARE} \]