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Title Page.

Induction of *AKR1C2* by Phase II Inducers: Identification of a Distal Consensus Antioxidant Response Element (ARE) Regulated by NRF2

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Running title page.

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Abstract: 266 words.

Introduction: 971 words.

Discussion: 2084 words.

d) Abbreviations: 3α-HSD, 3α-hydroxysteroid dehydrogenase (E.C. 1.1.1.50); AKR, Aldo-keto reductase; AP-1, activating protein 1; ARE, antioxidant response element; β-gal, β-galactosidase; β-NF, β-naphthoflavone; ChIP, chromatin immunoprecipiation; DDH, dihydrodiol dehydrogenase(s) (E.C. 1.3.1.20); DEPC, diethylpyrocarbonate; DHT, dihydrotestosterone; D-MEM, Dulbecco's-modified Eagle Medium; EA, ethacrynic acid; ELA, ellagic acid; EMSA, electromobility shift assay; GST, glutathione S-transferases (E.C. 2.5.1.18); HBAB, human bile acid binder; ILU, integrated light units; Luc, luciferase; NQO, NADPH Quinone Oxidoreductase (E.C. 1.6.99.2); PBS, phosphate buffer saline; PMSF, phenyl-methylsulfonyl fluoride; SSC,

Standard sodium citrate; THQ, *tert*-Butylhydroquinone; TPA, tumor promoting agents (Phorbol 12-myristate 13-acetate).

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Abstract.

AKR1C2, also referred to as the human bile acid binder (HBAB) and 3α-hydroxysteroid dehydrogenase type III, is a multifunctional oxidoreductase able to stereoselectively reduce steroids as well as oxidize or reduce polyaromatic hydrocarbons. This same protein was also identified by its robust induction by Phase II inducers in HT29 cells (Mol. Pharm. 48:639-647). In HepG2 cells, both *AKR1C2* and *AKR1C1* (97% sequence homology) were induced by Phase II inducers, but not the highly related *AKR1C3* and *AKR1C4* family members (84% sequence homology). We now report the initial characterization of *AKR1C2's* proximal promoter in HepG2 cell line and the identification of a potent enhancer-like element responsive to Phase II inducers located approximately 5.5 kb upstream from the transcription start site. DNA sequence analysis of this enhancer element revealed that it contained a consensus antioxidant response element (ARE), which was confirmed by mutation analysis. Treatment with Phase II inducers leads to increased accumulation of NRF2 in the nucleus, which was associated with increased binding to this ARE as determined by EMSA. Transient transfection with *Nrf2* increased the transcriptional activity of *AKR1C2's* ARE comparable to that observed with Phase II inducers. ChIP analysis also confirmed increased NRF2 binding to the ARE after induction by a Phase II inducer. The *AKR1C1* promoter also harbored this same ARE element in a highly homologous region, which was also bound by NRF2 in a ChiP analysis. No induction of *AKR1C2's* ARE was detected in $Nrf2^{-/-}$ fibroblasts. The regulation of *AKR1C2* by this distal ARE suggests that AKR1C2 detoxifies products of reactive oxidant injury, which has important implications for both hormone and xenobiotic metabolism.

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Introduction.

AKR1C2, also referred to as 3α-hydroxysteroid dehydrogenase (HSD) type III or the human bile acid binder, is a multifunctional enzyme that catalyzes the dehydrogenation or reduction of endogenous and exogenous planar compounds (Dufort et al., ; Hara et al., 1990; Stolz et al., 1984). We previously identified HBAB by its high affinity binding to bile salts, which was used to monitor its purification from human liver cytosol (Stolz et al., 1984; Takikawa et al., 1990). Others subsequently identified the same protein as either a 3α-HSD or a dihydrodiol dehydrogenases (DDH) (Hara et al., 1990; Khanna et al., 1995). DNA sequence analysis confirmed that AKR1C2 is a member of the Aldo-keto reductase (AKR) supergene family, an emerging group of evolutionarily conserved NADP(H) dependent oxidoreductase that resides in the cytosol (Jez and Penning, 2001)

In human liver, four AKR1C subfamily members are expressed, which metabolize both common and gene-specific substrates (Penning et al., 2000). Table 1 lists their respective enzymatic activities and their close sequence homology. AKR1C4 is considered to be the predominant family member responsible for catabolism of steroids because of its high catalytic activity however it is only expressed in the liver (Penning et al., 2000). In the prostate, AKR1C2 and AKR1C1 can regulate the intracellular levels of dihydrotestosterone (DHT) by respectively reducing it to either a 5α-androstane-3α,17β-diol (3α-diol) or 5α-androstane-3β,17β-diol (3β-diol), which are both weak androgens (Ji et al., 2003; Steckelbroeck et al., 2004). In prostate cancer, we observed a selective reduction of *AKR1C1* and *AKR1C2* expression in tumors as compared with paired normal tissues, which was associated with loss of DHT catabolism (Ji et al., 2003). Thus, AKR1C2 and possibly AKR1C1 may indirectly regulate the activity of the androgen receptor by promoting DHT's catabolism within prostatic cells and thereby

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modulating intracellular DHT levels. A similar finding was also observed in human breast cancer, in which selective loss of AKR1C1, which reduces progesterone to the weak progestine, 20α-dihydroxyprogestgerone, was found in tumors as compared to paired unaffected tissues (Ji et al., 2004; Lewis et al., 2004). Similar to prostate cancer, we predict that reduced AKR1C1 expression would also hinder progesterone metabolism and thereby augmenting the activity of the progesterone receptor (Ji et al., 2004).

Besides catabolizying steroids, polyaromatic hydrocarbons (PAH) are also substrates for AKR1C family members because of their DDH activity (Palackal et al., 2002; Pelkonen and Saarni, 1980). AKR1Cs catalyze the oxidization of non-K region trans dihydrodiols. These are initially produced by Cytochromes P_{450s} that form an arene oxide on the terminal ring of a PAH, which then undergo hydrolysis by epoxide hydrolyase to form non-K region trans dihydrodiols (Burczynski et al., 1999; Penning, 1993). If these trans dihydrodiols are not metabolized, they may undergo another round of epoxidation to form genotoxic carcinogens. AKR1C's DDH activity converts the trans dihydrodiol to a keto group, which then undergoes a rearrangement to form a catechol. In the presence of oxygen, this catechol forms an o-quinone, which are highly redox active and can consume reducing equivalents. This can ultimately lead to oxidative stress injury including oxidative DNA damage resulting in G to T transversions. When over expressed in cells, AKR1Cs increased the production of dimethylbenz[*a*]anthracene-3,4-dione, a potent mutagen from 7,12-dimethylbenz[*a*]anthracene-3,4-diol (Palackal et al., 2002). AKR1C2's ability to metabolize PAHs suggests that it is a part of the Phase II detoxification system, a diverse group of enzymes that metabolize xenobiotic as well as respond to oxidant injury. In fact, Ciaccio and co-workers also identified AKR1C1 and AKR1C2 by their robust induction in HT29, a human colon cancer cell line, after treatment with ethacryinic acid (EA), a model Phase II inducer (Ciaccio et al., 1993).

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Although AKR1C2 and AKR1C1 were identified by their response to Phase II inducers, the specificity of induction with regards to the other highly related family members or the molecular mechanism is not known. Phase II detoxification enzymes are regulated in part by a large group of structurally diverse compounds, which all share comparable chemical reactivity (Prochaska and Talalay, 1988). Recent studies have identified a shared cis-acting element, referred to as the antioxidant response element (ARE)/electrophile response element, which mediates coordinated induction of various genes that constitutes part of the Phase II detoxification system (Nguyen et al., 2004). The ARE cis-acting element is activated when bound by NRF2, officially referred to as the nuclear factor-erythroid-derived 2 related factor 2 (NFE2l2), which is a member of the Cap'n'collar (CNC) transcription factor family. These basic leucine zipper (b-zip) transcription factors co-dimerize with members of the short Maf family as well as other b-zip proteins leading to transcriptional activation of other genes that contain an ARE (Motohashi and Yamamoto, 2004; Nguyen et al., 2004).

In order to identify the cis-acting element responsible for AKR1C2 induction, we initially confirmed that *AKR1C1* and *AKR1C2* were induced in HepG2 cells by the Phase II inducer, EA, whereas *AKR1C3* and *ARK1C4* were not. This induction resulted from transcriptional activation and was dependent on both *de novo* protein and RNA synthesis. Using functional deletion analysis of the proximal and distal 5' flanking regions of *AKR1C2*, a consensus ARE cis-acting element was identified at approximately –5.5 kb upstream from the promoter, which was confirmed by mutation analysis. A highly homologous region (94% sequence identity) was also found approximately 6.3 kb upstream of the *AKR1C1* gene. EMSA demonstrated increased and selective binding to this ARE element by nuclear extract from cells treated with a Phase II inducer. After treatment with a Phase II inducer, ChIP analysis confirmed increased binding of NRF2 to *AKR1C2's* ARE and an identical sequence also present in

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AKR1C1. The basal luciferase activity of an *AKR1C2*'s ARE reporter construct was reduced and unresponsive to a Phase 2 inducer in $Nrf2^{-/-}$ fibroblasts. The finding of an ARE in the 5' flanking region of *AKR1C2* implicates an important link between steroid hormone and xenobiotic metabolism.

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Material and Methods.

Materials and Supplies - All chemical material purchased were of molecular biology grade or higher from the Sigma Corporation (St. Louis, MO) unless otherwise stated. Tissue culture supplies were purchased from GIBCO-BRL (Gaithersburg, MD) unless stated otherwise. Molecular biology reagents were purchased from GIBCO-BRL, Promega (Madison, WI) or Boehringer-Mannheim (Indianapolis, IN). All radioactive nucleotides were acquired from Dupont-NEN (Boston, MA).

Cell Culture - All cell lines were purchased from the ATCC (Rockville, MD). The human liver hepatoblastoma cell line HepG2 was maintained in DMEM supplemented with 10% fetal calf serum, and the human colon carcinoma HT29 cells were maintained in McCoy's 5 Medium supplemented with 4 mM L-glutamine, 10% fetal calf serum. Wildtype (WT) or and $Nrf2^{-/-}$ fibroblasts were maintained in Dulbecco's minimal essential medium/F12 medium supplemented with 10% FCS. All cell lines were grown at 37°C in a 5% CO_2 atmosphere. For induction experiments, exponentially growing cells were plated 24 h before treatment with agents dissolved in DMSO.

RNA Analysis of AKR1C Family Members - Total cellular RNA from treated or control cells was isolated (Chomczynski and Sacchi, 1987) and used for either Northern blot analysis or for real-time PCR. For Northern blot analysis, 10 μgs of total RNA was electrophoresed on a 1.2 % agarose gel at 3V/cm for 3 h before being transferred onto a Nitran membrane by capillary action and hybridized with a random primed [α-^{32}P]-dCTP labeled 1.2 kb *AKR1C1* or a *β-actin* cDNA probes (Stolz et al., 1991). Relative radioactivity was determined using an AMBIS Beta scanner (San Diego, CA). Relative expression of *AKR1C* family members was determined as described using a gene-specific real-time PCR, in which relative expression of individual family

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members were compared to the control gene, *RNase P*, whose expression was not altered by treatment with Phase II inducers (Ji et al., 2003).

Nuclear Runoff - Approximately 6.8 x 10^7 nuclei were harvested and incubated at 37°C for 30 min in 340 µl containing 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 80 mM KCl, 0.1 mM EDTA, 2.5 mM DTT, 0.1 U/µl of RNasin (Promega), 0.5 mM of ATP, GTP, CTP and 20 µl of [α-^{32}P] UTP (800 Ci/mmol). Elongation of RNA transcripts was terminated by incubation with 64 units of DNase I and 1 mM $CaCl_2$ at 37°C for 20 min followed by proteinase K (100 µg/ml) digestion. Nuclear RNA was isolated according to Chomczynski (Chomczynski and Sacchi, 1987) and separated from unincorporated nucleotide. Equal amounts of labeled RNAs (5 x 10^6 cpm/ml) were hybridized at 65°C for 3 days onto nylon filter previously dot-blotted with denatured plasmid DNAs (5 µg/dot) containing either *AKR1C1*, *β-actin*, or empty vector to control for non-specific binding. Filters were washed, exposed, and quantitated for radioactivity using AMBIS Beta scanner. Relative transcription rates for all *AKR1C* family members were normalized with those for non-induced gene, *β-actin*, after subtraction of non-specific hybridization to vector plasmid.

Construction of Deletion Mutation Constructs - Serial deletions of the *AKR1C2* promoter were generated in either the pGL2 or pGL3 luciferase reporter plasmid. In brief, a 13 kb EcoR I fragment from cosmid clone 8 (Lou et al., 1994) was digested with Kpn I, subcloned into the EcoR I and Kpn I sites of pGEM7(+) to generate two plasmids: pGEM6/4kb (-8819 to -4599) and pGEM6/8kb (-4599 to intron 3). A Hind III site was introduced +31 bp upstream of the initial ATG codon of *AKR1C2* by PCR with the antisense primer, 5'-cgt aag cTTCTGTCACTGGCCTGGTTA-3' (lower cases represent added sequence, and Hind III site is underlined) and a sense primer. The resulting PCR product was digested with Bgl II and Hind III

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(position -231 and +31) and inserted upstream of a luciferase reporter gene in the pGL2-Basic plasmid (Promega, Madison, WI) to generate the pluc-231 construct. The sequence was confirmed by DNA sequencing and used to generate a series of progressive deletion constructs of the proximal –5 kb region contained in p-4599 luc.

Constructs containing 5'-distal region of the *AKR1C2* were generated by first inserting a 4 kb BamH I fragment (-8.5 to - 4.6 kb) in either orientation downstream of the luciferase gene of the p231-luc and were named pluc-4600/8528 or pluc-8528/4600. A series of 5' and 3' deletion mutations of the -8.8 to -4.6 kb of the *AKR1C2* genomic region were generated by different combination of restriction enzyme digestions and the resulting fragments: pEBg (-8819 to -7619), pBgK (-7619 to -4599), pSK (-5622 to -4599), pSM (-5622 to -5584), pSF (-5622 to -5433), pSF/MK (-5622 to -5433 and -4770 to -4599); pNK (-5247 to -4599), or pMK (-4770 to -4599) were inserted in front of the homologous promoter contained in a new pluc-231 construct now using the pGL3-Basic plasmid (Promega, Madison, WI).

ptkluc constructs were generated by inserting -37 to +52 of the HSV thymidine kinase gene promoter (McKnight et al., 1981) in front of the luciferase reporter gene in pGL3-Basic, which was then used to generate the following heterologous promoter constructs: pWtkluc (-5594 to –5454), pABtkluc (-5552 to -5454), pBtkluc (-5517 to -5454), pAtkluc (-5552 to -5518), or pC/Btkluc (-5552 to -5594 and -5517 to -5454). Point mutations of the ARE were generated by insertion of double stranded mutated oligos in front of the tk minimal promoter.

Transient Transfection - HepG2 cells were transfected using either the DEAE-dextran method as described or Superfect Transfection Reagent (Qiagen, Valencia, CA) at 80% of confluency according to manufacture's instructions. Twenty-two to twenty-six h after transfection, cells were treated with indicated drugs or 0.1% DMSO as control, and harvested 18 to 24 h later. Equivalent molar amounts of the indicated plasmids were used with salmon sperm DNA as a

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carrier. Transfection efficiency was determined by comparing luciferase activity with either a 0.2 μg of *β-gal* expression plasmid (lacZ reporter gene driven by *β-actin* promoter kindly provided by Dr. L. Kedes) with β-gal assay was performed or a pTK-*Renilla* luciferase expressing plasmid. Luciferase activity assay was performed using the Dual-Luciferase Reporter 1000 Assay System (Promega, Madison, WI). Cells were washed twice with PBS and lysed with Passive Lysis Buffer. *Firefly* and *Renilla* luciferase activities were determined sequentially by the Luminoskan Ascent (ThermoLab Systems) with 20 μl of total cell lysates in 100 μl of Luciferase Assay Reagent followed by addition of 100 μl Stop & Glow Reagent per reaction. Relative luciferase activity of reporter gene was calculated and normalized.

For Nrf2 studies, HepG2 cells were transiently transfected using Superfect Transfection Reagent (Qiagen, Valencia, CA) at 80% of confluency according to the manufacture's instructions. Cells were plated in 12-well plates the day prior to transfection, incubated with 2.5 μg of total DNA per well in transfection solution overnight, and changed with fresh medium the following morning. DNA consisted of a mixture including 0.5 μg of reporter plasmid, pARE tkluc or pAREmu1 tkluc in combination with either various amounts of expression plasmid pcDNAI:Nrf2 or control vector including 2 ngs of pTK-RL, which was used as an internal control for transfection efficiency. Cells were treated with β-NF or DMSO 24 h after transfection and harvested 24 h later.

Similarly, WT or and $Nrf2^{-/-}$ fibroblasts (Leung et al., 2003) were transiently transfected with 1.0 μg of pARE tkluc or pAREmu1 tkluc reporter plasmids. pTK-RL was used as a transfection control. The next morning, transfected cells were treated with DMSO or 60 μM of tert-butylhydroquinone (THQ) and cells were harvested 8 hours later. Luciferase activity was determined as before.

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Nuclear Extract Preparation - HepG2 cells were maintained in DMEM supplemented with 10% FBS at 37^{o}C in a 5% CO_2 atmosphere. For induction experiments, exponentially growing cells were treated with DMSO (0.1% total volume) alone or β̃naphthoflavone (β-NF) dissolved in DMSO (final concentration 4 μM) for different lengths of time. Cells were washed with PBS, harvested, nuclear extracts prepared from HepG2 cells as described (Dignam et al., 1983) with protein concentrations determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis - Thirty μg of nuclear extracts were separated on 4-12 % gradient SDS-polyacrylamide gel, followed by transfering onto a nitrocellulose membrane using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories, Hercules, CA). Immunoblotting was carried out with Nrf2 antibodies diluted 1:200 (Santa Cruz Biotechnology, Stan Cruz, CA), followed by incubation with HRP-labeled secondary antibody (Amersham Biosciences, Piscataway, NJ). Membranes were developed using the ECL-Plus Western Blotting System and visualized by the Storm 860 Blue Fluorescence/Chemifluorescence Scanner (Amersham Biosciences, Piscataway, NJ).

Electrophoretic Mobility Shift Assays. - EMSA assays were performed using nuclear extracts prepared from HepG2 cells treated with DMSO or β-NF for 24 h. Complementary 28-base single-stranded oligonucleotides containing the ARE sequences of the human *AKR1C2* and *AKR1C1* were synthesized with the sense strand labeled with biotin at the 5'position (5'-TTGATGCAGTCAGGGTGACTCAGCAGCT–3'). The complementary strands were annealed and used as a probe. EMSA reactions were performed with 4 μl of nuclear extracts in a buffer system containing 10 mM Hepes-KOH, 27 mM KCl, 100 mM NaCl, 2 mM $MgCl_2$, 1.0 mM EDTA and 15% Glycerol. Poly[dI-dC] at 144 μg/ml was added to each reaction as a non-

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specific competitor. In competition experiments, 50- or 200-fold molar excess of unlabeled wild type or mutant probes (5'-TTGATGCAGTCAGGGTGACT**g**CA**t**CAGCT-3') were used. The reaction mixtures were incubated for 20 min at room temperature and fractionated on non-denaturing 5% polyacrylamide gels in 0.5 X TBE buffer. The binding reactions were then transferred to Biodyne B nylon membrane using capillary transfer in 20XSSC. Transferred DNA was then cross-linked to membrane using Stratalinker UV Crosslinker (Stratagene, La Jolla, CA). Detection of biotin-labeled DNA was performed using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL) following the manufacture's instructions. The membrane was finally exposed to X-ray film or captured by CCD camera (Bio-Rad Laboratories, Hercules, CA).

Chromatin immunoprecipitation assay - HepG2 cells were grown in DMEM supplemented with 10% FCS and were treated overnight with 4 μM of β-NF. ChIP assay was performed as described using the ChIP assay kit (Upstate Technology, Lake Placid, NY). Cross-linking was performed by adding formaldehyde (final concentration 1%) directly to the medium and after a 10 min incubation, cells were washed with cold PBS, harvested, and disrupted by sonication. The chromatin solution was diluted and pre-cleared with salmon sperm DNA-protein A-agarose for 1 h. The supernatant was then incubated overnight at 4^{o}C with either anti-Nrf2 antibody or pre-immune serum. The immune complex was then incubated with salmon sperm DNA-protein A-agarose for 1 h. Following stringent washes, the bound DNA was eluted from the immune complex, purified using Qiagen PCR purification kit, and resuspended in 30 μl ddH_2O. The amount of *AKR1C2* or *AKR1C1* containing the ARE were detected by a real time PCR technique using the following gene specific primers for *AKR1C2* 5'-CTATCTAGGAGTGGTCGCAAGGT-3' and 5'-TCTGCACTGTTTGTTATTTTACTATTGCT-3' or 5'-CCAGGAGTGGTCGCAAGGT-3' and 5'-CCCTACAATCTACTCGGGTTGATG-3' for *AKR1C1*, which were used to amplify the shared region of interest in combination with the

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probe 5'-TGCAAGCTGCTGAGTCACCCTGACTG-FAM on a Applied Biosystems 7900 HT system. Experiments were repeated three times and the results for DMSO and β-NF treated HepG2 cells are given as percentage of inputed DNA.

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Results.

Selective Induction of AKR1C Family Members by Phase II Inducers

We confirmed the prior studies of Ciaccio and co-workers that *AKR1C* expression is significantly increased after EA treatment, a prototypical monofunctional Phase II inducer, in HT29 or HepG2 cell lines (Ciaccio et al., 1994; Ciaccio et al., 1993) (data not shown). EA treatment caused an approximate 17-fold increase in hybridization to a radiolabeled *AKR1C1* probe that cannot differentiate between the four *AKR1C* family members. Similar results were also found after β-napthoflavone (β-NF) treatment, a Phase I and Phase II bifunctional inducer. *AKR1Cs* gene expressions were also induced after treatment with phenylbut-3-en-2-one (20 μM), or the peroxidant, *tert*-Butylhydroquinone (THQ) (20 μM) (data not shown). Approximately 40-fold increase in the AKR1C's immunoreactivity was detected in the cytosol of HT29 cells after EA treatment in agreement with its increased gene expression and prior reports (Ciaccio et al., 1993).

As prior studies suggested that *AKR1C2* was not inducible by Phase II inducers (Burczynski et al., 1999), gene-specific *AKR1C* real-time PCRs were utilized to identify those family members that were induced by these agents. As illustrated in Fig. 1 Panel A, only *ARK1C1* and *AKR1C2* were dose-dependently induced in HepG2 cells after β-NF treatment. Similar results were also found with EA treatment in this and other cell lines (data not shown). In Panel B, nuclear runoff data demonstrated approximately 6 to 10-fold increase in gene transcription of *AKR1C1* and *AKR1C2* in HT29 cells and a 4-fold increase in HepG2 cells as neither *AKR1C3* nor *AKR1C4* were induced in HepG2 cells by these treatments (Panel A). Increased stability of *AKR1C1* and *AKR1C2* mRNAs was also demonstrated in preliminary

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studies, which may also contributes to their striking induction by these Phase II inducers. As shown in Panel C, induction of *AKR1C1* and *AKR1C2* was dependent on both *de novo* protein and RNA synthesis in HT29 cells as either cycloheximide or actinomycin D were able to block their induction. This finding is consistent with prior studies that have demonstrated the need for protein synthesis for induction of other Phase II inducer responsive genes.

Preliminary Characterization of the AKR1C2 5' Flanking Region

The transcription start site for *AKR1C2* gene in HepG2 cells was previously mapped to 31 bp upstream of the initial methionine by using both primer extension and S1 mapping techniques (Lou et al., 1994). We confirmed that a previously characterized cosmid clone 8 contained the proximal flanking region of *AKR1C2* by comparing its sequence with another report and the human genome database (Nishizawa et al., 2000). 5' deletion constructs of the -4599 to +31 region were used to localize the proximal promoter region and to screen for a potential consensus ARE cis-acting element. As illustrated in Fig. 2, Panel A, deletion construct containing at minimum the –117 region resulted in a 40-fold or greater increase in luciferase reporter activity, as compared to the pluc-33 promoter, whose activity was only 5-fold greater than the pGL2 backbone plasmid. This finding suggest that the proximal promoter of the gene lies between –33 and –117. Within this region, a consensus C/EBP β binding site - TTGTGTAAG located at -110 to -102 was identified, which is present in the promoter of many genes that are well expressed in the liver (Xanthopoulos and Mirkovitch, 1993). The role of this element in mediating liver specific expression is currently under investigation. Constructs pluc-117 to pluc-2793 conferred comparable luciferase activity in HepG2 cells, whose activity decreased as more distal elements were included. Surprisingly, none of these constructs

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responded to EA treatment despite a proximal, consensus AP-1 like sequences located at –185 (data not shown), which share high sequence similarity to the ARE .

Localization of a Distal Antioxidant Response Element

The luciferase activity of the distal -8.5 to -4.6 kb region was compared to the homologous –231 promoter (pluc-231) to localize a putative ARE located beyond 4.6 kb. In Fig. 2 Panel B, a distal –8.5 to –4.6 kb fragment in either orientation placed in front of the homologous pluc-231 promoter, pluc-4600/8528 or pluc-8528/4600 resulted in an two-fold increase in luciferase activity as compared to the pluc-231 homologous promoter. In response to the Phase II inducers β-NF, EA, THQ or ellagic acid (ELA), a constituent of tree bark and grape skins, luciferase activity of either construct was increased by two to three-fold as compared to the proximal pluc-231 construct. Thus, the Phase II responsive element lies in the -4.6 to -8.5 kb region of the *AKR1C2* gene. TPA, a potent inducer of PKC activity and activator of AP-1 transcriptional activity, and hydrogen peroxide (Favreau and Pickett, 1993), were both able to increase the luciferase activity of all constructs suggesting that a consensus AP-1 site located at –185 from the transcriptional start site is functional.

Identification of the Phase II Responsive Element in AKR1C2 at –5.5 kb

Progressive deletions of the -8.8 to -4.6 kb region inserted proximal to the –231 promoter region were screened for both increase in basal activity as well as induction by Phase II inducers. As illustrated in Fig. 3, Panel A, HepG2 cells transfected with pBgK, pSK, pSF, or pSF/MK, which all share the -5622 to –5433 region, respectively increased basal luciferase activity by approximately 9, 30, 20, or 23-fold as compared to the pluc-231 construct. Treatment with either EA or β-NF further increased luciferase activity by 2 or 3 fold. Construct pSM, containing the

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-5622 to –5584 region was unresponsive indicating that -5583 to –5433 region is likely to harbor the Phase II responsive element. In Panel B, DNA sequence analysis of -5622 to –5433 revealed a consensus ARE binding sequence on the negative strand at positions –5552 to -5524, as identified in numerous Phase II responsive genes. The functional activity of this candidate ARE in *AKR1C2* was confirmed by creating 5' and 3' and internal deletion mutants for the region -5594 to - 5454, which were cloned in front of a minimal tk heterologous promoter. As shown in Fig. 3 Panel C, only the constructs containing the sequence -5552 to –5524 conferred both increased luciferase activity and induction by EA or β-NF treatments. The increase in transcription by two to five-fold is comparable with the nuclear run off data presented in Fig. 1 Panel B. To confirm that this region is responsible for induction by Phase II inducers in context of the entire flanking region, an intact -6,705 construct was cloned into the same luciferase construct. As shown in Panel D, intact -6.7 kb construct was responsive to treatment with three different Phase II inducers demonstrating that the intact region containing this element was responsive to Phase II inducers.

Mutational Analysis of AKR1C2's ARE

The sequence of the ARE identified in *AKR1C2* was compared to AREs found in other Phase II responsive genes. As illustrated in Fig. 4 Panel A, *AKR1C2's* ARE element, which is located on the negative strand, shares high sequence identity with a ARE consensus site found in other Phase II responsive genes (Wasserman and Fahl, 1997), a few of which are shown. *AKR1C2*'s ARE sequence is identical to that of *NQO1*. Subtle differences in the DNA sequence surrounding the core ARE site can modify the relative activity of the element (Nioi et al., 2003). Fig. 4 Panel B confirms that mutations in the consensus ARE site reduce both basal enhancer activity and responsiveness to EA or β-NF treatments. Truncation of the 5' portion of the

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extended ARE in construct 5'ARE significantly reduced the basal enhancer activity, but minimally inhibited the induction by Phase II inducers. In contrast, mutation of the proximal consensus A to C at position –5537 in the expanded consensus sequence (pAREmu3) significantly reduced enhancer activity as well as induction by EA or β-NF. Mutation of T to C at position –5541 in pAREmu1 construct inhibited induction by either inducer and eliminated almost all the enhancer activity. Conversion of the distal G to A at position –5548 in the consensus sequence reduced enhancer activity to a greater extent than responsiveness to the Phase II inducers. In construct pAREmu4, the 5' half of the ARE was converted to a consensus core ARE in the opposite orientation from the distal core sequence separated by 3 bps resulted in a doubling of both enhancer and induction of luciferase activity in response to EA or β-NF suggesting generation of two independent ARE binding sites.

Increased Binding of NRF2 to the AKR1C2's ARE Element After Phase II Inducer Treatment.

The binding of nuclear proteins to the ARE consensus sequence of *AKR1C2* was determined by electromobility shift assay (EMSA) using nuclear extract from HepG2 cells treated with β-NF. As AKR1C1 and AKR1C2 were the only *AKR1C*s family members responsive to these agents and are highly homologous, we compared the 190 bp sequence listed in Fig. 3 Panel B with the human genome (Build 35.1). The only homologous sequence identified was found with *AKR1C1* gene, which shared 94% sequence identity with this region and was located approximately 6.3 kb upstream from the gene. Fig. 5 Panel A demonstrates 100% sequence identity in the ARE identified in *AKR1C2* with *AKR1C1*, which was used to identify nuclear proteins that bound to the ARE. As illustrated in Panel B, nuclear proteins bind to a double stranded *AKR1C2* ARE present in nuclear extract of DMSO treated HepG2 cells.

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Treatment of HepG2 cells with β-NF for 24 hr increased the binding by two to three-fold. Excess unlabeled wild type ARE was able to competitively displace the labeled complex whereas a mutated unlabeled ARE was unable to do so. Note that non-specific binding to the complex is also observed in the EMSA.

As prior studies have demonstrated that NRF2 is essential for activation of the ARE consensus cis-acting element, we directly assessed if increased Nrf2 expression could activate the ARE luciferase reporter utilized in Fig. 4, Panel B. As illustrated in Fig. 6 Panel A, transient transfection of HepG2 cells with increasing amounts of a *Nrf2* expression plasmid caused a dose-dependent increase in the luciferase activity of the tk ARE luc construct, which approached that observed after treatment with β-NF. No activation was observed when using a mutated ARE, pAREmu1 tkluc, whose basal expression was approximately 6% of the wildtype element. As illustrated in Panel B, time-dependent increase in NRF2 immunoreactivity were detected in nuclear extracts from HepG2 cells treated with β-NF in agreement with increased binding observed in the EMSA in Fig. 5. In order to confirm the binding of NRF2 to the ARE element *in vivo*, a ChIP analysis was performed using a real-time gene specific PCR technique for *AKR1C1* or *AKR1C2* with a common probe. As shown in Fig. 6, Panel C, approximately four to five -fold increase in binding of NRF2 to either the *AKR1C2* or the presumed *AKR1C1* ARE was observed after β-NF treatment in HepG2 cells as compared to DMSO treated cells. To confirm that NRF2 is required for the induction by Phase II inducers, wildtype ARE, pARE tkluc was transfected into murine fibroblast deficient in *Nrf2* or wildtype fibroblasts. As illustrated in Panel D, pARE-tk luc activity in wildtype fibroblast doubled by treatment with 60 μM THQ whereas in $Nrf2^{-/-}$ cells, basal activity was reduced and was unresponsive to THQ treatment. Thus, Nrf2 is required for induction of *AKR1C2*'s ARE.

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Discussion.

AKR1C2 is a member of a highly related group of monomeric oxidoreductases that metabolize endogenous and xenobiotic hydrophobic compounds (Jez and Penning, 2001; Penning, 1997; Stolz et al., 1991). Structural analysis of AKR1C family members reveal a common eight-chain alpha/beta barrel structure in which the cofactor lies at the bottom of the barrel lined with hydrophobic residues. Sophisticated kinetic analysis by Penning and co-workers have determined which steps in the catalytic cycle are rate limiting for enzyme catalysis and the structural basis for stereospecificity of reduction (Heredia and Penning, 2004). For example, AKR1C1 mediates reduction of DHT predominately to a 3β-diol whereas AKR1C2, which shares 97% sequence identity with AKR1C1, generates only a 3α-diol because of the orientation of DHT in the binding pocket (Heredia and Penning, 2004). We originally isolated and purified AKR1C2 from human liver by its unique, high affinity binding for bile salts (Kd < 1 μM), which distinguished it from the other highly related AKR1C family members (Stolz et al., 1984; Takikawa et al., 1990). Our prior studies in both isolated rat hepatocytes and intact rat liver perfusion studies demonstrated that bile salts interact with the cytosolic 3α-HSD (Akr1c8), and that this interaction was essential for rapid transcellular movement of bile salts from the sinusoidal to the canalicular pole of the hepatocyte (Bahar and Stolz, 1999). Unlike Akr1c8, AKR1C2 has minimal 3α-HSD activity for bile salts (Takikawa et al., 1990).

Functional analysis of the proximal -4.6 kb 5' genomic region of *AKR1C2* in HepG2 cells identified a minimal promoter located between -32 and -111 bp. No consensus TATA binding site was identified in this region. A consensus binding site for C/EBP β, was identified, which has been observed in other genes expressed in the liver. Promoter analysis of the proximal

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flanking regions of *AKR1C3* and *AKR1C4*, which shares approximately 70% sequence identity with *AKR1C2*, have been evaluated. Unlike *AKR1C2*, the –1 to–666 region of the *AKR1C3* gene has the greatest luciferase reporter activity in HepG2 cells (Ciaccio et al., 1996). Potential C/EBP β and HNF-5 sites within this region may account for this enhancer activity in HepG2 cells. Two repressor elements were also contained in the regions encompassing -256 to -589 and -881 to -1161. It is not surprising that a 1.1 kb proximal region was unresponsive to EA as our real-time PCR studies failed to detect increased expression of *AKR1C3* in response to Phase II inducers (Ciaccio et al., 1994). Similar to *AKR1C3*, two distal elements in the proximal promoter region of *AKR1C4* contain elements that are responsible for maximal luciferase reporter activity in HepG2 cells (Ozeki et al., 2001). These sites interact with the transcription factors HNF-4 and HNF-1, both of which are associated with liver specific expression. Synergy between these two sites has been implicated as being responsible for the maximal promoter activity detected in HepG2 cells (Ozeki et al., 2002). This HNF-1 site is also responsible for liver specific expression as binding by vHNF-C inhibits *AKR1C4* expression in a kidney derived cell line. As hepatic expression of AKR1C4 varies widely between individuals, Kamataki has suggested that variations in HNF-1α, HNF-4α, and HNF-4γ levels may be responsible for this widely variable expression pattern (Ozeki et al., 2003).

To our knowledge, the localization of *AKR1C2* ARE is the third AKR family member found to have this element. In detailed studies in the murine aldose reductase, *Akr1b3*, two adjacent AREs and an AP1 sites were required for maximal response to cotransfected Nrf2 (Nishinaka and Yabe-Nishimura, 2005). Potential AREs were also identified by sequence analysis in the proximal promoter region of *Akr7a3*, but not characterized (Ellis et al., 2003). *AKR1C2*'s ARE is located approximately -5.5 kb from the transcriptional start site, which is one of the farthest location reported to date for any Phase II responsive genes. The identical sequence

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homology and the binding of Nrf2 to *AKR1C1*'s ARE after treatment with a Phase II inducer suggested that this element is also responsible for induction of AKR1C1 by Phase II inducers.

In the absence of Phase II inducers, the ARE by itself functions as a potent enhancer that increases basal expression of a tk minimal promoter by approximately 200-fold. *AKR1C2's* ARE shares the greatest sequence homology with *NQO1*. An interesting finding of our study was that the ARE is required for the enhancer activity of a neighboring cis-acting elements. In Fig. 3 Panel C, the –5594 to –5454 has almost a 3-fold greater luciferase activity than the –5553 to –5454 construct contained in pABtkluc consturct. As the pBtkLuc and pC/Btkluc constructs lack transcriptional activity, the region between -5594 and -5552 must contain an enhancer-like activity, which is dependent on the ARE. We speculate that the ARE may stabilize the transcription machinery thereby augmenting activity of surrounding enhancer elements.

Fig. 4 demonstrates the importance of conserved nucleotides in *AKR1C2*'s ARE sequence as confirmed by mutation analysis and EMSA studies. For the p5'ARE construct, a truncated ARE was able to enhance basal activity as well as retain responsiveness to Phase II inducers, but the overall activity was reduced by over six-fold. Mutations of the consensus ARE in the constructs pAREmu2 and pAREmu3 reduced the basal activity while maintaining some responsiveness to Phase II inducers. Mutation of T to C in pAREmu1 confirmed that it is essential for both enhancer activity and responsiveness to Phase II inducers. In contrast, elimination of C in pAREmu4 to generate a palindromic core ARE element separated by 3 bps resulted in a doubling of both basal activity and responsiveness to Phase II inducers. This and other studies demonstrate that subtle changes in the ARE consensus sequence can profoundly influence both basal expression and responsiveness to Phase II inducers (Nioi et al., 2003). EMSA studies in Fig. 5 confirmed that the ARE binds to nuclear factors in HepG2 cells, which is consistent with its enhancer-like activity in this cell line. The ARE may also be required for

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basal expression as well as response to Phase II inducers. In the *Nrf2* knock-out mice, the basal expression of specific genes such as *Epx*, epoxide hydrolase and *Nqo*, were reduced as well as their response to Phase II inducers where as basal expression of other responsive genes were unaffected such as the catalytic and regulatory subunits of *Gcs* (Thimmulappa et al., 2002).

Identification of Nrf2 as the key regulator of ARE's activation has allowed a detailed understanding of how it functions and its mechanism of regulation (Motohashi and Yamamoto, 2004). Typically, Nrf2 is retained within the cytosol by binding to Keap1, which is an actin-binding cytosolic protein. In response to oxidant stress or electrophils, redox sensitive cysteines in Keap1 release Nrf2 exposing a nuclear localization signal causing it to migrate into the nucleus. In the nucleus, Nrf2 co-dimerizes with short members of the Maf gene family as well as other b-zip proteins and binds to ARE elements leading to activation of gene transcription mediated in part by binding with CBP, the CREB binding protein (Motohashi and Yamamoto, 2004). In addition to electrophiles, Nrf2 can also be phosphorylated by a number of second messenger systems including Protein Kinase C and MAP kinase (Motohashi and Yamamoto, 2004; Nguyen et al., 2004). Nrf2 is known to have a short half-life and continually undergoes ubiquitin-dependent proteolysis (Motohashi and Yamamoto, 2004). Recent studies have demonstrated that Keap1 interacts with part of the ubiquination machinery, which may account for the rapid degradation of Nrf2 when bound to Keap1. The requirement for *de novo* synthesis of Nrf2 for the induction of *AKR1C1* and *AKR1C2* is consistent with our findings in Fig. 1 Panel C as a protein synthesis inhibitor was able to abrogate the induction.

Transcriptional activation of the ARE when bound by Nrf2 is a common feature for a diverse group of genes involved in metabolism, transport, production of reducing equivalents and DNA repair (Motohashi and Yamamoto, 2004). The *Nrf2* knockout mice have increased susceptibility to oxidant-induced liver injury and carcinogen induced gastric tumor formation

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demonstrating the critical role that Nrf2 has in orchestrating response to noxious injury (Motohashi and Yamamoto, 2004). Protective pathways are also regulated by this element. The selective induction of AKR1C2 and AKR1C1 in HepG2 cells suggest a difference in the physiological function for these particular family members as compared to the highly related AKR1C3 and AKR1C4. To date, no one has identified the molecular mechanism responsible for potent induction of AKR1C2 by Phase II inducers. Our EMSA and ChIP studies confirms that like other Phase II responsive genes, Nrf2 plays a critical role in the regulation of AKR1C2 by this distal, consensus ARE cis-acting element.

Fig. 7 reviews the known physiological functions of AKR1C2. In breast and prostate tumors, we previously observed a selective reduction in *AKR1C1 and AKR1C2* expression as compared to *AKR1C3* (Ji et al., 2004; Ji et al., 2003). We speculated that loss of AKR1C2 in prostate tumors would impair the catabolism of DHT to the weak androgen, 3α-diol and thereby augment androgen-dependent growth of tumor cells. In recent studies, we noted that freshly isolated prostatic tumors had a reduced capacity to metabolize radiolabeled DHT to 3α-diol as compared to paired normal tissue (manuscript in preparation). In breast cancer, selective reduction of AKR1C1 in tumor samples was also observed as compared to paired normal tissue (Ji et al., 2004). AKR1C1's 20α-HSD activity metabolizes progesterone to the weak progestin, 20α-dihydroxyprogeseterone. Similar to our findings in prostate cancer samples, reduced metabolism of progesterone could indirectly regulate the activity of the progesterone receptor. Taken together, specific AKR1C family member may function as pre-receptor regulators of the androgen or progesterone receptor by regulating the intracellular levels of DHT or progesterone. This mechanism of pre-receptor regulation by ligand catabolism is well-recognized for both the glucocorticoid and aldosterone receptors (Nobel et al., 2001).

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In contrast to these hormone dependent tumors, increased expression of *AKR1C1* and *AKR1C2* has been observed in tumors of the aero-digestive tract. Increased expression of *AKR1C1* and *AKR1C2* were noted by Hsu and co-workers in non-small cell lung cancers (Hsu et al., 2001). This greater expression of AKR1C1 and AKR1C2 had no prognostic significance, but was more frequently found in squamous carcinoma (Chen et al., 2002). Augmented expression of these same genes were also observed in esophageal tumors (Kazemi-Noureini et al., 2004). Increased expression of AKR1Cs in a lung carcinoma cell line can enhance the production of genotoxic carcinogens from PAH (Palackal et al., 2002). We therefore speculate that the observed increase in expression of AKR1C1 and AKR1C2 in aero-digestive tumors may enhance production of genotoxic carcinogens from PAH and thereby contribute to tumor formation at these sites.

Besides their role in steroid and PAH metabolism, Deng identified a new role for AKR1C1 and AKR1C2 in modifying chemotherapeutic resistance. They reported a selective induction of AKR1C1 and AKR1C2, but not other genes typically induced by Phase II inducers such as GST, in a cisplatin-resistant variant ovarian cancer cell line (2008/C13*) as compared to its parent cell line (Deng et al., 2004; Deng et al., 2002). Over expressing of only AKR1C1 or AKR1C2 in the parent cell line was sufficient to render it resistant to a platinum based chemotherapeutic agents. AKR1C1 over-expressed in cells derived from cervical, germ-cell, or lung carcinoma also exhibited greater resistance to cis-platinum as well as chemotherapeutic agents of other classes such as paclitaxel, vincristine, doxorubicin or melphan. The authors speculated that AKR1C1 or AKR1C2 may metabolize some unknown substrates or function as inhibitors of apoptosis, and thereby renders cells resistant to chemotherapeutic agents. These findings may have important implications for predicting a tumor's sensitivity to a specific chemotherapeutic regiment.

In summary, *AKR1C2* is transcriptionally up regulated by Phase II inducers mediated by an ARE cis-acting element located 5.5 kb upstream from the transcriptional start site. Because the ARE coordinates expression of enzymes involved with detoxification of toxic compounds, *AKR1C2* is predicted to be part of the cellular defense mechanisms protecting against oxidant induced injury. Increased AKR1C2 expression in tumors of the aero-digestive tract may enhance the tumorgenicity of airborne PAH, which are well-known risk factors for these tumors. For these tumors, the increased AKR1C2 may also render them more resistant to chemotherapeutic agents. In contrast to PAH dependent tumors, the reduced expression of *AKR1C2* in prostate cancer may ensure an adequate supply of trophic androgens thereby providing a selective advantage for proliferation of these malignant cells. The inducibility of *AKR1C2* by Phase II inducers has important implications for development of new therapeutic strategies for treatment or prevention of prostate cancer by enhanced catabolism of DHT.

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Footnotes.

a) Studies were supported by National Institutes of Health DK41014 (A.S.) and USC Center For Liver Disease Research 5P30DK048522.

b) Andrew Stolz, HMR 101A, 2011 Zonal Avenue, Los Angeles, CA 90033. astolz@usc.edu.

c) The work was presented in part as a poster: Stolz, A, Lou H, Du, S, and Ji, Q. Phase II Inducers Can Up Regulate AKR1C2 Expression by a Distal Antioxidant Response Element (ARE): Implications for chemoprevention of carcinogen induced tumors. Third Annual AACR International Conference on Frontiers in Cancer Prevention Research. 2004

d) AKR1C2 genomic sequence has been deposited in Genebank, Accession number DQ379983.

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Legends for figures.

Fig. 1. Induction of AKR1C2 by Phase II inducers is dependent on protein and RNA synthesis.

Panel A: Using a gene-specific real-time PCR, only *AKR1C1* and *AKR1C2* are dose-dependently increased by β-naphthoflavone (β-NF) treatment in HepG2 cells. Panel B: Nuclear-runoff studies confirm increased *AKR1C1* and *AKR1C2* gene transcription after EA treatment in HT29 or HepG2 cell lines. Panel C: Northern blot analysis of induction of *AKR1C1* and *AKR1C2* by Phase II inducers in HT29 cells is dependent on both protein and RNA synthesis as the inhibitors cyclohexamide (CHX) or actinomycin D (ACTD) are able to block their induction by EA.

Fig. 2. Promoter analysis of *AKR1C2* in HepG2 cells and localization of the Antioxidant Response Element between -4.6 and -8.6 kb.

Panel A: Deletion analysis of the *AKR1C2's* promoter demonstrates potent luciferase reporter activity in HepG2 cells with all fragments containing the –117 region. Not shown, no increase in relative luciferase activity was found after treatment with Phase II inducers. Panel B: Relative luciferase activity of the distal –4.6 to –8.6 kb region of *AKR1C2* in either orientation upstream to the pluc-231 homologous promoter (pluc-4600/8528 or pluc-8528/4600) is compared to pluc-231 proximal promoter construct, which is arbitrarily defined as 100. Inclusion of the more distal elements lead to a 2 or 3 fold increase in basal activity. These constructs responded to treatment with the following Phase II inducers: ethacrynic acid (EA) (40 μM), β-naphthoflavone (NF) (4 μM), tert-butyhydroquinone (THQ) (100 μM), or ellagic acid (ELA) (50 □M). The AP-1

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inducing agents - hydrogen peroxide (H_20_2) (5 mM), or Phorbol 12-myristate 13-acetate, the tumor promoting agent (TPA) (100 ng/ml) increased luciferase activity of all constructs.

Fig. 3. AKR1C2's Core Antioxidant Response Element (ARE) is located between -5550 to -5540.

Panel A: Deletion analysis of –4.6 to -8.6 kb region proximal to the pluc–231 promoter construct identifies a common region (-5622 to –5433) that increased both basal activity and responses to the Phase II inducers ethacrynic acid (EA) (40 μM) or β-naphthoflavone (β-NF) (4 μM) dissolved in DMSO (mean of 3 or more experiments ± S.D.). Panel B: DNA sequence analysis reveals an ARE consensus core sequence at -5550 to -5540 on the negative strand. Panel C: Deletion analysis of the –5594 to -5454 region in front of a heterologous tk promoter (ptkluc) confirms that the -5552 to -5524 contains the ARE (average of two independent experiments in triplicate). Panel D: Luciferase reporter activity of proximal –6.7 kb fragment inserted into the pGL3 reporter plasmid demonstrated increased relative luciferase activity in responses to the Phase II inducers ethacrynic acid (EA) (40 μM), or β-naphthoflavone (β-NF) (4 μM), or tert-butyhydroquinone (THQ) (100 μM) dissolved in DMSO as compared to DMSO treated control HepG2 cells (mean of 3 or more experiments ± S.D.).

Fig. 4. Mutation analysis of *AKR1C2*'s ARE

Panel A: The consensus and core ARE sequence of *AKR1C2* contains the Phase II responsive element on the minus strand and shares sequence homology with other Phase II responsive genes such as rat *Gst Pi*, *Gst Ya* and human and rat NADPH quinone oxidoreductase type I gene (*NQO*) as well as the consensus ARE sequence (Wasserman and Fahl, 1997). Panel B: Mutation

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of conserved nucleotides in the ARE consensus sequence reduces enhancer activity and response to Phase II inducers. Truncation of the core element reduces activity but not responsiveness of Phase II inducers whereas mutations within the core sequence reduce both enhancer activity and responsiveness to Phase II inducers. Generation of a palindromic core sequence in pAREmu4 increases both basal activity and responsiveness to Phase II inducers. Results are average of 2 independent experiments performed in triplicate.

Fig. 5. Electrophoretic mobility shift assay with the human AKR1C2 ARE

Panel A: Sequence of EMSA probe for *AKR1C2* identifies an identical sequence in the *AKR1C1* promoter region located approximately 6.3 kb upstream of exon 1 in the human genome (Build 35.1).

Panel B: Biotinylated double stranded *AKR1C2* containing the ARE was incubated for 20 min at room temperature with crude nuclear extracts prepared from HepG2 cells treated with DMSO alone (Ctrl) or β-NF (4 μM). In competition experiments, 50- or 200-fold molar excess of unlabeled wild type or mutant type probes were used to demonstrate specificity of binding. Shifted bands and free probe were as indicated by arrows while nonspecific binding was designated by *.

Fig. 6. NRF2 regulates *AKR1C2's* ARE element.

Panel A: Luciferase activity of the *AKR1C2* ARE reporter plasmid, pARE tkluc, increases after transient transfection with increasing amounts of an *Nrf2* expression plasmid (Average ± S.D. of three experiments). HepG2 cells were transfected with the empty vector or indicated amount (μg) of *Nrf2* expressing plasmid and the wildtype (WT) or mutated AKR (mut) pAREmu1-tk reporter plasmid. HepG2 cells were also transfected with pARE tkluc and treated with either

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DMSO or β-NF (4 μM). Two ngs of pTK-RL were co-transfected as an internal control for transfection efficiency for all studies. Relative luciferase activity was normalized to the activity of pARE tkluc treated with DMSO. Panel B: Treatment with 4 μM of β-NF was associated with a time-dependent appearance of NRF2 in the crude nuclear extracts of HepG2 cells. Nuclear extracts were isolated from HepG2 cells treated with DMSO alone (Ctrl) orβ-NF for 1, 3, 6, and 24 h, respectively, and NRF2 was detected with anti-Nrf2 antibody. Panel C: ChIP analysis confirms increased association of NRF2 to the ARE element of *AKR1C2* or the potential ARE in *AKR1C1* after treatment with 4 μM of β-NF as compared to untreated cells (Average ± S.D. of 3 experiments). The chromatin-transcriptional factor complexes were immunoprecipitated with anti-Nrf2 antibody. The DNA fragments from the precipitated complex were purified and subjected to real-time PCR using probe and primer pairs to specifically amplify the human *AKR1C2* or *AKR1C1* ARE. Results are presented as percentage of inputed genomic DNA. Panel D: Nrf2 is required for induction of *AKR1C2's* ARE. $Nrf2^{-/-}$ or wildtype fibroblasts were transiently transfected with pARE-tk construct and cells were treated with DMSO (control) or THQ (60 μM). As compared to wildtype fibroblast, basal expression of pARE-tk construct was reduced by 50% and was unresponsive to THQ treatment in $Nrf2^{-/-}$ fibroblasts.

Fig. 7. Physiological role of AKR1C2 and its dysregulation in tumors

Potential consequences of dysregulation of AKR1C2 in hormone-dependent and aero-digestive tumors. A functional ARE in the *AKR1C2* gene indicates a role in cellular detoxification. In cell lines, increased expression of AKR1C2 confers resistance to chemotherapeutic agents by an unknown mechanism that may include inhibition of apoptotic pathways or metabolism of unknown substrates .

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Tables.

Table 1: Predominant enzymatic activities of AKR1C Family Members

AKR Nomenclature	Alternate names	Enzyme activity	Sequence homology
AKR1C1	DDH 1, 20α-HSD	20α-HSD, 3β-HSD	98%
AKR1C2	Human Bile-acid binding protein (BABP), human bile acid binder (HBAB), DDH 2, 3α-HSD III	3α-HSD, 17β-HSD	100%
AKR1C3	17β-HSD V, DDH 3, 3α-HSD II,	17β-HSD	84%
AKR1C4	Chlordecone reductase, DDH 4	3α-HSD	83%

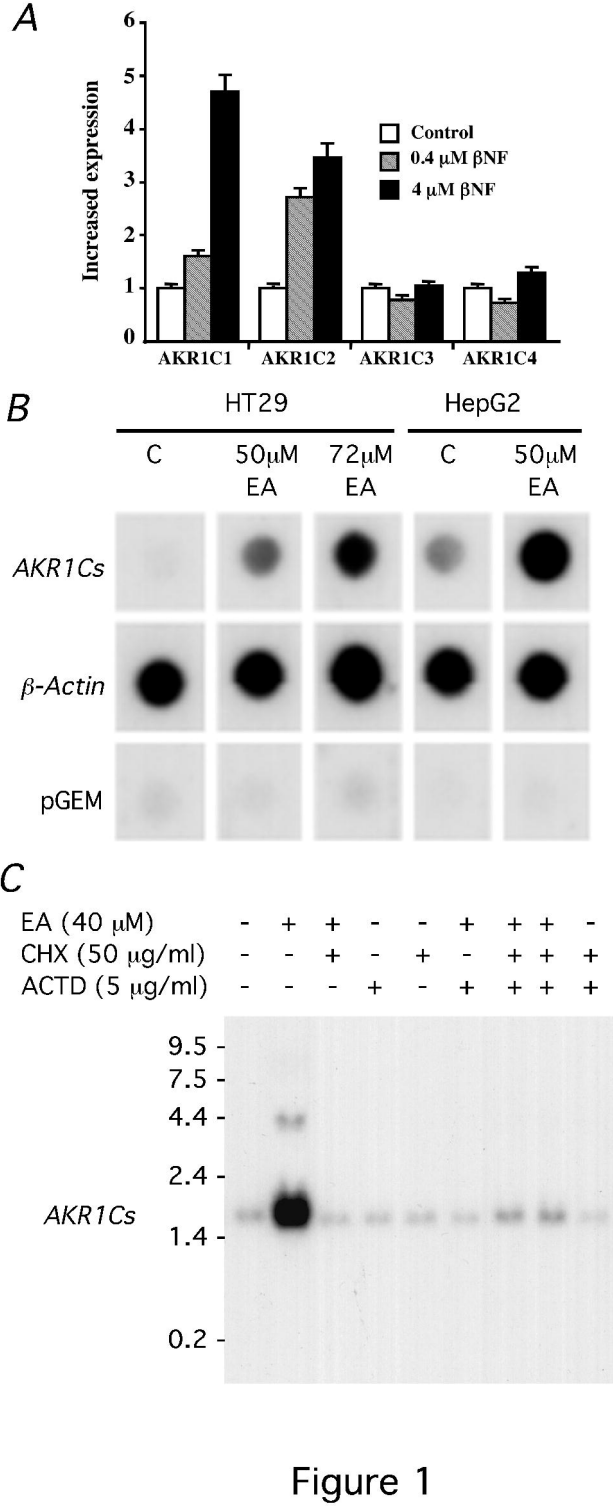
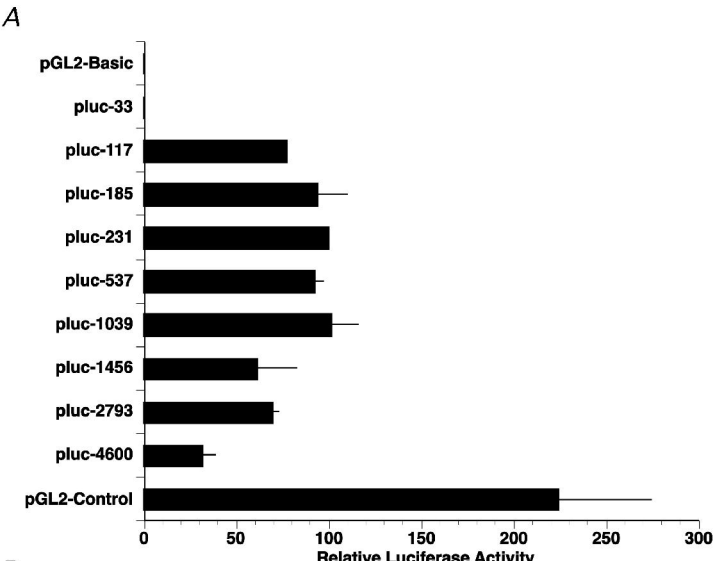

A
Increased expression
6
5
4
3
2
1
0
Control
0.4 μM βNF
4 μM βNF
AKR1C1
AKR1C2
AKR1C3
AKR1C4
B
HT29
HepG2
C
50μM EA
72μM EA
C
50μM EA
AKR1Cs
β-Actin
pGEM
C
EA (40 μM) - + + - - + + + -
CHX (50 μg/ml) - - + - + - + + +
ACTD (5 μg/ml) - - - + - + + + +
9.5
7.5
4.4
2.4
1.4
0.2
AKR1Cs

Figure 1


A
pGL2-Basic
pluc-33
pluc-117
pluc-185
pluc-231
pluc-537
pluc-1039
pluc-1456
pluc-2793
pluc-4600
pGL2-Control
0
50
100
150
200
250
300
Relative Luciferase Activity

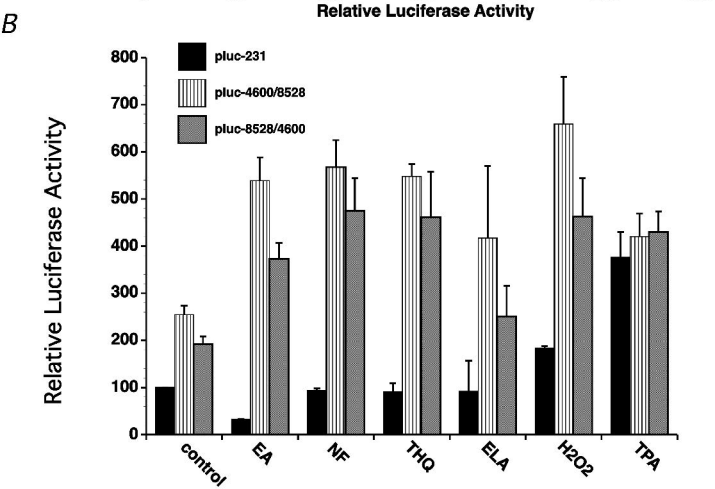

B
pluc-231
pluc-4600/8528
pluc-8528/4600
Relative Luciferase Activity
800
700
600
500
400
300
200
100
0
control
EA
NF
THQ
ELA
H2O2
TPA

Figure 2

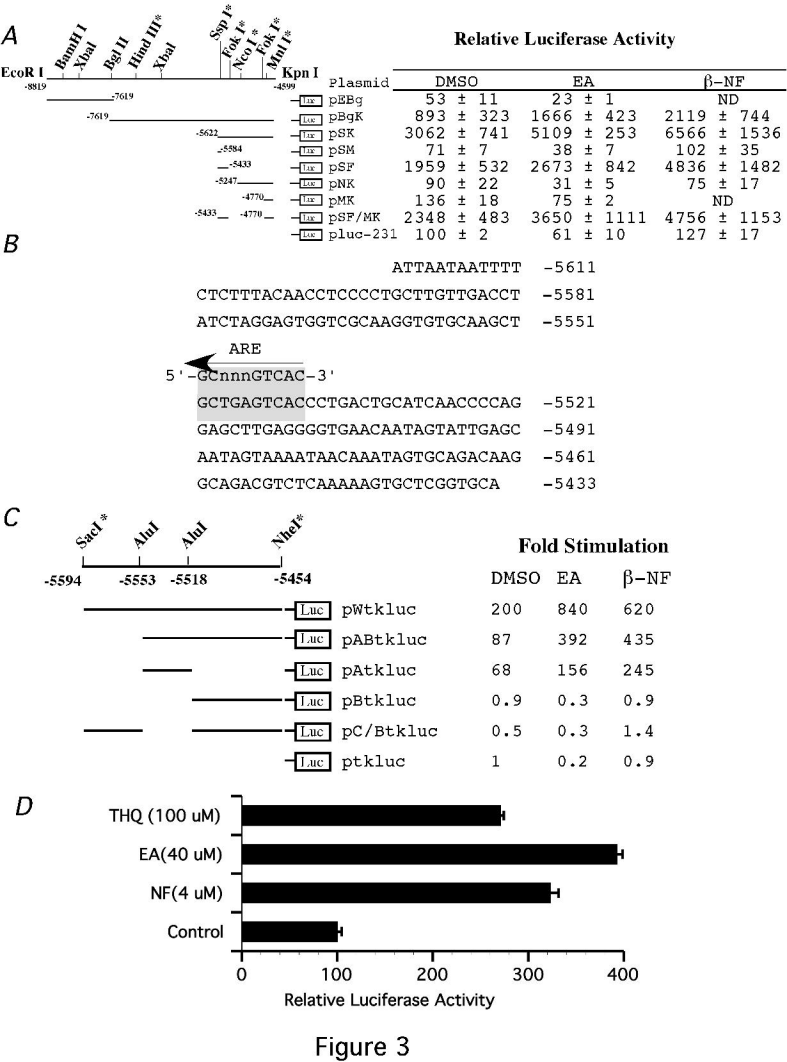

A
BamH I
XbaI
Bgl II
Hind III*
XhaI
Ssp I*
Fok I*
Nco I*
Fok I*
Mlu I*
EcoR I
Kpn I
-8819
-4599
-7619
-7619
-5622
-5584
-5433
-5247
-4770
-5433
-4770
Relative Luciferase Activity
Plasmid DMSO EA β-NF
pEBg 53 ± 11 23 ± 1 ND
pBgK 893 ± 323 1666 ± 423 2119 ± 744
pSK 3062 ± 741 5109 ± 253 6566 ± 1536
pSM 71 ± 7 38 ± 7 102 ± 35
pSF 1959 ± 532 2673 ± 842 4836 ± 1482
pNK 90 ± 22 31 ± 5 75 ± 17
pMK 136 ± 18 75 ± 2 ND
pSF/MK 2348 ± 483 3650 ± 1111 4756 ± 1153
pluc-231 100 ± 2 61 ± 10 127 ± 17
B
ATTAATAATTTT -5611
CTCTTTACAACCTCCCCTGCTTGTTGACCT -5581
ATCTAGGAGTGGTCGCAAGGTGTGCAAGCT -5551
ARE
5'-GCnnnGTCAC-3'
GCTGAGTCACCCTGACTGCATCAACCCCAG -5521
GAGCTTGAGGGGTGAACAATAGTATTGAGC -5491
AATAGTAAAATAACAAATAGTGCAGACAAG -5461
GCAGACGTCTCAAAAAGTGCTCGGTGCA -5433
C
SacI*
AluI
AluI
NheI*
-5594
-5553
-5518
-5454
Fold Stimulation
DMSO EA β-NF
pWtkluc 200 840 620
pABtkluc 87 392 435
pAtkluc 68 156 245
pBtkluc 0.9 0.3 0.9
pC/Btkluc 0.5 0.3 1.4
ptkluc 1 0.2 0.9
D
THQ (100 uM)
EA(40 uM)
NF(4 uM)
Control
0
100
200
300
400
Relative Luciferase Activity

Figure 3

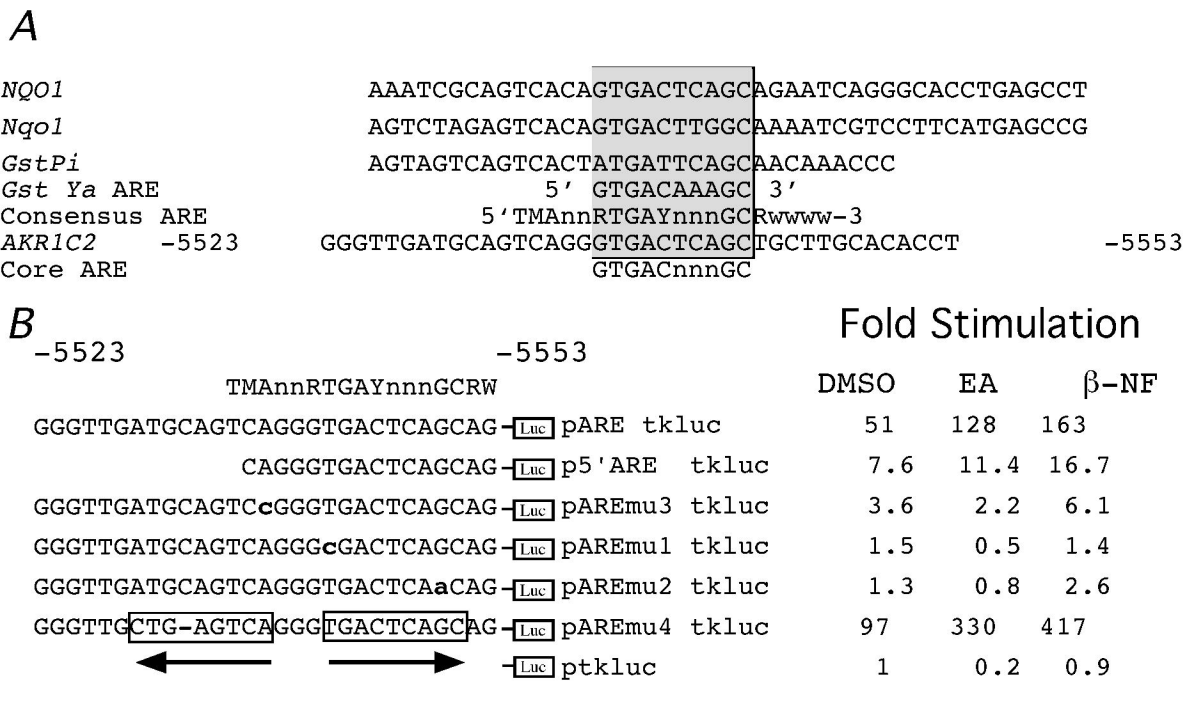

A
NQO1 AAATCGCAGTCACAGTGACTCAGCAGAATCAGGGCACCTGAGCCT
Nqo1 AGTCTAGAGTCACAGTGACTTGGCAAAATCGTCCTTCATGAGCCG
GstPi AGTAGTCAGTCACTATGATTCAGCAACAAACCC
Gst Ya ARE 5' GTGACAAAGC 3'
Consensus ARE 5'TMAnnRTGAYnnnGCRwwww-3
AKR1C2 -5523 GGGTTGATGCAGTCAGGGTGACTCAGCTGCTTGCACACCT -5553
Core ARE GTGACnnnGC
B
-5523 -5553
TMAnnRTGAYnnnGCRW
GGGTTGATGCAGTCAGGGTGACTCAGCAG-Luc pARE tkluc
CAGGGTGACTCAGCAG-Luc p5'ARE tkluc
GGGTTGATGCAGTCcGGGTGACTCAGCAG-Luc pAREmu3 tkluc
GGGTTGATGCAGTCAGGGcGACTCAGCAG-Luc pAREmu1 tkluc
GGGTTGATGCAGTCAGGGTGACTCAaCAG-Luc pAREmu2 tkluc
GGGTTGCTG-AGTCAGGGTGACTCAGCAG-Luc pAREmu4 tkluc
-Luc ptkluc
Fold Stimulation
DMSO EA β-NF
51 128 163
7.6 11.4 16.7
3.6 2.2 6.1
1.5 0.5 1.4
1.3 0.8 2.6
97 330 417
1 0.2 0.9

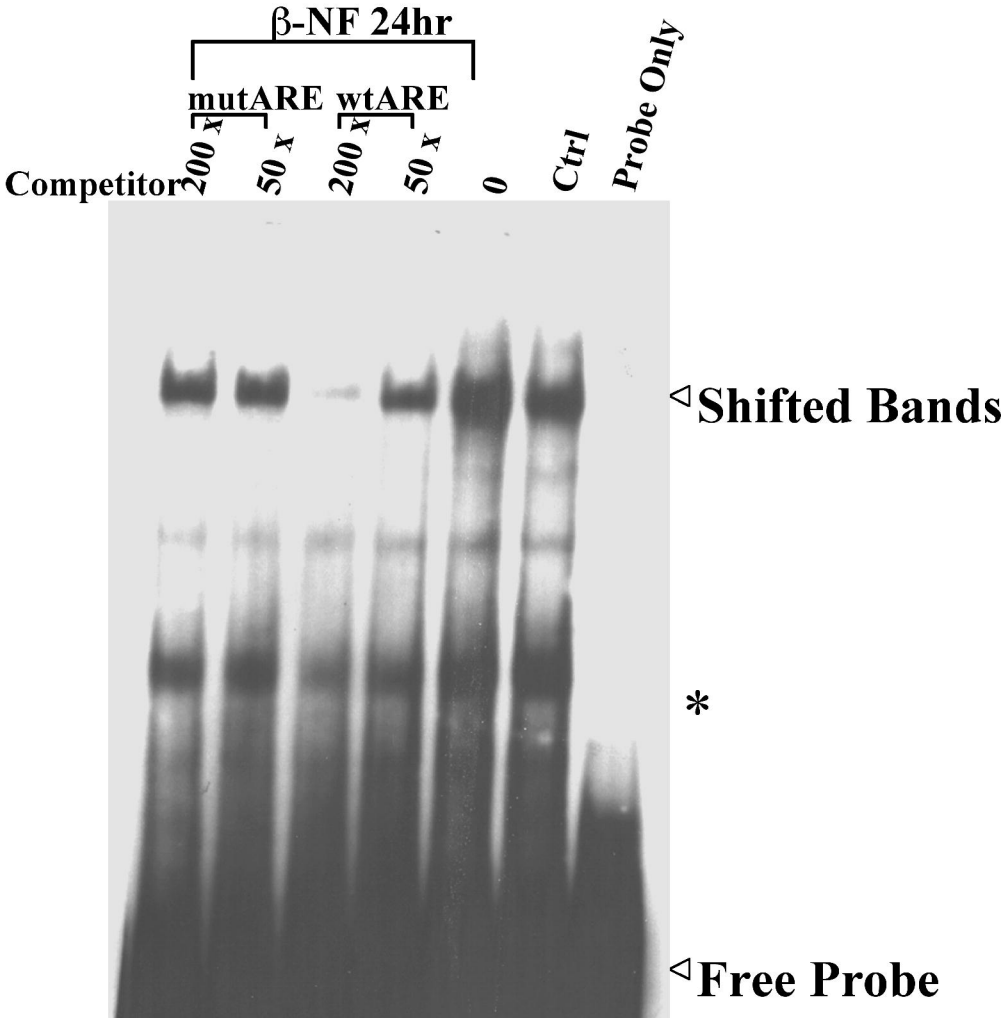
Figure 4

Figure 5

A

```
ARE Probe 1     TTGATGCAGTCAGGGTGACTCAGCAGCT   28
                ||||||||||||||||||||||||||||
4939287         TTGATGCAGTCAGGGTGACTCAGCAGCT   4939260 AKR1C2
                ||||||||||||||||||||||||||||
4991586         TTGATGCAGTCAGGGTGACTCAGCAGCT   4991613 AKR1C1
```

B



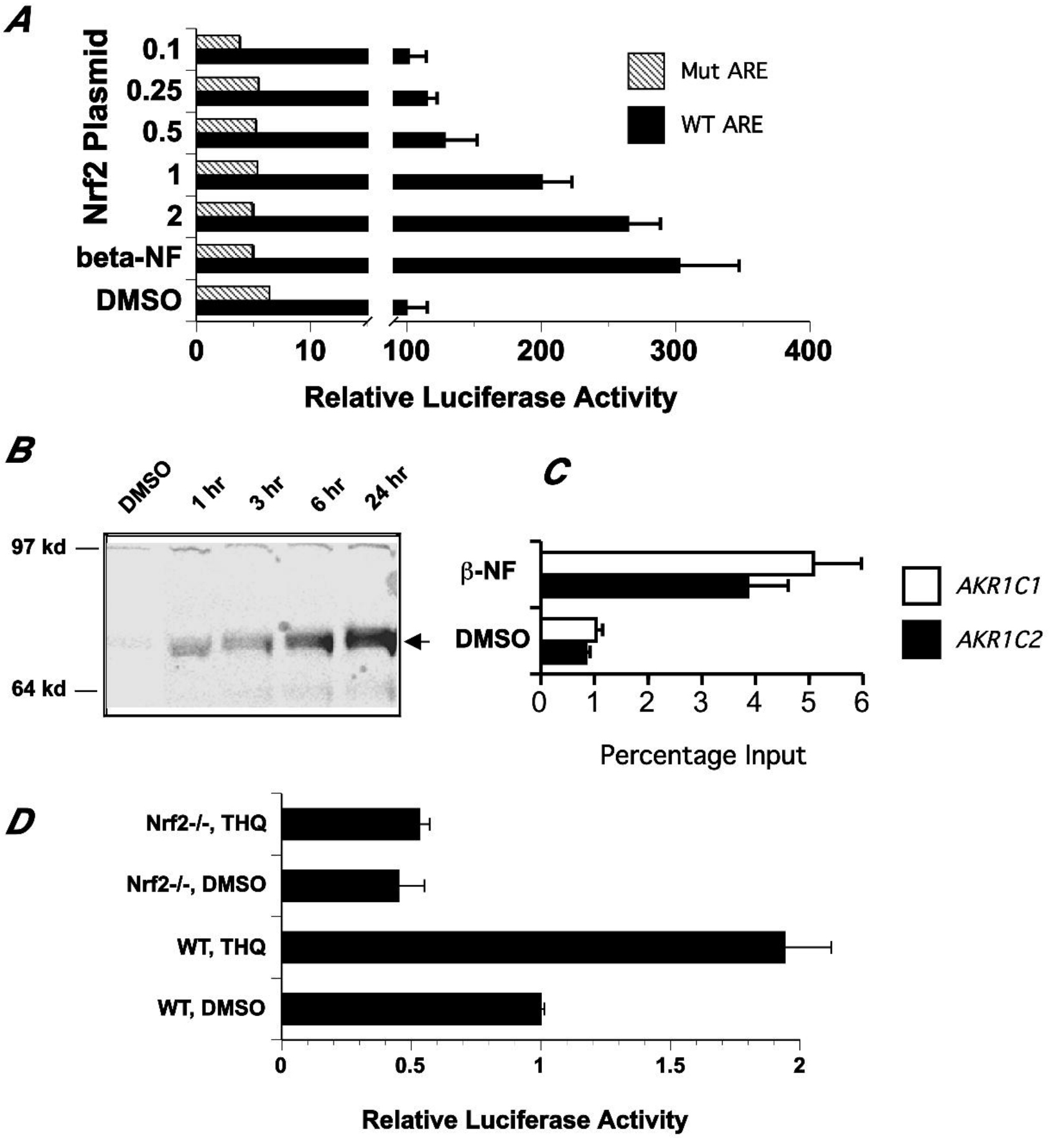

A
Nrf2 Plasmid
0.1
0.25
0.5
1
2
beta-NF
DMSO
Mut ARE
WT ARE
0
10
100
200
300
400
Relative Luciferase Activity
B
DMSO
1 hr
3 hr
6 hr
24 hr
97 kd
64 kd
C
β-NF
DMSO
AKR1C1
AKR1C2
0
1
2
3
4
5
6
Percentage Input
D
Nrf2-/-, THQ
Nrf2-/-, DMSO
WT, THQ
WT, DMSO
0
0.5
1
1.5
2
Relative Luciferase Activity

Figure 6

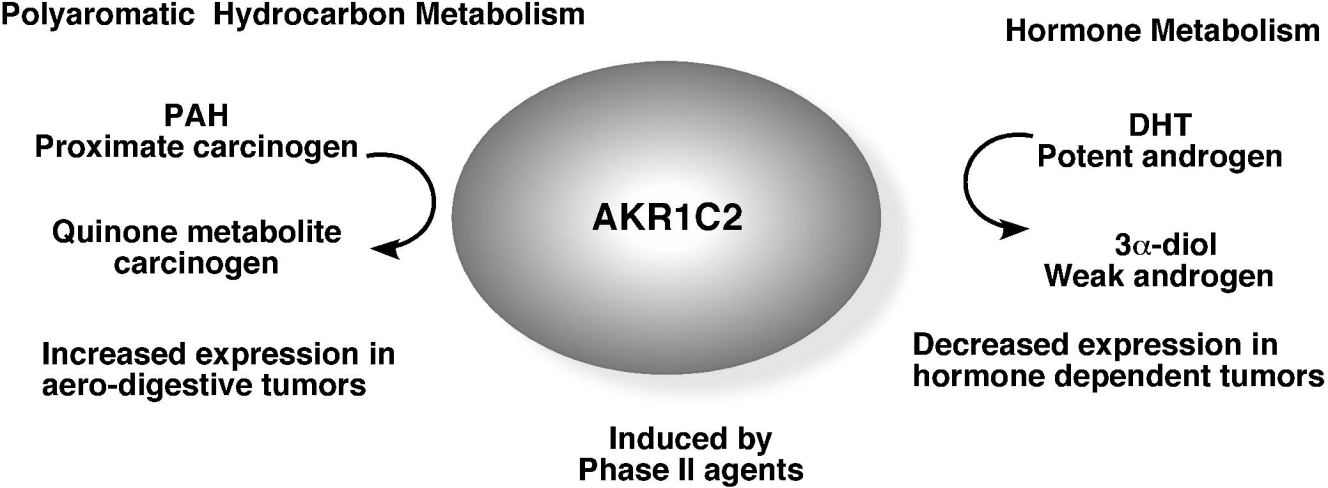

Polyaromatic Hydrocarbon Metabolism
Hormone Metabolism
PAH
Proximate carcinogen
Quinone metabolite
carcinogen
Increased expression in
aero-digestive tumors
AKR1C2
DHT
Potent androgen
3α-diol
Weak androgen
Decreased expression in
hormone dependent tumors
Induced by
Phase II agents

Figure 7