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**RETINALDEHYDE IS A SUBSTRATE FOR HUMAN ALDO-KETO REDUCTASES OF THE 1C SUBFAMILY**

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## SYNOPSIS

Human aldo-keto reductase 1C proteins (AKR1C1-AKR1C4) exhibit relevant activity with steroids, regulating hormone signaling at pre-receptor level. We here investigate the activity of the four human AKR1C enzymes with retinol and retinaldehyde. All the enzymes except AKR1C2 showed retinaldehyde reductase activity with low  $K_m$  values ( $\sim 1 \mu\text{M}$ ). The  $k_{\text{cat}}$  values were also low ( $0.18 - 0.6 \text{ min}^{-1}$ ), except for AKR1C3 with 9-*cis*-retinaldehyde whose  $k_{\text{cat}}$  was remarkably higher ( $13 \text{ min}^{-1}$ ). Structural modeling of the AKR1C complexes with 9-*cis*-retinaldehyde indicated a distinct conformation of Trp-227, originated by changes in residue 226, which may contribute to activity differences. This was partially supported by the kinetics of the AKR1C3 R226P mutant. Retinol/retinaldehyde conversion, combined with the use of the inhibitor flufenamic acid, indicated a relevant role of endogenous AKR1Cs in retinaldehyde reduction in MCF-7 breast cancer cells. Overexpression of AKR1C proteins depleted retinoic acid (RA) *trans*-activation in HeLa cells treated with retinol. Thus, AKR1Cs may decrease RA levels *in vivo*. Finally, by using lithocholic acid as an AKR1C3 inhibitor and UVI2024 as an RA receptor antagonist, we provide evidence that the pro-proliferative action of AKR1C3 in HL-60 cells involves the RA signaling pathway and that this is in part due to the retinaldehyde reductase activity of AKR1C3.

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**Short Title:** Specificity of human aldo-keto reductases 1C with retinaldehyde

**Key words:** aldo-keto reductase, retinaldehyde, retinoic acid, retinol, proliferation

**Abbreviation Footnote:**

ADH, alcohol dehydrogenase; AKR, aldo-keto reductase; AKR1B1, human aldose reductase; AKR1B10, human small intestine aldose reductase; AKR1C1-AKR1C4, human aldo-keto reductase 1C members; AKR1C7, bovine prostaglandin F synthase; MCF-7, breast adenocarcinoma epithelial cell line; MDR, medium-chain dehydrogenase/reductase; PPAR $\gamma$ , peroxisome proliferator-activated receptor gamma; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; SDR, short-chain dehydrogenase/reductase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

## INTRODUCTION

Retinol (vitamin A) and its derivatives retinaldehyde and retinoic acid (RA) are essential for the formation and maintenance of many body tissues, such as skin, bone, and vasculature, as well as for the visual cycle (retinaldehyde) and immune function. They also play a role in reproduction, embryonic growth and development. RA is a morphogen and a key factor in the development of different vertebrate tissues and organs due to its ability to promote differentiation and apoptosis (for review [1]). RA has also a role in several pathological conditions, such as skin diseases, premature birth and rheumatoid arthritis. Various human cancers have altered retinoid metabolism and low RA levels, which favor tumor progression [2].

Conversion of retinol to RA requires several oxidative steps (Scheme 1). Members of three oxidoreductase superfamilies have been implicated in the reversible oxidation of retinol to retinaldehyde [3, 4]. The participation of cytosolic alcohol dehydrogenases (ADHs) from the medium-chain dehydrogenase/reductase (MDR) superfamily and of microsomal short-chain dehydrogenases/reductases (SDRs) has been deeply studied [4]. More recently, some members of the aldo-keto reductase (AKR) superfamily, such as chicken AKR1B12 [5], human aldose reductase (AKR1B1) and human small intestine aldose reductase (AKR1B10) [6], were defined as a novel group of cytosolic enzymes that could contribute to retinoid redox conversions. Comparison of the kinetic properties between members of the three superfamilies indicates similar  $K_m$  values for retinol and retinaldehyde, while differences in  $k_{cat}$  values determine the catalytic efficiency. Based on the cofactor specificity, ADHs (NAD-dependent) may work in the oxidative direction, AKRs (NADPH-dependent) in the reductive direction, while SDRs show examples of both specificities [3, 4].

AKR1C1-AKR1C4 (or human hydroxysteroid dehydrogenases, HSD) are cytosolic NADP-dependent monomeric dehydrogenases composed of 323 amino acid residues. They share 86% sequence identity and correspond to: AKR1C1 (20 $\alpha$ (3 $\alpha$ )-HSD); AKR1C2 (type 3 3 $\alpha$ -HSD or bile-acid binding protein); AKR1C3 (type 2 3 $\alpha$ -HSD or type 5 17 $\beta$ -HSD); and AKR1C4 (type 1 3 $\alpha$ -HSD) [7]. The individual enzymes show different biochemical properties, such as substrate and inhibitor specificities, and expression patterns [8, 9]. The vast range of substrates used by human AKR1C members comprises steroids, prostaglandins, bile acids, *trans*-dihydrodiols of polycyclic aromatic hydrocarbons, and several endogenous and xenobiotic aldehydes and ketones. They are phase I drug-metabolizing enzymes for a variety of carbonyl-bearing compounds [7, 10, 11].

Transient transfection in COS-1 cells indicates that AKR1C enzymes function as ketosteroid reductases, transforming several steroid hormones or their precursors [7]. Thus, human AKR1C and members of other AKR subfamilies are involved in the pre-receptor regulation of nuclear (steroid hormone, peroxisome proliferator-activated, and orphan) receptors by controlling the local concentrations of their lipophilic ligands. AKR1C3 is one of the most interesting enzymes, as it participates in androgen, estrogen and prostaglandin regulation, favoring pro-proliferative signals [12]. *AKR1C* genes are highly conserved in structure and may be transcriptionally regulated by different hormones and oxidative stress [13].

Increasing evidence strongly supports the involvement of AKR1Cs, and especially AKR1C3, in cancer development. They are found elevated in different cancer types [14-16]. They can participate in hormone-dependent malignancies by altering the steroid hormone levels [17, 18]. AKR1C overexpression promotes apoptosis resistance and cell survival, and prevents differentiation [14]. AKR1Cs are involved in cancer

chemotherapeutic drug resistance and tobacco-related carcinogenesis [10, 19]. Finally, AKR1C3 inhibitors exhibit anti-neoplastic activity [20], and down-regulation of AKR1Cs by small interfering RNA led to significantly reduced cell viability [14, 21]. Several mechanisms have been proposed for the pro-proliferative activity of AKR1Cs, including the prostaglandin metabolism by AKR1C3, which eliminates the natural ligand for the peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) resulting in decreased differentiation. Other mechanisms, however, may exist [20].

Our initial work on the participation of AKR1Bs in the modulation of the anti-proliferative RA signaling pathway [22, 23] prompted us to investigate here the activity of AKR1Cs with retinol and retinaldehyde and their possible contribution in this pathway (Scheme 1). Moreover, evidence exists on a relationship between AKR1Cs and retinoids. Thus, AKR1C enzymes are overexpressed in prostate and breast cancers [18], where RA levels are low [2]. Moreover, AKR1C3 overexpression has been shown to be induced by RA in HL-60 leukemia cells [24], and AKR1C3 inhibitors promote differentiation of HL-60 cells in response of RA treatment, while overexpression of AKR1C3 reciprocally desensitizes the cells to RA [25].

In the present study human AKR1Cs have been characterized for their ability to metabolize RA precursors, retinol and retinaldehyde. In addition, the structural determinants of the different specificity for retinol/retinaldehyde within the AKR1C members have been investigated. Finally, using inhibitors and a RA receptor (RAR) antagonist, we have studied the role of AKR1C3 in mediating RA-dependent cell proliferation.

## EXPERIMENTAL

*Cloning, expression and purification of AKR1C1-AKR1C4.* AKR1C1 and AKR1C4 cDNA sequences were obtained from the Mammalian Gene Collection (MGC) clones provided by LGC Promochem (MGC:8954 IMAGE:3877178 for AKR1C1; MGC:22581 IMAGE:4734943 for AKR1C4) and were PCR amplified using two primers containing restriction sites (*Eco*RI and *Xho*I for AKR1C1; *Sal*I and *Xho*I for AKR1C4) at their 5' ends. Double digestion with these enzymes allowed cloning into the prokaryotic expression vector pGEX-4T-2 (GE Healthcare). AKR1C2 cDNA was obtained from MGC clones provided by Open Biosystems (MGC:70847 IMAGE:3877666). For AKR1C3, a pBluescript SK+ vector containing its cDNA was kindly donated by Dr. T. Nagase, Kazusa DNA Research Institute, Chiba, Japan. Both AKR1C2 and AKR1C3 cDNA sequences were PCR amplified and cloned into prokaryotic expression vector pET-30 Xa/LIC (EMD Biosciences), as indicated by the manufacturer.

For transfection experiments, AKR1C3 and AKR1C4 cDNAs were subcloned into mammalian expression vector pcDNA3.1/V5-TOPO (Invitrogen), as indicated by the manufacturer. AKR1C1 was transfected as pCMV-Sport6-AKR1C1 (MGC:8954 clone), and pcDNA3.1/V5-TOPO-LacZ was included as a control with the corresponding TOPO TA Expression Kit (Invitrogen). Primer sequences used for subcloning are described in the Supplementary data.

AKR1C1-AKR1C4 were expressed and purified as described [3]. *E. coli* BL21 cells were transformed with pGEX-4T-2 or pET-30 Xa/LIC containing the cDNA for each enzyme. Liquid cultures in 2xYT medium were incubated at 25°C until OD<sub>600</sub> = 0.6 was reached. For induction, 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside (IPTG, Roche Molecular Biochemicals) was added and cells were further incubated at 22°C for



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15 h. The AKR-GST protein constructs were purified using the affinity resin Glutathione-Sepharose 4B (GE Healthcare). After washing with phosphate-buffered saline buffer (PBS), elution of AKR enzymes was performed by thrombin digestion (10 U/mg protein, GE Healthcare) in the same buffer, for 15 h at room temperature. pET-30 Xa/LIC provides a (His)<sub>6</sub> tag in the N-terminal of enzyme. AKRs were purified by affinity chromatography using Chelating Sepharose<sup>®</sup> Fast Flow resin (GE Healthcare) with bound Ni<sup>2+</sup>. After washing with 60 mM imidazole (Sigma), 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9, the enzyme was eluted by 0.06-1 M imidazole gradient in 0.5 M NaCl, 20 mM Tris/HCl, pH 7.9. The absorbance at 280 nm of each fraction was analyzed using a Varian Cary 400 spectrophotometer. Finally, the purification buffer was changed with Amicon ultra device (Millipore) to 100 mM potassium phosphate, pH 7.0.

*Site-directed mutagenesis, expression and purification of AKR1C3 R226P and R226Q mutants.* AKR1C3 R226P and R226Q mutants were obtained by using wild-type AKR1C3 cDNA cloned into pET30-Xa/LIC (Novagen) as a template, based on the QuikChange<sup>®</sup> Site-Directed Mutagenesis Kit (Stratagene).

We designed two sets of two primers each to introduce each mutation: 5'-TCTCAACGAGACAAACCATGGGTGGACCCGAACCTCCC-3' and 5'-GTTCCGGGTCCACCCATGGTTTGTCTCGTTGAGATCCC-3' for AKR1C3 R226P; 5'-TCTCAACGAGACAAACAATGGGTGGACCCGAACCTCCC-3' and 5'-GTTCCGGGTCCACCCATTGTTTGTCTCGTTGAGATCCC-3' for AKR1C3 R226Q. Mutated nucleotides are underlined. The reactions were performed in a DNA thermal cycler (MJ Research) with *Pfu* Turbo DNA polymerase (Stratagene). PCR products were incubated with *Dpn*I at 37°C for 60 min. This treatment ensured the digestion of the *dam*-methylated parental strand. The resulting nicked circular mutagenic strands were transformed into *E. coli* BL21, where bacterial DNA ligase repaired the nick and allowed normal replication to occur. Before expression, the mutated DNAs were completely sequenced to ensure that unwanted mutations were absent. Expression and purification of AKR1C3 R226P and R226Q mutants were performed as indicated above.

*Protein analysis.* SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by the Coomassie<sup>®</sup> Brilliant Blue (Sigma) stain technique, was used to check protein purity. Protein concentration was determined by a dye binding assay (Bio Rad).

*Enzyme Kinetics.* Activity under standard conditions was measured spectrophotometrically [9] to follow the purification procedure and to check enzyme concentration before each kinetic experiment. Standard assay was performed with 75 µM androsterone (Sigma) for AKR1C4, 25 µM 9,10-phenanthrenequinone (Sigma) or 1 mM 1-acenaphthenol (Sigma) for AKR1C1, AKR1C2 and AKR1C3, in 100 mM potassium phosphate, pH 7.0, at 25°C. Reduction of androsterone and 9,10-phenanthrenequinone used 0.2 mM NADPH as a cofactor, while oxidation of 1-acenaphthenol was measured with 2.3 mM NAD<sup>+</sup>.

Activity with retinol and retinaldehyde (Sigma) in the presence of bovine serum albumin was performed in 90 mM potassium dihydrogen phosphate, 40 mM potassium chloride, pH 7.4, at 37°C, in siliconized glass tubes as reported [3], and the products were analyzed by HPLC. Kinetic constants were expressed as the mean ± S.E.M of at least three independent determinations. A saturating concentration of cofactor, 0.2 mM NADPH for retinaldehyde reduction and 2.3 mM NADP<sup>+</sup> for retinol oxidation, was used.

**HPLC analysis.** After organic extraction [3], retinol and retinaldehyde were separated by chromatography on a Spherisorb S3W column (4.6 × 100 mm, Waters) in hexane:methyl-*tert*-butyl ether (96:4, v/v) mobile phase, at a flow rate of 2 ml/min using Waters Alliance 2695 HPLC. Elution was monitored at 350 nm with a Waters 2996 photodiode array detector. Commercially available standards (Sigma) were employed to identify the retinoid compounds.

**Computer modeling.** Initial structures of the ternary complexes of AKR1C1 (PDB 1MRQ), and AKR1C3 (PDB 1RY0) with NADP<sup>+</sup> and 9-*cis*-retinaldehyde, as well as the ternary complex with all-*trans*-retinaldehyde, were built by homology with the closely-related AKR1B10 ternary complex (PDB 1ZUA), which was previously studied in our group [22]. As a first approximation, the substrate positions were refined with the AUTODOCK 3.0 program to optimize the protein-substrate interaction energy, keeping the protein fixed (see [22] for methodological references). To allow the substrate to accommodate into the binding pocket, the resulting structures were fully relaxed and submitted to molecular dynamics (MD) simulation using the AMBER7 program. The Cornell *et al.* force-field for the protein and the TIP3P for the water solvent were used. Point charges for the substrate were derived according to the RESP procedure. The following protocol was used in the MD simulations: (i) solvent equilibration and energy minimization of the whole system (ii) 100 ps using constant particle number, volume and temperature (NVT) dynamics at 100 K, fixing the protein backbone, the substrate and the cofactor; (iii) 100 ps NVT dynamics at 100 K, 200 K and 300 K, successively; (iv) one ns under NPT dynamics at 300 K and 1 atm. Once the system was equilibrated, the simulations were extended by 9 ns for AKR1C3:all-*trans*-retinaldehyde, AKR1C3:9-*cis*-retinaldehyde and AKR1C1:all-*trans*-retinaldehyde using the NAMD v2.6 program. Protein-substrate interaction energies per residue were computed using the MM-GBSA program included in the AMBER7 package.

**Retinaldehyde reductase activity in MCF-7 cells.** MCF-7 breast carcinoma cells were grown on 6-well plates in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Incubation was performed at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>:95% air.

To study retinol/retinaldehyde transformation by endogenous enzymatic activities, cells were incubated in the presence of retinaldehyde or retinol. The effect of an AKR1C inhibitor was analyzed by adding flufenamic acid (Sigma) in dimethyl sulfoxide (DMSO, never exceeding 0.1% of the medium volume). After 30-min incubation, cells were rinsed twice in ice-cold PBS and harvested by scraping into 200 µl 0.002% (w/v) SDS. Cell suspensions were stored frozen at -80°C. The thawed suspensions were sonicated in an ice bath to complete cell lysis. Retinol and retinaldehyde were extracted and analyzed as described above.

Retinol/retinaldehyde conversion was expressed as the percentage of retinaldehyde (or retinol) that was reduced to retinol (or oxidized to retinaldehyde) from the retinaldehyde (or retinol) that had entered the cell. Thus the measure for the conversion of retinaldehyde is:

$$100 \times (\text{pmol cell-retinol} + \text{pmol medium-retinol} / (\text{pmol cell-retinol} + \text{pmol medium-retinol} + \text{pmol cell-retinaldehyde}))$$

**Analysis of RA with a luciferase reporter vector in HeLa cells.** HeLa cells were grown on 24-well plates in Dulbecco's modified Eagle's medium supplemented with 10% (v/v)



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FBS (Invitrogen). For transient transfection, 0.75  $\mu$ g of each vector were added and mixed with Lipofectamine Plus™ (Invitrogen) according to the manufacturer's instructions. After 4 h, fresh medium was added and cells were incubated overnight.

pDR5/RARE-Luc reporter plasmid (PathDetect® *cis*-Reporting Systems, Stratagene), which contains a RA Response Element (RARE), (AGGTCANNNNN)<sub>5</sub>, upstream of the luciferase gene, or pCIS-CK, a negative control reporter vector containing the luciferase gene without *cis*-acting elements, was transfected into HeLa cells, along with the corresponding AKR expression vector (Supplementary data, Table S1). Baseline in *trans*-activation assays was determined by double transfection with pDR5/RARE-Luc plasmid (pDR5-Luc) and pcDNA3.1/V5-His-TOPO/LacZ (pLacZ) (Invitrogen). This control was performed to discard unspecific *trans*-activation effects due to plasmid cotransfection, which can potentially affect reporter gene expression [26]. Then, cells were incubated with 10  $\mu$ M all-*trans*- or 9-*cis*-retinol for 24 h (to allow the cells to synthesize RA) and lysed with the Reporter Lysis Buffer (Promega). As a positive control, cells were treated with 1  $\mu$ M all-*trans*- or 9-*cis*-RA for 24 h. Luciferase activity was quantified by measuring luminescence with the Luciferase Assay System with Reporter Lysis Buffer kit (Promega). Data were normalized as relative light units (RLU) per mg of protein [23, 27]. Protein concentration was determined by a dye binding assay (Bio Rad).

*Assessment of Cell Proliferation in HL-60 cells.* HL-60 cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) heat-inactivated FBS (Invitrogen). To study cell proliferation, experimental cultures were seeded at  $1 \times 10^5$  cells/ml in 96-well plates and incubated with the recommended medium for 3 days along with different treatments. Retinol and retinaldehyde stock solutions were prepared in ethanol, while lithocholic acid (an AKR1C3 inhibitor [28]) and UVI2024 (an RAR antagonist [29]) (Sigma) were diluted in DMSO. The concentration of ethanol and DMSO were set at 0.3% and 0.2 % (v/v), respectively, in the culture medium. Then, XTT reagent (Roche) was added, incubated for 90 min at 37°C, and optical density was measured as indicated by the manufacturer.

*Statistical analysis.* Data were analyzed with Sigmapstat (Systat Software Inc.) with one-way analysis of variance. Statistical significance was set at a *p* value of less than 0.05.

## RESULTS

*Kinetic analysis of human AKR1C enzymes with retinol and retinaldehyde.* AKR1C1-AKR1C4 were purified to homogeneity, as verified by SDS-PAGE analysis and Coomassie Blue staining. Specific activities with 9,10-phenanthrenequinone were 0.6, 2.0 and 1.5 U/mg for AKR1C1, AKR1C2 and AKR1C3, respectively. Specific activity of AKR1C4 was 0.2 U/mg with androsterone. These values are comparable to those previously reported [9, 30].

Kinetic constants (Table 1) were determined in detergent-free solutions and by HPLC analysis under well-established conditions [3]. With all-*trans*-retinaldehyde, AKR1C3 and AKR1C4 exhibited low *K<sub>m</sub>* and *k<sub>cat</sub>* values, while AKR1C1 displayed very low activity and AKR1C2 was inactive. None of the enzymes could oxidize all-*trans*-retinol.

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With the 9-*cis*-retinoid isomers, AKR1C2 was inactive, but kinetics could be studied with all other enzymes. AKR1C3 was by far the most efficient 9-*cis*-retinaldehyde reductase with a  $k_{\text{cat}}/K_m$  value 50- to 60-fold higher than those of AKR1C1 and AKR1C4. While  $K_m$  values were similarly low (0.4-0.8  $\mu\text{M}$ ) for the three enzymes, the differences were due to  $k_{\text{cat}}$  (Table 1). AKR1C3 was the only AKR1C active with 9-*cis*-retinol, although with a very low  $k_{\text{cat}}$  value (0.26  $\text{min}^{-1}$ ).

AKR1B10 kinetic constants are also included to compare subfamily 1C with the up to now reportedly most active AKR with retinaldehyde. AKR1B10 and AKR1C3 are the best retinaldehyde reductases but with remarkably distinct specificities. AKR1B10 is the most efficient AKR with all-*trans*-retinaldehyde, with a 100-fold higher catalytic efficiency than AKR1C3, while AKR1C3 is the best 9-*cis*-retinaldehyde reductase, with a 25-fold higher catalytic efficiency than AKR1B10.

*Computer modeling of 9-cis-retinaldehyde and all-trans-retinaldehyde bound to AKR1C1 and AKR1C3.* Structural features that could explain the kinetic differences with retinaldehyde isomers were investigated by computer modeling. Models of the ternary complexes of AKR1C1 and AKR1C3 with NADP<sup>+</sup> and either the 9-*cis* or all-*trans* retinaldehyde substrate, were built using a combination of docking and MD simulations (Fig. 1)

The overall structure of each of the four complexes did not change significantly during the MD simulations (average root mean square deviation, r.m.s.d, value for backbone atoms was less than 1.3 Å), and the substrates remained bound to the protein. However, slight but yet important changes were observed when comparing the structure of AKR1C1 and AKR1C3 complexes with 9-*cis*-retinaldehyde. As clearly seen in Figure 1A, the position and the orientation of the bound 9-*cis*-retinaldehyde substrate, especially of the cyclohexene ring, are different for each complex, which results in distinct binding modes. Analysis of the ligand-protein interactions (Fig. 1B and C, and Supplementary data, Fig. S1) provided the main residues involved in the binding of 9-*cis*-retinaldehyde to AKR1C1 and AKR1C3 (Table 2). Differences are observed in both positions and residues involved. Some non-conserved residues lining the ligand are hydrophobic and larger in AKR1C1 but hydrophilic and smaller in AKR1C3: Ile→Ser-129, Leu→Ser-308, Ile→Ser-310. In addition, a close view of the binding pocket of the AKR1C1:9-*cis*-retinaldehyde and AKR1C3:9-*cis*-retinaldehyde complexes (Fig. 1B and C) shows a remarkable difference in the orientation of Trp-227, located in loop B. In AKR1C1:9-*cis*-retinaldehyde, loop B is displaced towards the entrance of the binding pocket (Fig. 1A) and, therefore, Trp-227 is interacting with the retinaldehyde cyclohexene ring (Fig. 1B). In contrast, loop B in AKR1C3:9-*cis*-retinaldehyde faces the inner part of the binding pocket, and Trp-227 interacts with the isoprenoid chain of the compound (Fig. 1C). Therefore, the orientation of Trp-227 affects the substrate positioning and this could be at the origin of the observed kinetic differences between AKR1C1 and AKR1C3 with 9-*cis*-retinaldehyde. In the ternary complexes of the AKR1C1 and AKR1C3 enzymes with all-*trans*-retinaldehyde, the substrate molecule adopts a very similar position and orientation (Fig. 1A and Supplementary data Fig. S1), which is different from the 9-*cis*-isomer orientation, probably due to the larger volume of the former. In addition, the orientation of Trp-227 and loop B in both AKR1C1:all-*trans*-retinaldehyde and AKR1C3:all-*trans*-retinaldehyde complexes is very similar to those in the AKR1C1:9-*cis*-retinaldehyde model. This is consistent with the low activity of all the enzymes, including AKR1C3, with the all-*trans*-retinaldehyde substrate. Trp-227 had been proposed to be critical in positioning the substrate for a productive catalysis in AKR1Cs [31, 32]. Even though Trp-227 is conserved in all AKR1C

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enzymes, its preceding residue (position 226) is not. AKR1C1, having a proline at position 226, shows a more rigid and tighter loop B than AKR1C3, which has an arginine. Therefore, residue 226, by orientating Trp-227, could be determinant for the 9-*cis*-retinaldehyde positioning into the active site, and in turn, for defining substrate specificity in the human AKR1Cs enzymes.

*Site-directed mutagenesis.* To investigate the contribution of the conformational changes imposed by residue 226 on the remarkable kinetic differences between AKR1C1 and AKR1C3, mutants AKR1C3 R226P and R226Q were prepared, and their kinetics were analyzed (Table 1). The AKR1C3 R226P mutant, built to mimic the poorly retinaldehyde-active AKR1C1, changed the kinetic constants in the direction predicted by our models. Activities with 9-*cis*-retinaldehyde revealed a 3-fold decrease in the  $k_{\text{cat}}$  value, while the  $K_m$  value did not vary. A similar change was observed towards all-*trans*-retinaldehyde, with a 2.3-fold decrease of the  $k_{\text{cat}}$  value. The AKR1C3 R226Q mutant exhibited changes in both  $K_m$  and  $k_{\text{cat}}$  values, resulting in a decrease of the catalytic efficiency similar as that of the AKR1C3 R226P mutant. In contrast, kinetics with 9,10-phenanthrenequinone did not significantly change in the mutants (Table 1), emphasizing the distinct binding of the retinaldehyde substrates.

*AKR1Cs metabolize retinaldehyde in MCF-7 breast carcinoma cells.* AKR1C3 is expressed at high levels in mammary gland [9] and it is also expressed in breast carcinoma epithelial cell line MCF-7, both at mRNA and protein levels [33]. MCF-7 cells are also known to express AKR1C1 (showing very low activity with retinaldehyde isomers) and AKR1C2 (inactive) [33, 34]. Thus, MCF-7 was chosen as a model to study cellular retinaldehyde reductase activity by endogenous AKR1Cs. Cells were analyzed by immunoblotting against AKR1C1 and AKR1C3 antibodies which confirmed the expression of AKR1C1 and AKR1C3 (Supplementary data, Fig. S2).

MCF-7 cells were incubated, in separate experiments, with all-*trans* and 9-*cis*-retinaldehyde and their retinoid content was analyzed. No production of retinyl esters or RA was detected after 30-min incubation, but a significant conversion to retinol was observed (Fig. 2). MCF-7 showed high capacity for the reduction of all-*trans*-retinaldehyde (90% conversion of the retinaldehyde incorporated into the cell) and a lower capacity for 9-*cis*-retinaldehyde reduction (30% conversion). The contribution of AKR1Cs to retinaldehyde metabolism was estimated with the use of flufenamic acid, an effective inhibitor for AKR1C1-1C3 [35]. Considering the extremely low activity of AKR1C1 and the inactivity of AKR1C2 with retinaldehyde, it could be assumed that, in the MCF-7 cellular model for endogenous retinaldehyde reductase activity, flufenamic acid would act mainly as an AKR1C3 inhibitor. Titration experiments (Supplementary data, Fig. S3) were used to check the dose for which activity differences could be observed. With respect to all-*trans*-retinaldehyde conversion (Fig. 2), it decreased by 30% upon incubation with flufenamic acid, a percentage that would correspond to the AKR1Cs activity, while the remaining percentage is therefore due to other reductases [36].

With regard to 9-*cis*-retinaldehyde (Fig. 2), the inhibition by flufenamic acid indicates that most of the 9-*cis*-retinaldehyde reductase activity (more than 80%) of the MCF-7 cells is provided by the AKR1Cs. The contribution of AKR1C3 should be higher than that of AKR1C1, due to the higher catalytic efficiency of the former. Incubation with 10  $\mu\text{M}$  9-*cis*-retinol resulted in a very low retinaldehyde production (1%), nearly the same percentage as that for the incubation in the presence of

flufenamic acid (Fig. 2). Consistent with previous reports [7], the studied AKR forms appear to function preferably as reductases in whole cells.

*Overexpression of AKR1Cs reduces RA trans-activation in HeLa cells.* HeLa cells were transiently transfected with a RARE reporter (RAR can bind either all-*trans* or 9-*cis*-RA) alone or in combination with *AKR1C1*, *AKR1C3* or *AKR1C4* cDNA, subsequently treated with all-*trans* or 9-*cis*-retinol for 24 h (to allow the cells to synthesize RA), and luciferase activity was measured as reported [23]. For all-*trans*-retinol treatment, transfection of *AKR1C1*, barely active with the all-*trans* isomer (Table 1), did not affect *trans*-activation. In contrast, *AKR1C3* and *AKR1C4* transfection produced a 2-fold decrease in *trans*-activation (Fig. 3A). For the 9-*cis*-retinol treatment, *AKR1C1* caused a 2-fold decrease in *trans*-activation, while *AKR1C3* and *AKR1C4* overexpression resulted in a 20-fold decrease (Fig. 3B).

Thus, the *in vivo* activities of *AKR1C3* and *AKR1C4* with both retinaldehyde isomers, and that of *AKR1C1* with the 9-*cis*-isomer, produced inhibition of RA biosynthesis. In general, the results of *in vivo* experiments, *e.g.*, the retinol/retinaldehyde metabolism in MCF-7 cells and the RA *trans*-activation in HeLa cells, were consistent with the kinetic constants of the AKR1C enzymes (Table 1).

*RAR-dependent decrease of HL-60 leukemia cell line proliferation by AKR1C3 inhibition.* The relationship between the retinaldehyde reductase activity of *AKR1C3* and cell proliferation, through decrease of RA biosynthesis, was studied in HL-60 leukemia cell line. These cells contain *AKR1C3* [25] and the enzymatic systems for RA synthesis [37, 38]. RA induces their differentiation [37] and *AKR1C3* overexpression has been shown to desensitize HL-60 cells to RA and vitamin D<sub>3</sub>, acting as a suppressor of cell differentiation [25]. In the present work, cells were treated for 3 days, in separate experiments, with 9-*cis*-retinol, *AKR1C3* inhibitor lithocholic acid [28] or RAR antagonist UVI2024 (or BMS493) [29], and cell proliferation was followed with the XTT assay (Fig. 4). In the control without 9-*cis*-retinol, lithocholic acid was not able to decrease cell proliferation. Addition of 1 and 2.5  $\mu$ M 9-*cis*-retinol barely showed an anti-proliferative effect, while a significant decrease was detected at 5 and 10  $\mu$ M 9-*cis*-retinol. When 9-*cis*-retinol and lithocholic acid were added together, the proliferation decrease was significantly enhanced, especially at 10  $\mu$ M 9-*cis*-retinol, in which lithocholic acid provoked a 23% decrease with respect to the treatment with 9-*cis*-retinol alone. The RAR antagonist was used to check whether the RAR pathway was involved in this decrease. In the control without 9-*cis*-retinol, no significant changes were detected by treatments with lithocholic acid alone or by lithocholic acid along with the antagonist. However, in all the experiments in the presence of 9-*cis*-retinol the proliferation decrease corresponding to the *AKR1C3* inhibitor treatment was completely rescued by the RAR antagonist (Fig. 4). This suggests that *AKR1C3* inhibition results in a decrease of cell proliferation, which is specifically triggered through the RAR pathway.

To check the specificity of the treatments, additional control incubations were performed (Supplementary data, Fig. S4). HL-60 cell treatment with 1  $\mu$ M 9-*cis*-RA provoked nearly 40% decrease in the proliferation rate, as expected from a previous report [38], which was not further enhanced by addition of lithocholic acid. Partial recovery of the proliferation was observed with the addition of UVI2024. In experiments with 1 and 5  $\mu$ M 9-*cis*-retinol, in the presence (Fig. 4) and absence (Supplementary data, Fig. S4) of lithocholic acid, the RAR antagonist was able to recover 100% proliferation in all cases. This supports the specificity of the treatments



and points to a direct link between retinaldehyde reductase activity of AKR1C3, RAR pathway and proliferation.

## DISCUSSION

Aldo-keto reductases have been recently revealed as capable of reducing retinaldehyde *in vitro* and *in vivo*. This activity was demonstrated in subfamily 1B (AKR1B1, AKR1B10 and AKR1B12) [3, 5, 6, 23], in bovine lung prostaglandin F synthase (AKR1C7) and in the rat AKR1C15 [39, 40]. In the present work we have further demonstrated that, except for AKR1C2, all other members of the human AKR1C subfamily (AKR1C1, AKR1C3 and AKR1C4) exhibit significant retinaldehyde reductase activity. Kinetic studies on the active AKR1Cs show low  $K_m$  values for both all-*trans* and 9-*cis*-retinaldehyde, in the same order of magnitude as those of previously studied AKRs, SDRs and ADHs [3]. In fact,  $K_m$  values for retinaldehyde isomers are among the lowest of the AKR1Cs with any physiological substrate. The  $k_{cat}$  values are in general relatively low, similar to those of aldose reductase (AKR1B1) [3]. As an exception, AKR1C3 is an efficient 9-*cis*-retinaldehyde reductase, with a  $k_{cat}$  value more than 10-fold higher than those of the other members of the subfamily. In contrast, AKR1C3 displays a low  $k_{cat}$  for all-*trans*-retinaldehyde ( $0.60 \text{ min}^{-1}$ ), far from the highest value ( $27 \text{ min}^{-1}$ ) found in AKR1B10. Indeed, AKR1C3 is among the most efficient human 9-*cis*-retinaldehyde reductases, comparable to some SDR enzymes [4, 41]. Moreover, although comparison with non-retinoid substrates is difficult because of different methodologies used, 9-*cis*-retinaldehyde is among the physiological substrates most efficiently reduced by AKR1C3 [7, 12].

*Predicted structural features for the specificity of AKR1Cs with retinaldehyde isomers.* Ligand docking and MD simulations have been used to compare the binding of retinaldehyde isomers to AKR1C1, a low-activity enzyme, and AKR1C3, a high-activity enzyme. The complexes obtained were very similar in their overall structure, but some clear differences at the binding-site region could be observed. The most relevant was that of residue Trp-227, at loop B, which adopted different conformations in the complexes, and that was unique for the AKR1C3:9-*cis*-retinaldehyde complex. This observation matched up with the relevant role of this residue, proposed to be critical in positioning the substrate for a productive catalysis in AKR1Cs [31, 32]. Structural analysis suggested that a likely cause for these different conformations could be residue 226, a Pro in AKR1C1 and an Arg in AKR1C3, which results in a more rigid and tighter loop B in AKR1C1. The importance of this residue was partially supported by a moderate drop of the catalytic efficiency of AKR1C3 R226P and R226Q mutants. This suggests that the orientation of loop B, and particularly Trp-227 influenced by residue 226, participates in the proper positioning of the substrate for AKR1C3 catalysis, although other structural features must have a contribution. Thus, the wider and more hydrophilic site of AKR1C3 may also facilitate the suitable binding of 9-*cis*-retinaldehyde. The lower activity of AKR1C3 with all-*trans*-retinaldehyde may be explained by loop B conformation found in the complex with this isomer, which is similar to that found in the AKR1C1:9-*cis*-retinaldehyde complex.

*Function of AKR1Cs on the control of cellular retinoid levels.* Breast adenocarcinoma cells (MCF-7) were chosen as a model for an *in vivo* study of retinaldehyde reductase activity by AKR1Cs because they express high amounts of AKR1C1 and AKR1C3 [33, 34] and because they exhibit very low retinol oxidation activity, down-regulated retinol esterification and low retinaldehyde oxidation [36, 42, 43].

The all-*trans*-retinaldehyde reduction was found to be very high in MCF-7 cells. About 30% of this activity was due to AKR1Cs, particularly to AKR1C3. The reductive metabolism of 9-*cis*-retinaldehyde was lower, but AKR1C3 and AKR1C1 were responsible for most of this activity. These results indicate that *in vitro* retinoid-active AKR1Cs are also active *in vivo*, and that when present in a given tissue they may play a major role in retinaldehyde reduction. We have further demonstrated that this *in vivo* activity has a strong influence on the RA synthesis pathway. Thus, *trans*-activation studies on HeLa cells indicate that overexpression of AKR1C1, AKR1C3 or AKR1C4 notably decreases RA biosynthesis from added all-*trans*-retinol, and especially from 9-*cis*-retinol. Probably, AKR1C overexpression upsets the balance between oxidative and reductive enzymes acting on RA synthesis, in such a way that increased retinaldehyde reductase activity deprives RAR of its ligand.

AKR1Cs have recently been emerging as proteins whose enzymatic activity may be involved in regulating the local concentration at the pre-receptor level of lipophilic ligands which bind nuclear receptors and trigger transcriptional response. Members of AKR1A, 1B and 1D subfamilies seem to exert also this function [12, 13]. Regulation of the RA synthesis pathway is crucial, because retinoid receptor expression is generally ubiquitous, while RA synthesis is limited both spatially and temporally. This leads to the conclusion that signaling events may be initiated by RA synthesis [44]. There are many enzymes involved in the reversible first step of the pathway (Scheme 1) that exhibit different specificity for the retinol/retinaldehyde isomers and for the oxidation/reduction direction of the reaction. This complementarity and sometimes redundancy suggest a precise fine tuning of this limiting step, which provides the retinaldehyde levels for the final and irreversible oxidation to RA [3, 23, 45]. We have demonstrated that AKR1B1, AKR1B10 [3, 22, 23], AKR1C1, AKR1C3 and AKR1C4 are active retinaldehyde reductases that can decrease retinaldehyde and RA levels *in vivo*, and therefore, that are capable of displaying pre-receptor regulation of the RAR and RXR nuclear receptors.

AKR1C3 is a highly active 9-*cis*-retinaldehyde reductase which could play a relevant function in the control of 9-*cis*-RA signaling. An unsolved issue, however, is the role of 9-*cis*-RA. Although it is well proved that it can activate RAR and RXR receptors, it has not been detected *in vivo* without previous addition of retinoids [46] except for one case [47]. Two possibilities are currently debated [46]: 1) 9-*cis*-RA is only present at active concentrations in localized regions and/or only transiently present, which precludes detection. The existence of several enzymes that use preferentially 9-*cis*-retinoids ([4, 36], present work), the report of significant levels of an active 9-*cis*-RA derivative (9-*cis*-4-oxo-13,14-dihydro-RA) [48], and the recent detection of 9-*cis*-RA in pancreas [47] may support this possibility. 2) Vitamin A toxicity and teratogenicity may be linked to generation of 9-*cis*-RA and abnormal activation of RXR-RAR or RXR-RXR controlled genes. In both cases the role of AKR1C3 may be relevant, in the control of physiological 9-*cis*-RA signaling and in the prevention of excessive 9-*cis*-RA formation following administration of elevated amounts of retinol.

*AKR1C3 may promote cell proliferation through down-regulation of RA biosynthesis.* The participation of AKR1C3 in cancer development is well proven, especially because



its metabolism of receptor ligands results in a proliferative effect [18]. In fact it has been demonstrated that AKR1C3 inhibitors could enhance antiproliferative activity by potentiating the PPAR $\gamma$  pathway through an indirect increase of 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15 $\Delta$ -PGJ<sub>2</sub>), a natural ligand for PPAR $\gamma$  [25]. However it has been also observed that PPAR $\gamma$ -independent mechanisms exist [20, 49]. Using the HL-60 cell line, we here provide evidence that the proliferative effect of AKR1C3 may be in part linked to the RA signaling pathway. We found that 9-*cis*-retinol exhibits antiproliferative effect, which must be a consequence of its conversion to 9-*cis*-RA. Our hypothesis was that this conversion should be in part blocked by the endogenous AKR1C3 retinaldehyde reductase activity. The fact that the AKR1C3 inhibition further increases the antiproliferative effect of 9-*cis*-retinol (Fig. 4), supports this possibility. Finally, the addition of an RAR antagonist completely reverses the effect of the AKR1C3 inhibition, supporting that part of the known proliferative effect of AKR1C3 might be through its retinaldehyde reductase activity, resulting in down-regulation of RA synthesis. Thus, AKR1C3 activity may deplete the antagonists of both PPAR $\gamma$  and RA receptors, resulting in inhibition of differentiation and increase of proliferation [20, 37, 49].

In conclusion, we have demonstrated a significant retinaldehyde reductase activity of human AKR1C enzymes, especially of AKR1C3. Although further research could be performed with additional approaches, such as the use of small interfering RNA, evidence presented with the use of inhibitors suggests that the RA signaling pathway would be involved in the pro-proliferative effect of AKR1C3.

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**Table 1. Kinetic constants of AKR1C1-AKR1C4, AKR1C3 mutants and AKR1B10 with retinoids and 9,10-phenanthrenequinone**

Substrate and parameter	AKR1C1	AKR1C2	AKR1C3	AKR1C3 R226P	AKR1C3 R226Q	AKR1C4	AKR1B10
<b>All-trans-Retinaldehyde</b>							
$K_m$ ( $\mu\text{M}$ )			$1.4 \pm 0.3$	$1.9 \pm 0.2$	$1.7 \pm 0.3$	$0.31 \pm 0.03$	$0.6 \pm 0.1^b$
$k_{\text{cat}}$ ( $\text{min}^{-1}$ )	L.A.	N.A.	$0.60 \pm 0.04$	$0.26 \pm 0.01$	$0.43 \pm 0.02$	$0.24 \pm 0.01$	$27 \pm 1^b$
$k_{\text{cat}}/K_m$ ( $\text{mM}^{-1} \cdot \text{min}^{-1}$ )			$430 \pm 100$	$140 \pm 16$	$260 \pm 50$	$790 \pm 90$	$45000 \pm 7600^b$
<b>9-cis-Retinaldehyde</b>							
$K_m$ ( $\mu\text{M}$ )	$0.48 \pm 0.08$		$0.40 \pm 0.06$	$0.37 \pm 0.04$	$0.75 \pm 0.08$	$0.80 \pm 0.18$	$0.7 \pm 0.1^b$
$k_{\text{cat}}$ ( $\text{min}^{-1}$ )	$0.18 \pm 0.01$	N.A.	$13 \pm 0.5$	$4.3 \pm 0.1$	$9.7 \pm 0.3$	$0.40 \pm 0.03$	$0.9 \pm 0.1^b$
$k_{\text{cat}}/K_m$ ( $\text{mM}^{-1} \cdot \text{min}^{-1}$ )	$370 \pm 70$		$32500 \pm 5000$	$11500 \pm 1360$	$12900 \pm 1500$	$500 \pm 120$	$1300 \pm 160^b$
<b>9-cis-Retinol</b>							
$K_m$ ( $\mu\text{M}$ )			$0.30 \pm 0.04$				
$k_{\text{cat}}$ ( $\text{min}^{-1}$ )	N.A.	N.A.	$0.26 \pm 0.01$	N.A.	N.A.	N.A.	N.D.
$k_{\text{cat}}/K_m$ ( $\text{mM}^{-1} \cdot \text{min}^{-1}$ )			$850 \pm 110$				
<b>9,10-Phenanthrenequinone</b>							
$K_m$ ( $\mu\text{M}$ )	$0.7 \pm 0.2^a$	$1.1 \pm 0.4^a$	$1.2 \pm 0.1$	$1.6 \pm 0.4$	$1.1 \pm 0.1$		
$k_{\text{cat}}$ ( $\text{min}^{-1}$ )	$35 \pm 6^a$	$110 \pm 18^a$	$55 \pm 1.4$	$66.4 \pm 3.6$	$40.5 \pm 0.9$	N.D.	N.D.
$k_{\text{cat}}/K_m$ ( $\text{mM}^{-1} \cdot \text{min}^{-1}$ )	$48000 \pm 16000^a$	$100000 \pm 40000^a$	$43800 \pm 4800$	$41100 \pm 9500$	$37200 \pm 4100$		

Activities were determined in 90 mM potassium dihydrogen phosphate, 40 mM potassium chloride, pH 7.4, at 37°C (with retinoids) or at 25°C (with 9,10-phenanthrenequinone). To calculate  $k_{\text{cat}}$  values, the  $M_r$  value used for all the proteins was 41.5 kDa. L.A., low activity (0.56 nmol·min<sup>-1</sup>·mg<sup>-1</sup> measured with 5  $\mu\text{M}$  all-*trans*-retinaldehyde); N.A., no activity or lower than 0.5 nmol·min<sup>-1</sup>·mg<sup>-1</sup> measured with 5  $\mu\text{M}$  all-*trans*-retinaldehyde; N.D., not determined. <sup>a</sup>Data taken from Ref. [30]. <sup>b</sup>Data taken from Ref. [22].



**Table 2. Comparison between residues that define the 9-*cis*-retinaldehyde-binding pocket in AKR1C1 and AKR1C3 proteins**

<b>Residue</b>	<b>54</b>	<b>55</b>	<b>86</b>	<b>117</b>	<b>129</b>	<b>226</b>	<b>227</b>	<b>306</b>	<b>307</b>	<b>308</b>	<b>310</b>	<b>311</b>
<b>AKR1C1</b>	L	Y	W	H	I	P	W	L	T	L	I	F
<b>AKR1C3</b>	L	Y	W	H	S	R	W	F	N	S	S	F

AKR1C1 and AKR1C3 binding-pocket residues are highlighted in bold. Identical residues are shaded in grey. Residue 226, which is found to determine the orientation of Trp-227, is also included.

## LEGENDS TO FIGURES

**Scheme 1. Retinoic acid metabolic pathway.** Retinol can be reversibly stored to retinyl esters by the actions of lecithin-retinol acyltransferase (LRAT) and retinyl ester hydrolase (REH). Alternatively, retinol can enter the RA synthesis pathway. Firstly, it is oxidized to retinaldehyde by either alcohol dehydrogenase (ADH) or retinol dehydrogenase (RDH), using NAD as a cofactor. In this reversible step, retinaldehyde can be reduced to retinol through the action of NADPH-dependent RDH or AKR. Retinaldehyde can also be irreversibly oxidized to RA by retinaldehyde dehydrogenases (RALDH). RA can initiate a signalling event through binding to nuclear RA receptors (RAR and RXR) that regulate transcription of target genes. RA can be further oxidized to 4-hydroxyretinoic acid by cytochrome P450 isoforms (CYP26) which is the first step of RA degradation and inactivation. Adapted from ref. [4].

**Figure 1. Computer molecular models of complexes of AKR1C1 and AKR1C3 with retinaldehyde.** (A) Superimposition of the 9-*cis*-retinaldehyde complexes with AKR1C1 (green) and AKR1C3 (orange), and of the all-*trans*-retinaldehyde complexes with AKR1C1 (blue) and AKR1C3 (red). Corresponding loop B conformations are highlighted. (B) 9-*cis*-retinaldehyde in the binding pocket of AKR1C1. (C) 9-*cis*-retinaldehyde in the binding pocket of AKR1C3. Residues shown are those relevant for substrate binding. Distances corresponding to conserved (in red) and non-conserved (in orange) relevant interactions are indicated.

**Figure 2. Retinaldehyde reductase activity of AKR1C enzymes in MCF-7 cells.** MCF-7 cells were incubated for 30 min with 10  $\mu$ M all-*trans*-retinaldehyde or 10  $\mu$ M 9-*cis*-retinaldehyde, in the absence or presence of 100  $\mu$ M flufenamic acid (AKR1C inhibitor). Incubations with 10  $\mu$ M 9-*cis*-retinol, with or without 100  $\mu$ M flufenamic acid, are also shown. Cellular retinol and retinaldehyde content was measured by HPLC. Data were expressed as percentage of conversion of the retinoid uptaken by cells (reduced retinaldehyde or oxidized retinol). Results are expressed as the mean  $\pm$  S.E.M. of at least three determinations. \* $P < 0.05$  vs no inhibitor.

**Figure 3. Effect of retinaldehyde reductase activity of AKR1C enzymes on RA *trans*-activation in HeLa cells.** RA *trans*-activation was evaluated through transient transfection of DR5-Luc RA Response Element in HeLa cells, which have RA synthesis capability. Cells were transfected with a reporter vector (pCIS-CK as a negative control or with pDR5-Luc) and an expression vector (containing each AKR1C1-AKR1C3 cDNA or the *lacZ* gene). Incubation was performed for 24 h with (A) 10  $\mu$ M all-*trans*-retinol or (B) 10  $\mu$ M 9-*cis*-retinol. RA (10  $\mu$ M) was used as a positive control. Data were normalized as relative light units (RLU) per mg of protein. Results are expressed as the mean  $\pm$  S.E.M. of at least three determinations. \* $P < 0.05$  vs LacZ.

**Figure 4. HL-60 cell proliferation levels resulting from treatments with 9-*cis*-retinol, AKR1C3 inhibitor lithocholic acid (Litho) and RAR antagonist UVI2024.** HL-60 cells were cultured for 3 days in the absence or presence of various 9-*cis*-retinol (9-*cis*-ROL) concentrations, lithocholic acid (20  $\mu$ M) and UVI2024 (1  $\mu$ M). Cell proliferation was measured by XTT reagent (Roche) and expressed as percentage vs untreated cells. Data are the mean  $\pm$  S.D. of three experiments performed in triplicate.

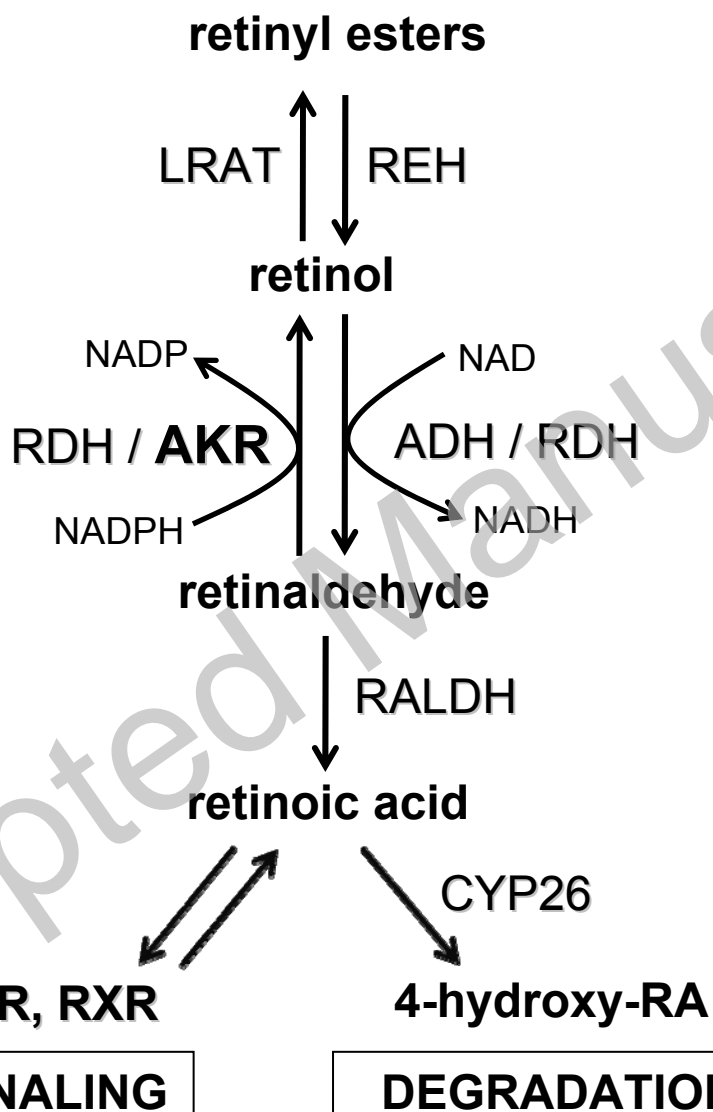
*Specificity of human aldo-keto reductases 1C with retinaldehyde*

\* $P < 0.05$  vs 9-*cis*-ROL alone; #  $P < 0.05$  vs 9-*cis*-ROL plus lithocholic acid, ‡  $P < 0.05$  vs untreated cells.

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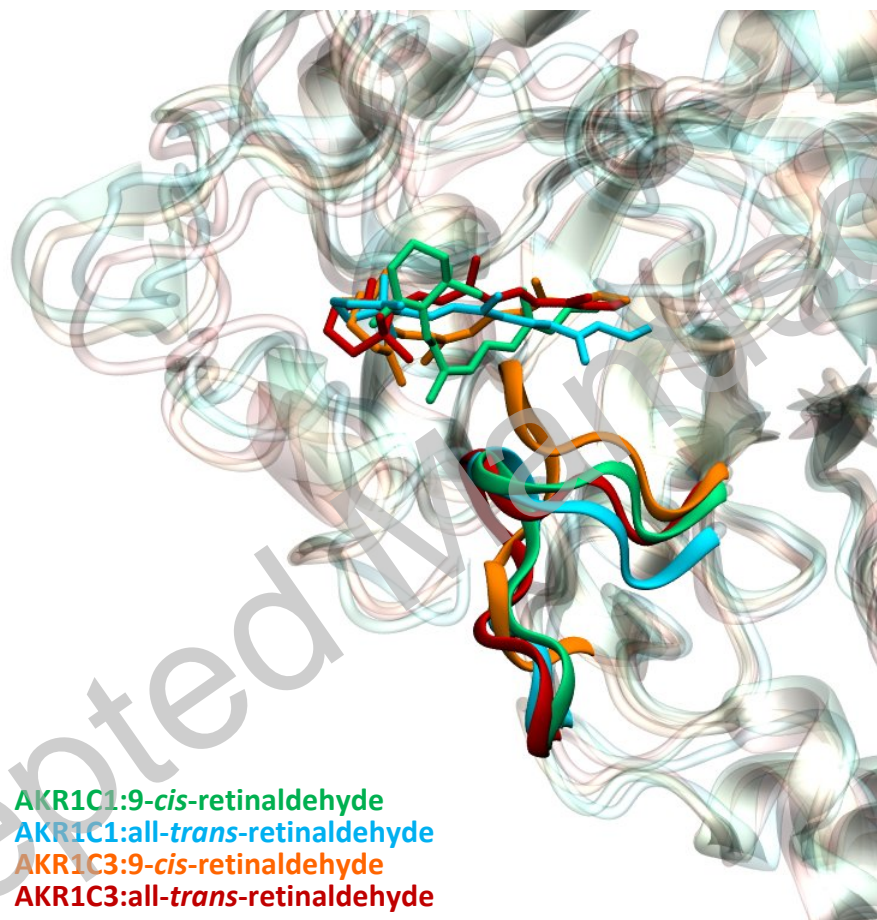
THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20111286

# Scheme 1



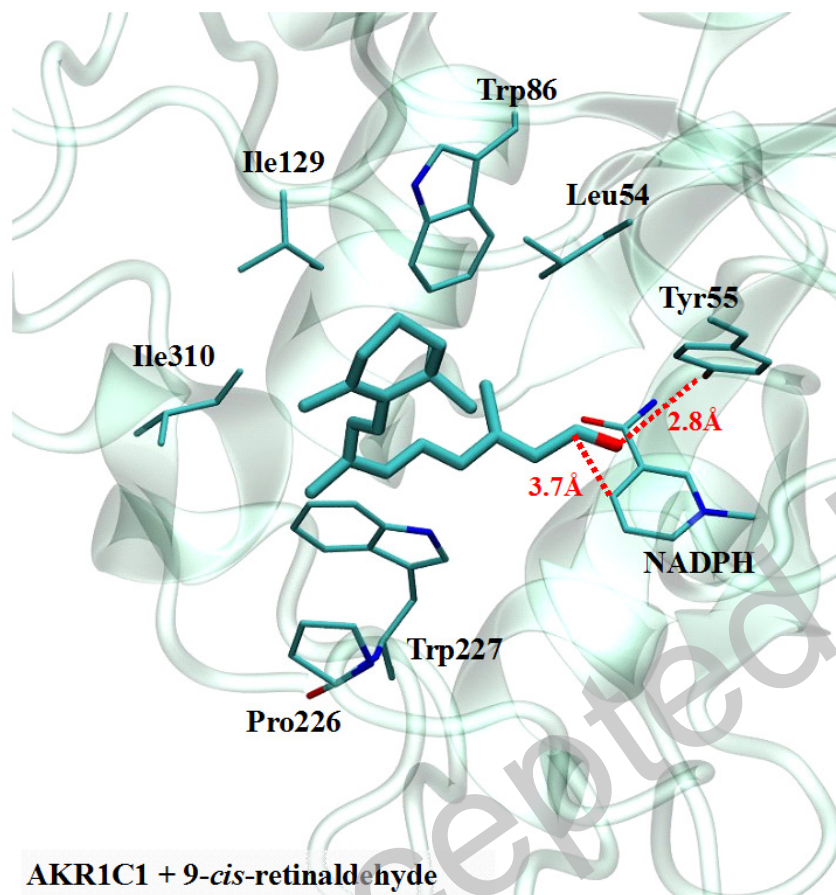
# Figure 1

A

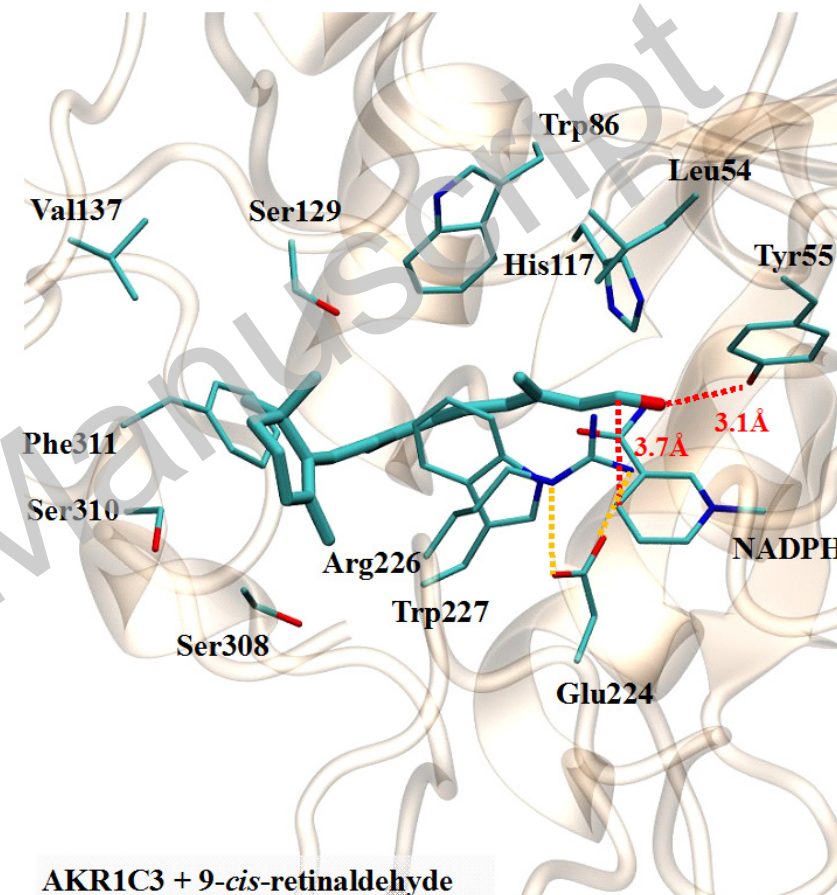




**B**



**C**





**Figure 2**

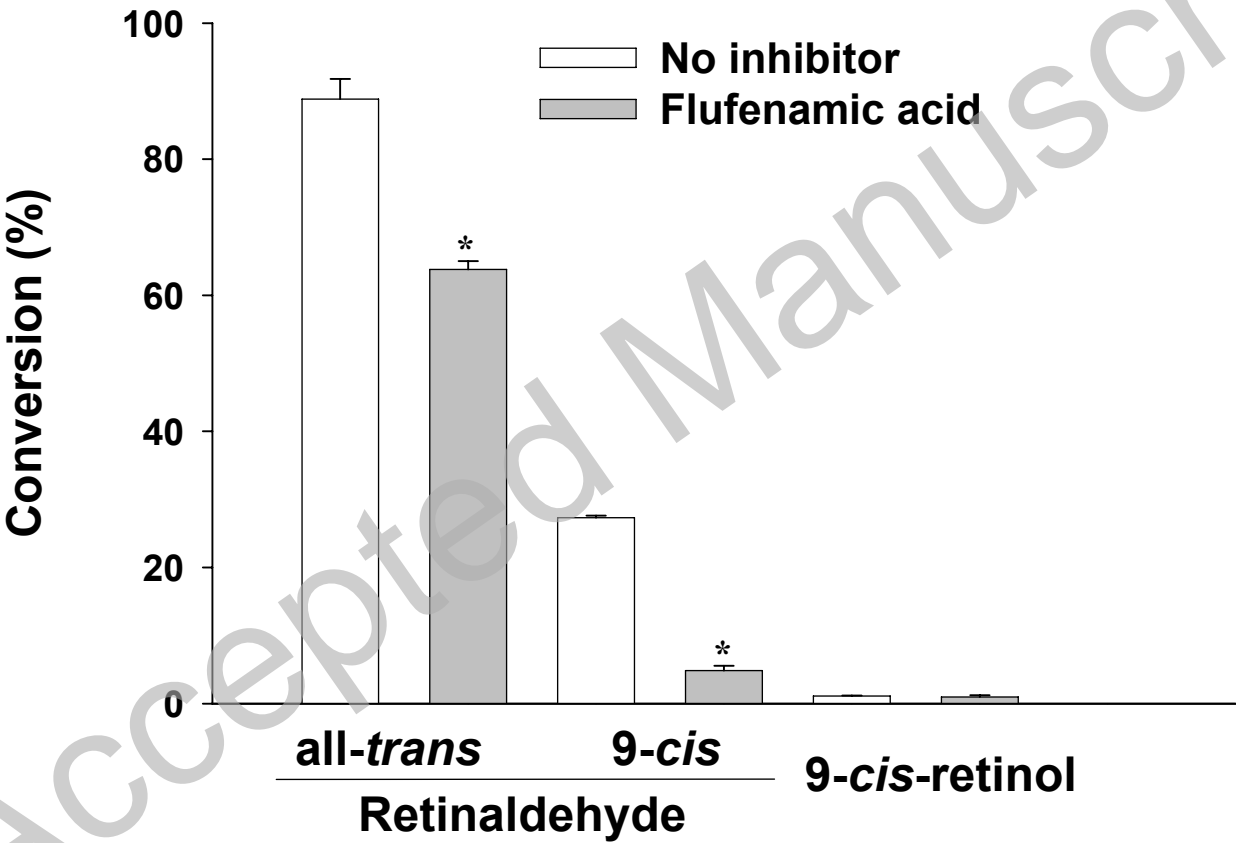
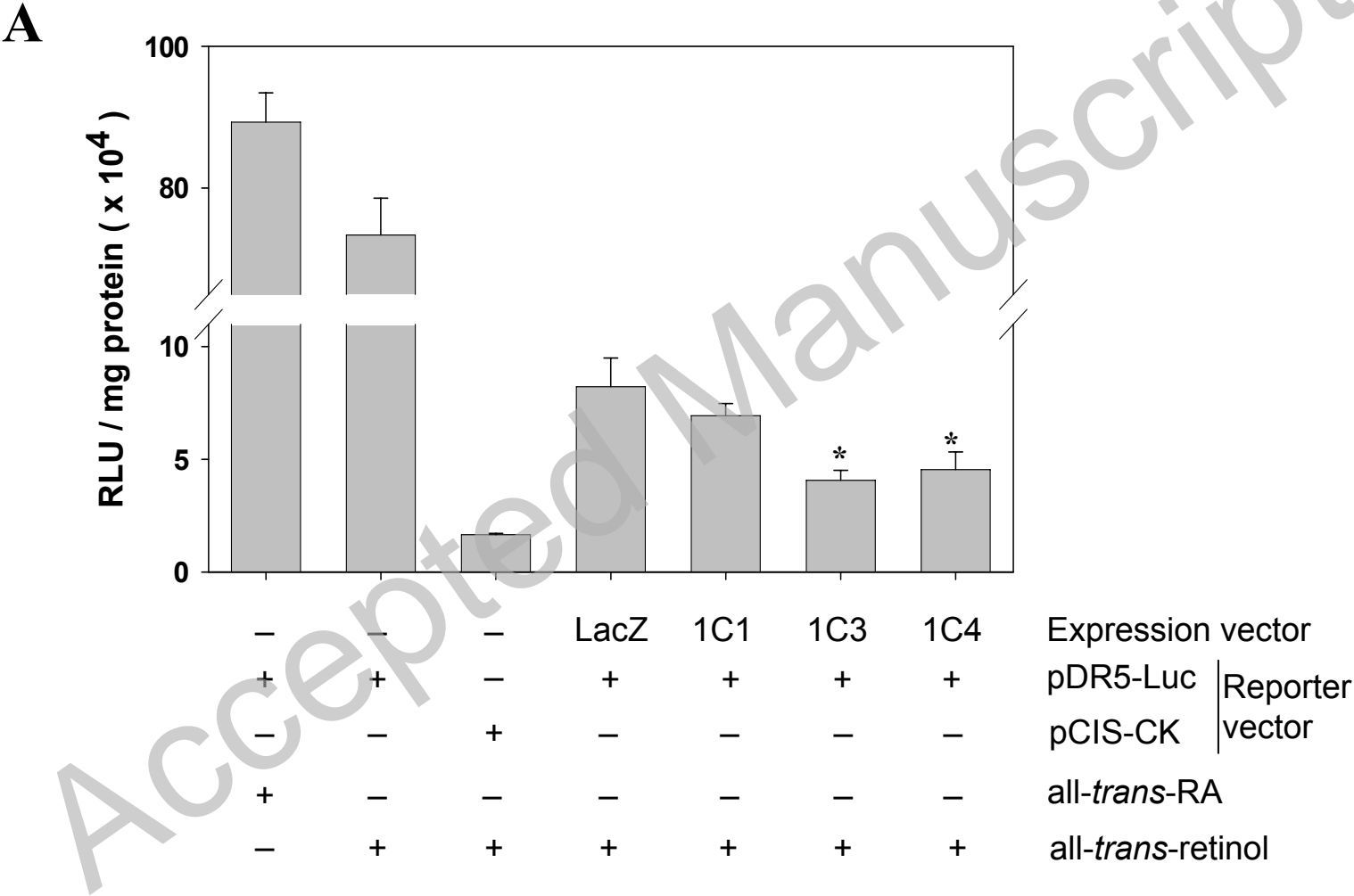
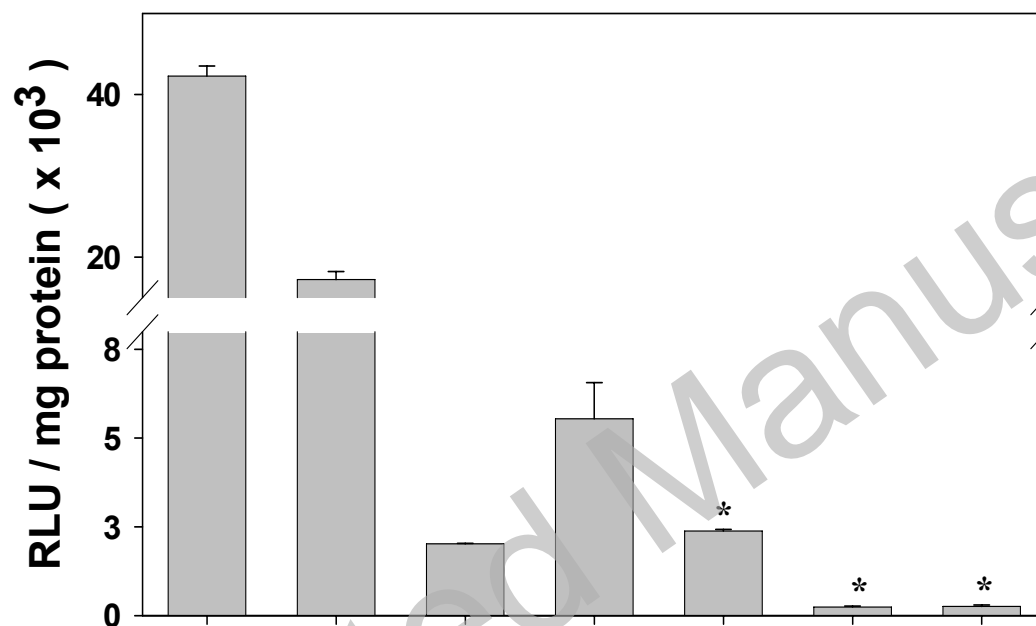


Figure 3



# B



-	-	-	LacZ	1C1	1C3	1C4	Expression vector
+	+	-	+	+	+	+	pDR5-Luc   Reporter
-	-	+	-	-	-	-	pCIS-CK   vector
+	-	-	-	-	-	-	9- <i>cis</i> -RA
-	+	+	+	+	+	+	9- <i>cis</i> -retinol

**Figure 4**

