Acid Derivatives of Pyrazolo[1,5-*a*]pyrimidine as Aldose Reductase Differential Inhibitors

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Summary

Aldose reductase, (AKR1B1), the key enzyme of the polyol pathway, plays a crucial role in the development of long term complications affecting diabetic patients. Nevertheless, the expedience of inhibiting this enzyme to treat diabetic complications has failed, due to the emergence of side effects from compounds under development. Actually, AKR1B1 is a Janus-faced enzyme which, besides ruling the polyol pathway, takes part to the antioxidant defence mechanism of the body. In this work we report the evidence that a class of compounds, characterized by a pyrazolo[1,5-*a*]pyrimidine core and a ionisable fragment, modulates differently the catalytic activity of the enzyme, depending on the presence of specific substrates like sugar, toxic aldehydes and glutathione conjugates of toxic aldehydes. The study stands out as a systematic attempt to generate aldose reductase differential inhibitors (ARDIs), intended to target long term diabetic complications while leaving unaltered the detoxifying role of the enzyme.

Introduction

Aldose Reductase (AKR1B1), the first enzyme of the so-called polyol pathway, is acknowledged as the critical checkpoint for the main pathological changes affecting over time the nervous, renal, cardiovascular and ocular systems of diabetic patients. Accordingly, its inhibition has been pursuing since decades as a useful therapeutic strategy to prevent the onset of diabetic complications (Del Corso et al., 2008).

However, despite the efforts to obtain effective aldose reductase inhibitors (ARIs) (Grewal et al., 2016), a true and widespread ARI therapy is not yet an established fact and epalrestat (Figure 1) is the only agent successfully marketed in Japan, India and China for the treatment of diabetic neuropathy (Li et al., 2016).

Products that appear to be promising during pre-clinical investigations often fail to proceed any further, showing uncertain results in clinical trials with humans or even leading to adverse side effects. These latter, in particular, have been recently correlated to the complex physiological role played by the enzyme (Alexiou et al., 2009).

Actually, besides ruling the polyol pathway, AKR1B1 takes part to the antioxidant defence mechanisms of the body, being highly efficient in reducing toxic aldehydes, including 4-hydroxy-2,3-nonenal (HNE), arising in large quantities from pathological conditions connected with oxidative stress (Srivastava et al., 1999). At the same time, AKR1B1 catalyses the reduction of glutathione conjugates of unsaturated aldehydes, showing in most cases a catalytic efficiency higher than that displayed against the parent free aldehyde. In this regard, special appears the case of the 3-glutathionyl-4-hydroxynonanal (GS-HNE), whose reduction product 3-glutathionyl-dihydroxynonane (GSDHN), was shown to be responsible for cytotoxicity through activation of the NF-κB signalling pathways, thus further muddling up the physiological role of this enzyme (Ramana et al., 2010; Ramana et al., 2011).

Due to this Janus-faced nature, the expedience of inhibiting AKR1B1 to prevent and treat long-term diabetic complications has been debated for long and remains controversial.

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To overcome the deadlock, we recently launched the concept of Aldose Reductase Differential Inhibitors (ARDIs) as a novel class of compounds able to selectively inhibit the catalytic activity of the enzyme depending on the specific substrates is going to be transformed (Del Corso et al., 2013). This approach, which may be in principle adopted for any enzyme able to act on different substrates (Cappiello et al., 2014), appears truly tailored for AKR1B1 which, besides being an aspecific enzyme, is also not permissive (Balestri et al., 2017).

Differently from ARIs developed till now, characterized by high but unspecific binding affinity against the target enzyme, ARDIs should show a differential intra-site binding affinity, being able to inhibit the catalytic activity of AKR1B1 against glucose and GS-alkanals while leaving unaltered, or, in any case less affected, the reductive activity of the enzyme towards toxic hydrophobic aldehydes. Accordingly, using an ARDI, it is possible in principle to prevent hyperglycemia-induced cell injury without affecting the antioxidant defense.

When thinking to differential inhibition, the choice of the substrate to be used for *in vitro* studies is not a trivial task. In this regard, on the bases of the peculiar non hyperbolic inhibition exerted on the enzyme by aldose hemiacetals (Balestri et al., 2015a), D,L glyceraldehyde usually adopted for inhibition studies becomes rather inadequate as a mimicking aldoses substrate. Thus when the aim of the test is to evaluate a differential inhibition, different physiological substrates as glucose or structurally related molecules (Balestri et al., 2015b), HNE and GS-HNE, chosen as examples of lipid aldehydes and glutathionyl-aldehyde adducts metabolized by the enzyme, respectively, should be used.

Screening an in house collection of previously developed compounds, characterized by different but customary pharmacophoric elements for AKR1B1 molecular recognition like the hydantoin ring (Da Settimo et al., 2005), the phenolic fragment (La Motta et al., 2007) and the carboxylic moiety (Da Settimo et al., 2001; Da Settimo et al., 2005; La Motta et al., 2008; Cosconati et al., 2009), we identified the 7-oxo-4,7-dihydropyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid **5a** (Figure 1) as a

promising ARDI, being able to intervene on glucose and GS-HNE reduction, catalysed by AKR1B1 from bovine lens, more efficiently than on HNE reduction (Del Corso et al., 2013).

With this in mind, and aiming to develop a class of effective ARDIs, we undertook the synthesis of a number of pyrazolopyrimidine derivatives and investigated their functional efficacy on the L-idose, HNE and GS-HNE reduction catalysed by the human recombinant enzyme, hAKR1B1.

Results and Discussion

Moving from the lead, 7-oxo-4,7-dihydropyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid (**5a**, Figure SI-1), identified as a promising ARDI towards the bovine lens enzyme (Del Corso et al., 2013), we synthesized a number of parent derivatives characterized by suitable substituents in position 4 of the heterocyclic core, to verify the influence of electron-withdrawal, electron-release and steric bulk on the differential intra-site binding affinity of the compounds. *In vitro* inhibitory tests on the synthesized compounds were firstly carried out exploiting L-idose as the substrate, chosen as a viable alternative to the physiologically relevant D-glucose. Actually, although similar in terms of structural rearrangement, the C-5 epimer of D-glucose turns out to be more useful to investigate the activity of the enzyme, as it offers a significantly higher concentration of the free aldehyde form when compared to the parent compound (Balestri et al., 2015b).

While **5a** exhibited an IC_{50} value of approximately 0.5 mM and **5b,c** were devoid of any appreciable inhibitory action on hAKR1B1 ($IC_{50} > 1$ mM), the IC_{50} values of all the other molecules in inhibiting L-idose reduction were of the same order of magnitude, ranging from approximately 60 μM to 200 μM. Afterward, focusing on the most active compounds (**5a**,**d-j**,**l**, Figure SI-1), the same assays were repeated using HNE as the substrate, to investigate their ARDI efficacy. The resulting differential inhibitory activity between L-idose and HNE reduction (DI, %) is shown in Figure 1.

It is noteworthy that **5a**, which inspired the ARDI library being a promising differential inhibitor when tested on the bovine lens enzyme (Del Corso et al., 2013), in the case of the human recombinant enzyme used in the present study shows a reduced inhibitory activity and doesn't exhibit any

differential inhibitory action between L-idose and HNE reduction. Even though disappointing at the first glance, the observed activity is not a peculiar event, being well documented that AKR1B1 from different sources is possibly differently targeted by inhibitory molecules, with the human placental enzyme appearing as the less susceptible to inhibition (Kador et al., 1980; Morjana et al., 1989; Kador et al.; 1985; Lee et al.; 2010).

In this respect, it is well conceivable that, considering the peculiar structural restrictions required for a molecule to act as an ARDI, differences in differential inhibition susceptibility between different enzyme forms have certainly more chances to take place. In any case, moving from **5a**, suitable decoration of the pyrazolopyrimidine scaffold made it possible to recover ARDIs features, as shown in Figure 1.

In particular, the presence of a *p*-chloro atom on the 4-benzyl ring leads to an appreciable result and **5e**, showing a DI value of 16%, turns out to be the best differential inhibitor of the whole series. Changing the less bulky, more electron-attracting chloro atom into the bulkier, less electron-attracting bromine one, as in **5f**, halved the DI value (DI: 8.44%), and the insertion of the even bulkier trifluoromethyl group carried out the negative trend, as **5g** showed a DI value of 2.6%. Finally, **5h**, showing a DI value of 1.4% and featuring the ortho-fluoro para-bromo substitution pattern typical of known ARIs such as ponalrestat, ranirestat, and minalrestat, turned out to be the less effective among this subseries.

Insertion of the electron-donating methyl group in the same position of the 4-benzyl ring retains a significant differential profile of inhibition (**5i**, DI: 11.0%), and the same was also true when the pendant phenyl ring was replaced by the bulkier naphthalene core (**5j**, DI: 11.3%). On the contrary, the presence of a methylene spacer between the main pyrazolopyrimidine liphophilic area and the additional aromatic ring in position 4, as in **5l**, turned upside down the ADRI profile, as the compound rather preferentially blocks *h*AKR1B1, even though slightly, when processing HNE instead of L-idose (DI: -4.03%).

Classical powerful ARIs like epalrestat and sorbinil were also tested as ARDIs. However, as reported in Figure 1, their differential inhibitory ability appears negligible. This result clearly demonstrates that structural requirements defined to date to obtain potent ARIs are not helpful in ARDIs' design and development, and novel pharmacophoric elements must be unveiled to get to effective and potent differential derivatives.

The inhibitory profile of the most effective ARDI (**5e**) and of a compound (**5g**), displaying a comparable inhibitory power but a rather modest differential inhibitory ability, was furthered. Besides being differentially active between L-idose and HNE reduction, **5e** turned out to exert its inhibitory action also toward the reduction of GS-HNE, thus proving to fulfil thoroughly the functional requirements of an ARDI. The inhibition curves referring to the three substrates, reported in Figure 2A, show that the reduction of L-idose and GS-HNE is similarly affected by the inhibitor and that the effect is more pronounced than that on HNE reduction. This is not the case of **5g** (Figure 2B) which inhibits AKR1B1 irrespectively of the used substrate. The lack of any differential inhibitory action exerted by epalrestat and sorbinil towards the reduction of all the three adopted substrates is again clearly evident in Figure 2C, in which the dose/effect response for the two classical ARIs are also reported. The emerging IC_{50} values related to L-idose, HNE and GS-HNE reduction (Table 1) are consistent with a differential inhibitory action exerted only by **5e**.

To unveil the pattern of interaction with the target enzyme, a detailed kinetic analysis of the inhibition action of **5e** was accomplished, using as negative controls for differential action **5g**, displaying a rather comparable IC_{50} to **5e**, and the powerful ARIs epalrestat and sorbinil. Results reported in Figures SI-1, SI-2, SI-3 and SI-4 allow to determine the inhibition models of action and the relative apparent dissociation constants for the binary enzyme/inhibitor complexes ($K_i^{(app)}$) and for the ternary enzyme/substrate/inhibitor complexes ($K_i'^{(app)}$) with respect to the different substrates.

For both inhibitors **5e** and **5g**, it appears a significant preferential targeting of the free enzyme ($K_i'^{(app)}/K_i^{(app)} > 1$) at an extent that the inhibitory action become very close to a competitive

inhibition, as for **5e** acting on GS-HNE reduction and **5g** acting on HNE reduction. The measured $K_i^{(app)}$ values (Table 2), especially for **5e**, appears affected by the nature of the substrate undergoing reduction. In this regard, **5e** seems to target the free enzyme more efficiently on L-idose and GS-HNE reduction, approximately two fold and four fold respectively, with respect to the HNE reduction; an evidence that may be invoked as the basis of the observed differential inhibitory action of this compound.

On the contrary, the apparent K_i values of **5g** are rather similar for all the three tested substrates. The same occurs for epalrestat and sorbinil, which display a mixed type of inhibition and an uncompetitive type of inhibition, respectively. As shown in Figures SI-3 and SI-4, the targeting of these powerful inhibitors to hAKR1B1 appears to occur irrespectively of the substrate undergoing reduction.

It is important to point out that the absolute values of the inhibition constants reported in Table 2, for **5e** and **5g**, necessarily obtained in the presence of DMSO, must be corrected taking into account the differential inhibition displayed by DMSO on the L-idose and HNE reduction catalyzed by hAKR1B1 (Misuri et al., 2017). In particular, the apparent kinetic constants for the tested compounds should be divided for a factor generally defined as $\{1 + ([DMSO]/K_{DMSO})\}$, in which $[DMSO]$ refers to the concentration of DMSO present in the assay and K_{DMSO} refer to the specific dissociations constants measured for DMSO either acting as competitive inhibitor of L-idose reduction or acting as mixed type inhibitor of the HNE reduction. Thus, for L-idose reduction, only the $K_i^{(app)}$ value will be reduced by factor of 1.85, while the $K_i^{(app)}$ and $K_i'^{(app)}$ values measured when HNE was used as substrate, will be reduced by a factor of 1.75 and 1.53, respectively. No correction is required for the apparent inhibition constant referring to GS-HNE reduction, being DMSO ineffective on the reduction of this substrate (Misuri et al., 2017). With such an approach, the competitive inhibitory efficiency of **5e** towards L-idose and GS-HNE reduction appear to be expressed by essentially the same K_i value (67 ± 4 µM and 70 ±16 µM, respectively) which is less than one half of the K_i value measured for HNE reduction.

Significance

In this work we presented a series of pyrazolo[1,5-*a*]pyrimidine derivatives, developed as aldose reductase differential inhibitors (ARDIs). Among the synthesized compounds, derivative **5e**, 4-(4-chlorobenzyl)-7-oxo-4,7-dihydropyrazolo[1,5-*a*]pyrimidine-6-carboxylic acid (Figure 1), emerged as a viable lead, proving to exert its inhibition toward the catalytic activity of *hAKR1B1* with a preferential action against both L-idose and GS-HNE with respect to HNE. It clearly represents an original compound which, differently from traditional ARIs developed to date, is intended to target long term diabetic complications while leaving unaltered the detoxifying role of the enzyme. Further studies in animal models will prove its robustness as prototypical drug candidate, exploitable to prevent the onset and progression of hyperglycemia-induced complication without the emergence of the adverse side effects commonly shown by known ARIs.

Acknowledgements

Author Contributions

F.B. accomplished the kinetic characterization study. R.M. and F.B. expressed and purified the target protein. M.C. conceived and applied a kinetic models-based approach for validation of ARDIs effectiveness. L.Q., V.C., and S.S. conceived and conducted the chemical synthesis of the inhibitors. A.D.C., U.M., F.D.S. and C.L.M. devised the work project. A.D.C. and C.L.M. wrote the paper. All the authors analysed and discussed the achieved results and reviewed the manuscript.

Declaration of Interests

The authors declare no competing interests.

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Main Figures and Tables Titles and Legends

Figure 1. Differential Inhibition of pyrazolo[1,5-*a*]pyrimidine derivatives, epalrestat and sorbinil on *h*AKR1B1.

4-Substituted-7-oxo-4,7-dihydropyrazolo[1,5-*a*]pyrimidine derivatives, **5a**,**d-j**,**l**, were tested as differential inhibitors at the concentration of 70 µM using either 0.8 mM L-idose or 0.04 mM HNE as the substrates, in the presence of 8 mU of purified *h*AKRB1. Bars (differential inhibition, DI) indicate the difference between the percentage inhibition observed using L-idose as the substrate and the percentage inhibition using HNE as the substrate. Values represent the mean from at least three independent measurement and error bars (if not visible are within the symbol size) refer to the standard deviation of the mean. Epalrestat (epa) and sorbinil (sorb), adopted as negative controls differential inhibitors, were used at a concentration of 0.1 µM and 1.0 µM, respectively.

Figure 2. Inhibition effectiveness of compounds **5e, 5g,** epalrestat and sorbinil on the *h*AKR1B1-catalyzed reduction of the following substrates: L-idose (0.6 mM, diamonds), HNE (0.04 mM, circles) and GS-HNE (0.04 mM, triangles).

(A) The enzyme activity in the presence of compound **5e** was determined at the indicated inhibitor concentrations. Values represent the mean from at least three independent measurement and error bars (if not visible are within the symbol size) refer to the standard deviation of the mean.

(B) The enzyme activity in the presence of compound **5g** was determined at the indicated inhibitor concentrations. Values represent the mean from at least three independent measurement and error bars (if not visible are within the symbol size) refer to the standard deviation of the mean.

(C) The enzyme activity in the presence of epalrestat (open symbols) and sorbinil (closed symbols) was determined at the indicated inhibitor concentrations. Values represent the mean from at least three independent measurement and error bars (if not visible are within the symbol size) refer to the standard deviation of the mean.

Table 1. Comparison of IC$_{50}$ values of **5e**, **5g**, sorbinil, and epalrestat for AKR1B1-dependent reduction of different substrates.

Table 2. Inhibition kinetic parameters related to **5e**, **5g**, sorbinil, and epalrestat as emerging from the kinetic analysis of depicted in Figures SI-1 and SI-2.

STAR Methods

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Concettina La Motta (concettina.lamotta@unipi.it).

Experimental Model and Subject Details

E. coli XL-1 Blue competent cells and *E. coli* BL21(DE3)pLysS cells were cultured in Luria Bertani (LB) broth at 37°C.

Methods Details

Chemistry reagents and Experimentation

3-Aminopyrazolo, diethyl 2-(ethoxymethylene)malonate, and the suitably substituted alkyl halides, used to obtain the target inhibitors, were from Alfa Aesar, Aldrich and Fluka. The carboxylic acid derivatives, **5a-l**, were synthesized as outlined in Scheme SI-1. Melting points were determined using a Reichert Köfler hot-stage apparatus and are uncorrected. Routine nuclear magnetic resonance spectra were recorded in DMSO-d_6 solution on a Varian Gemini 200 spectrometer, operating at 200 MHz, and on a Bruker 400 spectrometer, operating at 400 MHz. Evaporation was performed in vacuo (rotary evaporator). Analytical TLC was carried out on Merck 0.2 mm precoated silica gel aluminium sheets (60 F-254). Purity of the target inhibitors, **5a-l**, was determined by HPLC analysis, using a Merck Hitachi D-7000 liquid chromatograph (UV detection at 242 nm) and a Discovery C18 column (250 mm x 4.6 mm, 5 μm, Supelco), with a gradient of 40% water and 60% methanol and a flow rate of 1.4 mL/min. All the compounds showed percent purity values ≥95%.

Synthesis of Ethyl 7-Oxo-4,7-dihydropyrazolo[1,5-*a*]pyrimidine-6-carboxylate, 3. The commercially available 3-aminopyrazole **1** (1.00 mmol) was dissolved in 10 mL of glacial acetic acid. Diethyl ethoxymethylenemalonate **2** (0.24 mL, 1.20 mmol) was then added and the solution was refluxed under stirring for 2 hours (TLC analysis). After cooling at room temperature, the reaction mixture was treated with crushed ice and the white solid that precipitated was collected by filtration,

washed with water and purified by recrystallization from MeOH (yield: 95%). P.f.: 295 °C. ^{1}H-NMR (δ, DMSO-d$_6$): 1.30 (t, 3H), 4.00 (s, 1H), 4.20 (q, 2H), 6.33 (d, 1H), 7.50 (d, 1H), 8.33 (s, 1H).

General Procedure for the Synthesis of Ethyl 4-Substituted-7-oxo-4,7-dihydropyrazolo[1,5-*a*]pyrimidine-6-carboxylate Derivatives, 4a-l. The appropriate alkyl halide (1.20 mmol) was added to an ice-cooled solution of ethyl 7-oxo-4,7-dihydropyrazolo[1,5-*a*]pyrimidine-6-carboxylate **3** (1.00 mmol) and K$_2$CO$_3$ (1.20 mmol) in dry DMF. After addition, the reaction mixture was heated at T=90 °C under stirring until the disappearance of the starting material (TLC analysis). After cooling at room temperature, the solvent was removed under reduced pressure and the resulting crude material was treated with crushed ice. The white solid that precipitated was collected by filtration, washed with water and purified by recrystallization from the suitable solvent (Table SI-1).

General Procedure for the Synthesis of 4-Substituted-7-oxo-4,7-dihydropyrazolo[1,5-*a*]pyrimidine-6-carboxylic Acid Derivatives, 5a-l. A suspension of ethyl ester, **4a-l** (1.00 mmol) in 3 mL of 5% NaOH was heated at T=100 °C with stirring until a solution was achieved (TLC analysis). After cooling at room temperature, the solution was filtered and acidified with concentrated hydrochloric acid under ice-cooling. The white solid that precipitated was collected by filtration, washed with water and purified by recrystallization from the suitable solvent (Table SI-2).

Enzymatic assay

The activity of human placental aldose reductase (*h*AKR1B1) was determined at 37°C, following the decrease in absorbance at 340 nm due to NADPH oxidation (ε$_{340}$: 6.22mM^{-1} cm^{-1}) (Balestri et al., 2016). The standard assay mixture (700 µL) contained 0.25 M sodium phosphate buffer pH 6.8, 0.4 M ammonium sulfate, 0.5 mM EDTA, 0.18 mM NADPH, and 4.7 mM glyceraldehyde. The rate of NADPH oxidation in the absence of the substrate was subtracted as a blank. One unit of enzyme activity is the amount that catalyzes the conversion of 1 µmol of substrate/min in the above assay conditions. The above assay conditions were also adopted when L-idose, HNE or GSHNE (at the indicated concentrations) were used as substrates in inhibition studies.

Expression and purification of AR

The human recombinant enzyme was expressed in *E. coli* cells as previously described (Balestri et al., 2015b). Total RNA was extracted from human placenta with TRI®Reagent, according to the manufacturer's protocol. cDNA was prepared from total RNA by reverse transcription, using 200 units of SuperScript™ III Reverse Transcriptase and 0.5 µg of an oligo-dTprimer in a mixture (50 µL total volume) containing 0.5 mM of each dNTP, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT) and 0.1 mg/mL bovine serum albumin in 50 mM Tris–HCl, pH 8.3. After 60 min at 50°C the incubation mixture was used for PCR amplification or stored at -20°C. Aliquots of 1 µL of crude cDNA were amplified in a Bio-Rad Gene Cycler™ thermocycler, using 2.5 units of *T. aquaticus* DNA polymerase, 1 mM of each dNTP, 1 µM of each PCR primer, 50 mM KCl, 2.5 mM $MgCl_2$ and 0.1 mg/mL BSA in 10 mM Tris–HCl, pH 8.3, containing 0.1% (v/v) Triton X-100. At the 5' end, we used the specific primer: 5'-CAT ATG GCA AGC CGT CTC CTG CTC AA-3', corresponding to the sequence encoding the first six amino acids of the mature protein and containing an Nde I restriction site for ligation into the expression vector, which at the same time provided the ATG codon for an additional methionine in position 1. At the 3' end the specific primer: 5'-GAA TTC TCA AAA CTC TTC ATG GAA-3' encoded the last five amino acids, followed by a stop codon and an Eco RI restriction site. After an initial denaturation step at 95°C for 5 min, 35 amplification cycles were performed (1 min at 95°C, 30 s at 50°C, 1 min at 72°C) followed by a final step of 7 min at 72°C. An amplification product of about 900 bp was obtained, in agreement with the expected size (948 bp). The crude PCR product was ligated into a pGEM vector without further purification, using a 1:5 (plasmid:insert) molar ratio and incubating the mixture overnight, at room temperature. After transformation of *E. coli* XL-1 Blue competent cells with the ligation product, positive colonies were selected by PCR using the plasmid's primers SP6 and T7 and grown in LB/ampicillin medium. DNA was extracted using the Plasmid MiniPrep Kit and custom sequenced at Eurofins MWG. pGEM plasmid containing the appropriate sequence was digested with Nde I and Eco RI restriction enzymes for 2 h at 37°C and the digestion product was separated on agarose gel. The obtained fragment was purified from gel using QIAEX II Extraction kit and ligated into the expression vector pET30,

previously linearized with the same enzymes. The resulting plasmid was sequenced and shown to encode the mature protein.

For expression of recombinant protein, the pET-30 vector, containing the sequence encoding *h*AKR1B1, was used to transform *E. coli* BL21(DE3)pLysS cells. Protein expression was induced by adding IPTG to a final concentration of 0.4 mM when the culture had reached a value of O.D.$_{600}$=0.8. Cells were grown overnight at 37°C, then harvested by centrifugation and sonicated. The enzyme was purified from the cell extract by three chromatographic steps. In particular, the cell extract (approximately 0.3 g of proteins) in 0.1 M sodium phosphate buffer pH 7 containing 2 mM DTT (standard buffer) was applied to a column (30 x 3.5 cm) of DE-52 resin, previously equilibrated in standard buffer, and eluted by the same buffer at a flow rate of 15 ml/h monitoring on each fraction the absorbance at 280 nm until the value declined to approximately 0.5. A linear gradient of NaCl ranging from 0 to 120 mM in standard buffer was then applied allowing the enzyme to elute at approximately 100 mM salt concentration. Active fractions were pooled, concentrated and dialyzed against standard buffer devoid of DTT on Ultracel® 10 kDa ultrafiltration membrane, and then applied on a 23x3.5 cm column containing Matrex Orange A resin, previously equilibrated in standard buffer devoid of DTT and eluted in the same buffer at a flow-rate of 15 ml/h until the absorbance values at 280 nm fit the baseline. The enzyme was then eluted by supplementing the elution buffer of 0.1 mM NADPH. The fractions containing hAR activity were pooled, supplemented of 2 mM DTT and concentrated on Ultracel® 10 kDa ultrafiltration membrane up to 7 mL.

The sample was then applied on a Sephadex G-75 column (80x2.6 cm) previously equilibrated in standard buffer and eluted at a flow rate of 15 ml/h.

The fractions exhibiting enzymatic activity were tested for purity by SDS-Page electrophoresis using the silver stain nitrate technique (Laemmli, 1970; Wray et al., 1981), pooled, and concentrated on a YM10 membrane. The purified human recombinant enzyme (5.3 U/mg of protein) was stored at -80°C in standard buffer supplemented of 30% (w/v) glycerol. Immediately before use, the enzyme was extensively dialyzed against standard buffer devoid of DTT.

Protein concentration determination

The protein concentration of enzyme preparations was evaluated by the Bradford method (Bradford, 1976).

Inhibition assay

The sensitivity of AKR1B1 to inhibition was tested in the presence of inhibitors dissolved at proper concentrations in DMSO; the concentration of DMSO was kept constant at 1 % (v/v). IC_{50} values were determined by non linear regression analysis by fitting the data to the equation describing one site competition in a log-dose inhibition curve. The analysis was performed using standard statistical software (GraphPad Instat version 6.0, San Diego, CA). Each curve was generated using at least five concentrations of inhibitors and each concentration was tested at least in triplicate.