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STRUCTURE-BASED DESIGN OF AN INHIBITOR MODELED AT THE SUBSTRATE ACTIVE SITE OF ALDOSE REDUCTASE

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Abstract: This study presents the first successful example of structure-based drug design on aldose reductase in the extant literature. Starting from the structure of the modeled complex of aldose reductase with a pyridazinone acetic acid inhibitor that we previously disclosed, using the tools of molecular modeling for structure manipulation and molecular mechanics for energy minimization, we were able to design and synthesize a new analog that showed remarkably improved activity. We hope that a proper account of the most important enzyme-inhibitor interactions revealed by this study will allow, in the future, the design of new lead compounds having structures unrelated to carboxylic acids.

Structure-based drug design has become increasingly important in the rational process of discovery of new analog and lead compounds.[1-3] The availability of experimentally determined structures of complexes from protein crystallography in combination with the powerful tools of theoretical molecular modeling have provided unique opportunities to better understand structure-activity relationships, suggest new ligands to synthesize, develop models and make predictions. In particular, structural information can be used to preserve the critical interactions with the protein, while modifying the ligand to interact more effectively with the target and to occupy accessory sites, resulting in better potency and specificity.

In the present communication we go into the strategy, and present the results, of a molecular modeling study that successfully allowed us to design a potent inhibitor of aldose reductase (alditol:$NADP^+$ oxidoreductase, EC 1.1.1.21, ALR2), the first enzyme in the "polyol pathway" which converts glucose to sorbitol and is apparently involved in the etiology of late onset diabetic complications.[4,5]

In a previous publication we reported on the synthesis, biological evaluation and molecular modeling of a new series of tricyclic pyridazinones as selective aldose reductase inhibitors.[6] These compounds belong to the structural class of carboxylic acid inhibitors which, together with the spirohydantoines, constitute the most important classes of ARIs developed to date. In that publication we proposed the structure of a ternary complex, obtained by docking, molecular mechanics and molecular dynamics simulations, formed by ALR2, the cofactor $NADP^+$ and one of the most interesting inhibitor of our series taken as a model (**Ia**).[6]

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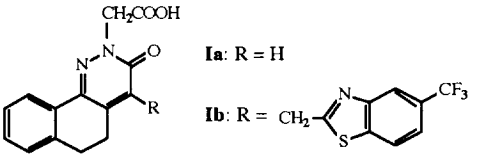
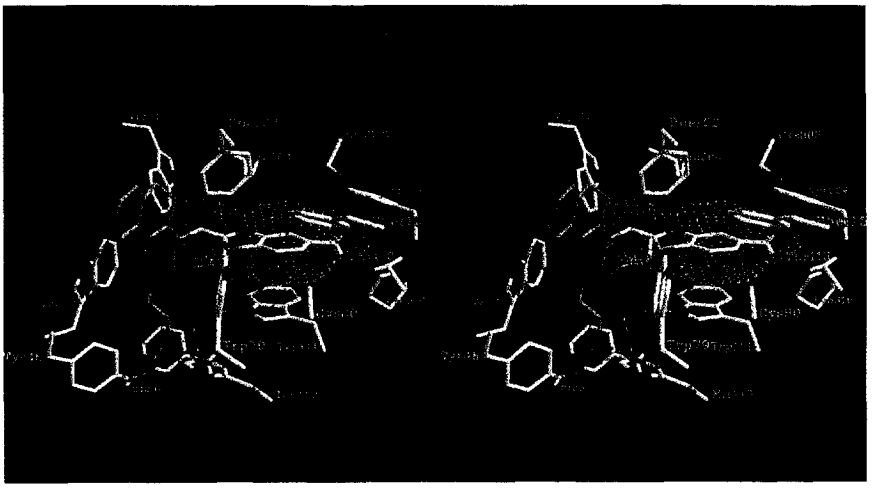


The complex was constructed starting from the coordinates of the human ALR2 holoenzyme by docking the carboxylate of the inhibitor in the so-called "anionic site" [7] of the enzyme formed by the nicotinamide ring of $NADP^+$ and the aminoacidic side chains of Tyr48, His110 and Trp111 (the substrate site). In this regard, the non-competitive or uncompetitive type of inhibition displayed by ARIs, including tricyclic pyridazinones[6], induced some uncertainty on the location of the ARIs binding site. However, even though on the basis of affinity labeling experiments an alternative binding site different from both the substrate and the cofactor site has been proposed[8], a convincing explanation for the apparent lack of competitive behaviour based on the mechanism of action of ALR2 has been given [9,10] which supports, even for inhibitors which appear as non competitive, an interaction at the substrate active site. Such an apparent failure of the kinetic approach in discriminating between different types of inhibition makes the experimental structural studies the ultimate tools able to unequivocally define the site of binding. Accordingly, the decision to dock the carboxylate of the inhibitor **Ia** into the substrate active site was prompted by the experimental evidence that the carboxylate of the potent inhibitor zopolrestat occupies this site in the crystal structure of its complex with ALR2 and $NADP^+$.[11]

Since the discovery of zopolrestat it has been hypothesized, on the basis of SAR studies, that there must be an accessory binding site on aldose reductase with strong affinity for benzothiazoles.[12,13] In the crystal structure of zopolrestat complexed with ALR2 and $NADP^+$, the carboxylate of the inhibitor occupies the anionic site, and the benzothiazole side chain occupies a hydrophobic pocket lined by Trp111 and Leu300. On the contrary, in our modeling of the complex of pyridazinone **Ia** with ALR2, the inhibitor did not participate in binding the benzothiazole site. Therefore, it is now very interesting to see whether the pyridazinones can be functionalized with a benzothiazolyl moiety specifically designed to fit this site, while maintaining the carboxylate in the anionic site. The structure previously obtained from the modelling of the complex of **Ia** clearly suggests that the substituent has to be introduced at position 4 of the pyridazinone. Accordingly, the 5-(trifluoromethyl)-benzothiazol-2-yl-methyl fragment was modeled at position 4 of the pyridazinone (compound **Ib**), and the energy of the complex with ALR2 and $NADP^+$ was minimized with molecular mechanics.[14] If the hypothesis that our inhibitors bind at the anionic site was indeed correct, and if the previously calculated structure of the complex of **Ia** with ALR2 was a faithful representation of the enzyme-inhibitor complex, we could expect the occupation of both the anionic and the benzothiazole sites of ALR2 to lead to an increase in binding affinity.

Figure 1 illustrates a selection of residues (3Å) interacting with the inhibitor in the structure of the minimized complex. The results show that the inhibitor is still able to bind the anionic site (dotted hydrogen bonds with Tyr48, His110 and Trp111), its carbonyl still hydrogen bonds Cys298 and, very interestingly, it places the benzothiazole fragment in the site suitable for interaction. This last fragment is in contact with Trp79, Cys80, Thr113, Phe115, Phe122, Trp219, Cys298, Leu300, Cys303, Tyr309 and Pro310, all conserved among human, bovine and porcine aldose reductases.

Figure 1: Selection of residues interacting with the inhibitor **Ib** (yellow) in the structure of the minimized complex with ALR2 (cross-eyed stereoview). The Van der Waals surface of the benzothiazole fragment of **Ib** is displayed as dots, and the hydrogen bonds of the carboxylate of the inhibitor with Tyr48, His110 and Trp111 are drawn as dashed lines.



The comparison of the interaction energies of this inhibitor with those previously reported for the unsubstituted compound[6] reveals a substantial gain in interaction energy with several aminoacids (Table 1).[15] While the interaction energies of the inhibitors **Ia** and **Ib** with the residues Tyr48 and His110 of the anionic site and with Cys298 are almost unaffected by substitution with the benzothiazolyl moiety, there is a net increase in interaction energy mainly attributable to the residues having contacts with the benzothiazole (Table 1). Of particular interest is Trp111, because it is hydrogen bonded to the carboxylate of the inhibitor (as in the unsubstituted compound) but also stacks the benzothiazole ring of the inhibitor. The decomposition of the interaction energy of **Ib** with Trp111 into its electrostatic and Van der Waals components (Table 1) indicates that the increase in interaction energy with respect to **Ia** is due to the Van der Waals term (-11.0 Kcal/mol *vs.* -2.5 Kcal/mol), a finding that suggests that the stacking of the benzothiazole with Trp111 is an important interaction in the benzothiazole binding site.

Table 1: Interaction energies (Kcal/mol) between the inhibitors **Ia** and **Ib** and some selected protein residues in the minimized structures of their complexes with ALR2

	Ia	*Ib*		*Ia*	*Ib*
Tyr48	-14.8	-14.6	Trp79	-1.7	-3.0
His110	-17.7	-17.7	Cys80	-0.2	-0.7
Cys298	-6.1	-6.0	Phe115	0	-1.5
Trp111[a] - tot	-14.2	-20.4	Phe122	-3.8	-4.6
- VdW	-2.5	-11.0	Leu300	-1.9	-2.5
- Elec	-11.2	-9.2	Cys303	-0.9	-2.2
Trp20	-6.0	-8.6	Tyr309	-1.6	-3.7
Trp219	-3.7	-2.7	Pro310	-1.3	-1.2

a) The interaction energies of **Ia** and **Ib** with Trp111 have been decomposed into their Van der Waals (VdW) and electrostatic (Elec) components.

Taking into account conformations, it is known that the structure of the ALR2 holoenzyme cannot accomodate zopolrestat unless a change in conformation is induced in the NADPH-bound enzyme.[11] In particular, significant conformational changes were reported for a loop (residues 121-135) and a short segment (residues 298-303).[11] Similarly, in our previous molecular dynamics simulations of the structure of the unsubstituted compound **Ia** in complex with ALR2 a substantial motion of these chains occurred, especially for the loop, which showed the highest root-mean square deviations (rmsd) from the structure of the holoenzyme.[6] In the present calculations of the structure of the complex with **Ib** a much more pronounced conformational change of the segment 298-303 occurs; this probably arises from the fact that Leu300 partially occludes the benzothiazole binding site in the holoenzyme, so that a rearrangement of the whole segment is required to better accomodate the benzothiazole of the inhibitor. Table 2 compares the rmsd values of the structures of the loop and the segment of the complexes of **Ia** and **Ib** with the corresponding chains in the X-ray structures of the holoenzyme and the zopolrestat-bound enzyme. As far as the conformation of the loop is concerned, the complex of **Ib** turns out to be more similar to the zopolrestat-bound form than to the holoenzyme (rmsd values of 1.27 *vs* 1.78, respectively). The complex of **Ia** shows much more similar values of rmsd (1.28 *vs* 1.20). As for the conformation of the segment, the complex of **Ia** is much more similar to the holoenzyme than to the zopolrestat-bound form (0.73 *vs* 1.52, respectively); on the contrary, **Ib** is more similar to the zopolrestat-bound form than to the holoenzyme (1.30 *vs* 1.65, respectively). Therefore, the complex of **Ib** resembles the conformation of the zopolrestat-bound form much more closely than the conformation of the holoenzyme.

Table 2: Root-mean square deviations (rmsd) of loop 121-135 and of segment 298-303 among the structures of the holoenzyme(H), the zopolrestat-bound enzyme (Z) and the complexes modeled with **Ia** and **Ib**.

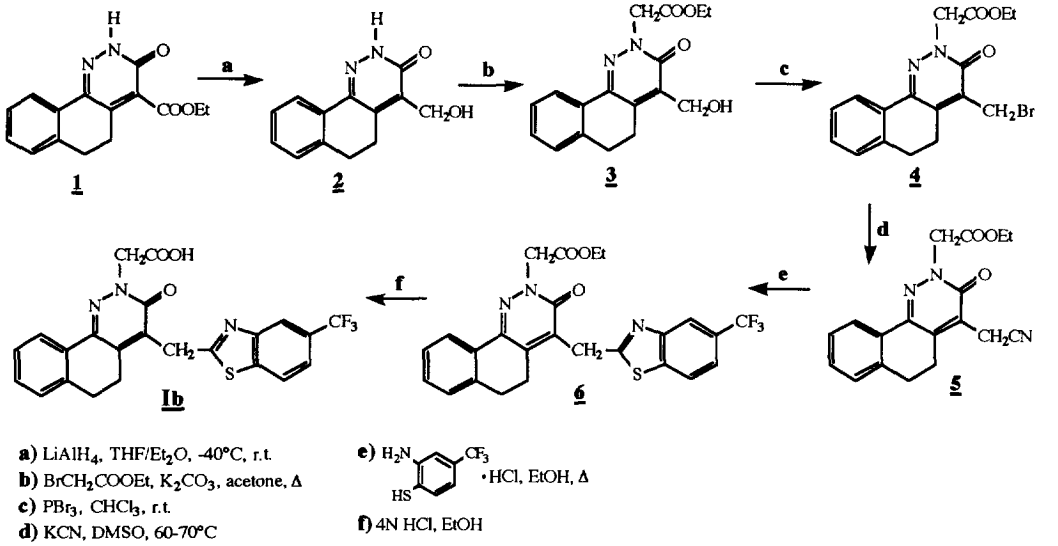
	loop 121-135		**segment 298-303**	
	H[a]	*Z*[b]	*H*[a]	*Z*[b]
Ia[c]	1.20	1.28	0.73	1.52
Ib[d]	1.78	1.27	1.65	1.30

Only the Cα-carbons were used in the evaluation of the rmsd, since only these are included in the coordinates of the complex with zopolrestat in the Protein Data Bank (entry code 1MAR). *a.* Crystal structure of the Holoenzyme (Wilson et el., *Science* 1992, 257, 81-84); *b.* Crystal structure of the Zopolrestat-bound enzyme (ref. 11); *c.* and *d.*, modeled structures of the complexes of Ia (ref.6) and Ib (present work), respectively.

The foregoing analysis indicates both a very good complementarity of **Ib** with the anionic and benzothiazole sites of ALR2 and a favourable interaction energy; therefore, **Ib** is a valid candidate for the purposes previously stated.

Accordingly, the inhibitor **Ib** was synthesized as shown in Scheme 1.(**Ib**: mp=241°C. ^{1}H-NMR δ: 2.8-3.1 (m, 4H); 3.2 (s, 2H); 4.6 (s, 2H); 7.2-7.4 (m, 3H); 7.6-7.8 (m, 1H); 7.8-8.0 (m, 1H); 8.2-8.4 (m, 2H). $C_{23}H_{14}F_3N_3O_3S$ (C,H,N,S)).

Scheme 1: Synthesis of compound **Ib**:



The activity of compound **Ib** was tested for the inhibition of bovine lens ALR2. The results are extremely positive since the new pyridazinone **Ib** turns out to be about two orders of magnitude more active than the parent unsubstituted compound against highly purified bovine lens ALR2. Indeed, the IC_{50} of **Ib** results 0.15±0.03μM, against the IC_{50} of 12±3μM measured for **Ia**.[16] Moreover, it is interesting to report that, while **Ia** is inactive[6] against S-glutathionyl-ALR2, a modified enzyme form[17,18] present in the lens in conditions of oxidative stress[19] and less susceptible to inhibition by classical ARIs,[17] **Ib** shows good inhibitory activity also for this modified form with an IC_{50} of 17±4μM.

Encouragingly, the finding that the prediction obtained by the theoretical modeling of the complex has, indeed, ended up with a remarkably improved inhibitory activity is a confirmal that ligand-enzyme interactions are sufficiently well described in our model. Further evidence will be provided by a structure-activity study on a series of new derivatives (work in progress). Moreover, we do hope that our studies will enable, in the future, the design of compounds with structures even very different from carboxylic acids in an attempt of finding new leads.

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