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Selective binding of coumarin enantiomers to human α_1-acid glycoprotein genetic variants

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Abstract—Coumarin-type anticoagulants, warfarin, phenprocoumon and acenocoumarol, were tested for their stereoselective binding to the human orosomucoid (ORM; AGP) genetic variants ORM 1 and ORM 2. Direct binding studies with racemic ligands were carried out by the ultrafiltration method; the concentrations of free enantiomers were determined by capillary electrophoresis. The binding of pure enantiomers was investigated with quinaldine red fluorescence displacement measurements. Our results demonstrated that all investigated compounds bind stronger to ORM 1 variant than to ORM 2. ORM 1 and human native AGP preferred the binding of (*S*)-enantiomers of warfarin and acenocoumarol, while no enantioselectivity was observed in phenprocoumon binding. Acenocoumarol possessed the highest enantioselectivity in AGP binding due to the weak binding of its (*R*)-enantiomer. Furthermore, a new homology model of AGP was built and the models of ORM 1 and ORM 2 suggested that difference in binding to AGP genetic variants is caused by steric factors.

1. Introduction

Human α_1-acid glycoprotein (AGP, orosomucoid (ORM)), a member of the lipocalin family, is one of the most important glycoprotein components of blood plasma that binds both endogenous and exogenous ligands with a chemically diverse structure.[1,2] Although its precise biological function is yet unclear, AGP is suggested to play a role in immunomodulation.[3,4] Binding of drugs to AGP may have clinical importance as it influences both the pharmacokinetics and pharmacodynamics[5] of a drug. Moreover, under various pathological and physiological conditions the plasma concentration of AGP may increase up to three- or four-fold which results in an alteration in the binding of drugs and other ligands.[2,4]

The AGP molecule consists of a single polypeptide chain of 183 amino acids and of five asparaginyl linked glycans. Besides the high heterogeneity of glycans, the protein part has also been found to show polymorphism.[6] The variants are encoded by two different genes: The F1 and S variants are encoded by the alleles of the same gene, while the A variant is encoded by a different gene.[6] There is a difference of at least 22 amino acid residues between the F1–S (ORM 1) and A (ORM 2) variants, while F1 and S forms differ only in a few residues.[7] ORM 1 and ORM 2 variants were shown to possess different binding properties.[8–11] Warfarin is a selective ligand of ORM 1 variant of human AGP[9] and the possibility of high binding stereoselectivity was raised.[9] Nakagawa et al.[12] determined preference in binding of (*S*)-warfarin to ORM 1 variant. Furthermore, structurally related acenocoumarol binding on native AGP was found to have a stereoselectivity factor (K_S/K_R ratio) of 3 in favour of the (*S*)-enantiomer.[13,14]

The three-dimensional X-ray structure of AGP is unknown, therefore, little information is available about the binding mechanism. A three-dimensional model of AGP based on other members of the lipocalin family was presented in 1993[15] and refined in 2003.[16] Former docking calculations of some of the present authors based on the latter model yielded the unlikely result where warfarin was bound at the surface of AGP,[17] indicating that the model underestimates the size of the binding cavity.

Abbreviations: AGP, α_1-acid glycoprotein; ORM, orosomucoid; QR, quinaldine red; CE, capillary electrophoresis.

Keywords: AGP; Genetic variants; Acenocoumarol; Phenprocoumon; Warfarin; Capillary electrophoresis; Displacement study; Molecular modelling.

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In the current study, stereoselective binding of warfarin and two more structurally related anticoagulants, phenprocoumon and acenocoumarol (Fig. 1), to F1–S (ORM 1) and A (ORM 2) variants of AGP was investigated. Direct binding measurements with racemates and displacement study using pure enantiomers were carried out. It was anticipated that examination of the binding of these three compounds helps in revealing which functional group of the chiral molecule is responsible for interactions with AGP. Furthermore, a new homology model of ORM 1 and ORM 2 was constructed based on published X-ray structure of members of the lipocalin protein family in order to identify the interactions influencing the ligand binding properties of the genetic variants.

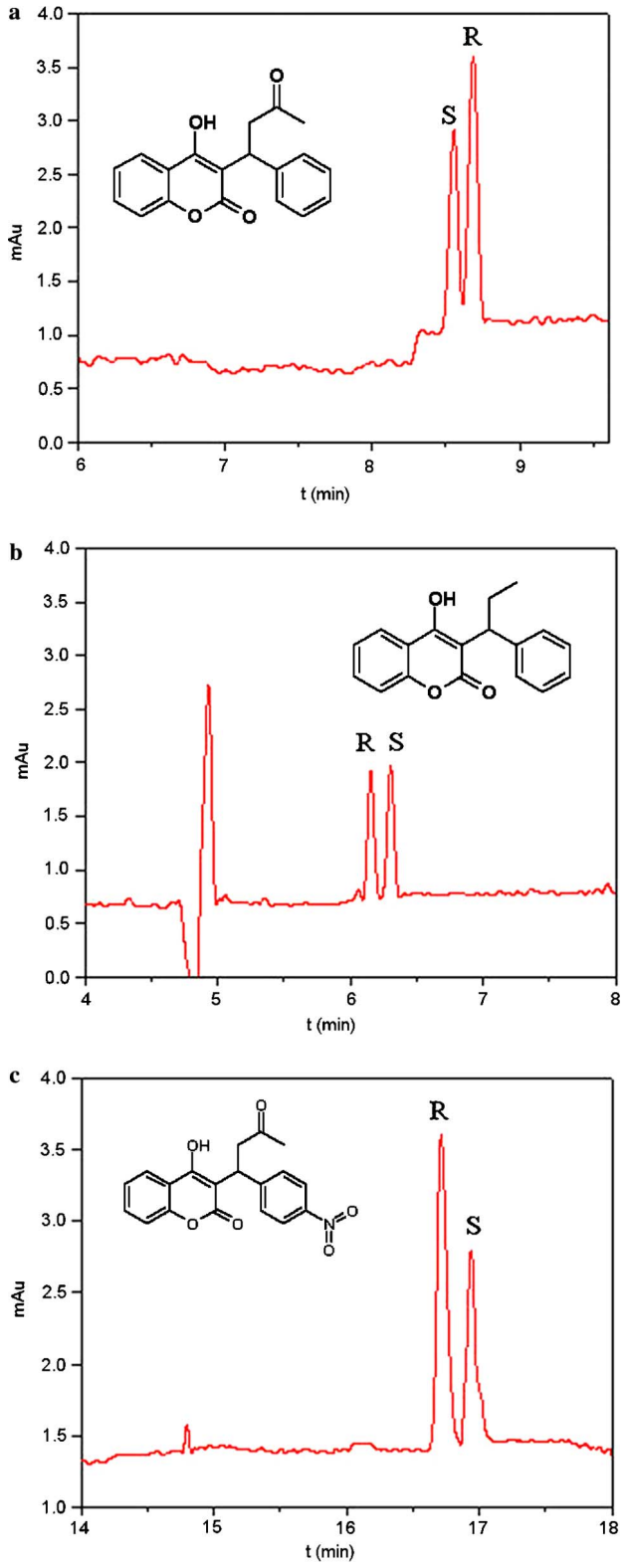

Figure 1. Electropherograms of the chiral separation of coumarin enantiomers in ultrafiltrates (**a**), warfarin (separation was achieved using random methylated β-cyclodextrin) (**b**), phenprocoumon (α-cyclodextrin was used as a chiral selector) (**c**), acenocoumarol (sulfopropylated β-cyclodextrin was used as a chiral selector).

2. Methods

2.1. Chemicals

rac-Warfarin, human AGP and quinaldine red (QR) was purchased from Sigma–Aldrich and Co. (St. Louis, MO). Sulfopropylated β-cyclodextrin, α-cyclodextrin and random methylated β-cyclodextrin were kindly donated by Cyclolab (Budapest, Hungary). *rac*-Acenocoumarol was obtained from Alkaloida Chemical Factory (Tiszavasvári, Hungary). Phenprocoumon enantiomers were generous gift of Hoffmann-LaRoche (Basel). All other chemicals were from Reanal, Hungary. Warfarin enantiomers were obtained using the resolution method of West et al.[18] Resolution of acenocoumarol was carried out as described previously.[13] The two main genetic variants of human AGP were separated following the method of Hervé et al.[19] producing about 70% F1–S (ORM 1) variant mixture and 30% of pure A (ORM 2) variant. Native AGP concentration was calculated by using a molecular mass value of 44,000. AGP variant solutions of the same concentration were prepared by adjusting them to the UV absorbance of native AGP solution.

2.2. Fluorescence measurements

Fluorescence measurements were carried out on a Shimadzu RF-1501 spectrofluorophotometer at room temperature (24 ± 1 °C), using quartz cuvette with 1 cm optical path length. Excitation wavelength was 495 nm and emission spectra were recorded at 530–630 nm, both bandwidths set at 10 nm. Aliquots of 2 ml AGP solutions (2 μM) were labelled with QR (2 mM stock solution in DMSO), 2 μM QR used for native AGP and ORM 1 variant, 4 μM for the ORM 2 variant. Titrations were performed with 2 mM coumarin stock solutions in Ringer buffer. Fluorescence intensities were corrected with that of the AGP solution, coumarins causing no disturbance.

2.3. Binding experiments

Coumarins were dissolved in 0.1 M NaOH and diluted with Ringer buffer, pH 7.4. The binding experiments were carried out at room temperature in Ringer buffer, pH 7.4. Coumarins and native AGP or its genetic variants were used at 50 μM concentration. Ultrafiltration was performed with an Amicon MPS-1 system using

YMT 30 membranes. Non-specific loss was checked by the filtration of protein-free solutions.

2.4. Capillary electrophoresis

Capillary electrophoresis measurements were performed on an Agilent CE system combined with a diode array UV–vis detector. On-line detection was used at 210 nm. Collection and evaluation of the data were performed using Agilent Chemstation software. Uncoated fused-silica capillary 48.5 cm (effective length 40 cm) × 50 μm was used throughout the study. The capillary was thermostated at 12.5 °C. Samples were introduced by electrokinetic injection using 30 kV for 3 s. The applied voltage was 30 kV for the electrophoretic separations. All sample solutions were filtered through a 0.2 μm filter. Migration orders of the enantiomers were checked using pure enantiomers.

2.5. Separation of coumarin enantiomers

In case of acenocoumarol, 120 mM Britton–Robinson buffer—consisting of 40 mM acetic acid, 40 mM phosphoric acid and 40 mM boric acid—prepared according to Britton[20] was used as running buffer at pH 5.0. Sulfopropylated β-cyclodextrin was used as a chiral selector at a concentration of 5.0 mM. Resolution of phenprocoumon enantiomers was carried out in 120 mM Britton–Robinson buffer at pH 7.0. α-cyclodextrin was used as chiral selector at a concentration of 15.0 mM. For separation of warfarin enantiomers random methylated β-cyclodextrin at 8.0 mM concentration was used as chiral selector dissolved in a 98% 50 mM phosphate buffer, pH 8.4/2% MeOH solution.

2.6. Protein modelling

Sequences of ORM 1 and ORM 2 were from the Protein Information Resource,[21] (NF00080879 and NF00081090). Multiple sequence alignments were performed by 'ClustalW' software using default parameters.[22] Amino acid similarity was determined using Blosum62 matrix. Homology model building of ORM 1 and ORM 2 was carried out using 'Nest'[23] and 'Loopy'[24] programs of the Jackal protein structure modelling package. The homology models were based on the experimentally determined structure of the major horse allergen, a member of the lipocalin protein family (taken from the Protein Data Bank,[25] PDB entry: 1EW3.[26]) Refinements of the resulting structures were carried out by 'Minst' program of the Jackal protein structure modelling package on a Silicon Graphics Octane workstation under Irix 6.5 operation system. The program Voidoo[27] was used to identify binding cavities and calculate cavity volumes for AGP.

'AutoDock 3.0'[28] was applied for docking calculations, using the Lamarckian genetic algorithm (LGA) and the 'pseudo-Solis and Wets' (pSW) methods. AutoDock calculates the free energy of binding in solvent using a scoring function, which was parameterized by experimental data of inhibition constants. The parameters included in Autodock are based on the 'assisted model building with energy refinement' (AMBER) force field.[29] Gasteiger–Hückel partial charges were applied both for ligands and proteins. Solvation parameters were added to the protein coordinate file and the ligand torsions were defined using the 'Addsol' and 'Autotors' utilities, respectively, in Autodock 3.0. The atomic affinity grids were prepared with 0.375 Å spacing using the Autogrid program for a $20 \times 20 \times 20$ Å box around the ligand binding site. Random starting positions, orientations and torsions (for flexible bonds) were used for the ligands. Each docking run consisted of 100 cycles. The number of evaluations was set to 1.5 million. Final structures with rmsd less than 2.0 Å were considered to belong to the same cluster. Best energy results of dockings were investigated. Intermolecular interactions were analysed by HBPLUS v 3.0, a hydrogen bond calculation program, the algorithm of which involves finding the positions of the hydrogen atoms and calculating the hydrogen bonds.[30]

3. Results

3.1. Ligand binding measurements by stereoselective analysis of the ultrafiltrates

Binding of racemates to AGP genetic variants was investigated by the ultrafiltration method. The determination of unbound ligand concentration and resolution of the free enantiomers in the ultrafiltrates was carried out by capillary electrophoresis method. Figure 1 shows the electropherograms of the coumarin enantiomers demonstrating that baseline separation was achieved for each investigated enantiomer pair. In Table 1, the free fractions of coumarin enantiomers in the ultrafiltrates are summarized. All ligands were found to possess a higher affinity towards ORM 1 than towards ORM 2 variant. Among the investigated coumarins, (*R*)-phenprocoumon displayed the highest affinity to ORM 2. All other ligands were found to bind to ORM 2 with a very low affinity—the bound fractions being less than 30%. In agreement with these findings, binding to native AGP (consisting of about 70% ORM 1 and 30% ORM 2) resembled binding to ORM 1 rather than to ORM 2 in enantioselectivity indicating only a minor role of ORM 2 in the binding of coumarins.

No significant enantioselectivity was observed in binding to ORM 2. In case of phenprocoumon, no enantiomeric selectivity was observed in binding to ORM 1 either. In contrast, (*S*)-enantiomers of warfarin and acenocoumarol were found to possess a higher affinity for ORM 1 than the corresponding (*R*)-enantiomer. The enantioselectivity value was 1.8 for warfarin, while acenocoumarol possessed the highest stereoselectivity (2.6) due to the much weaker binding of (*R*)-acenocoumarol as compared to its (*S*) enantiomers.

3.2. Displacement of quinaldine red

QR is a specific fluorescent probe for AGP.[31,32] This basic dye molecule has negligible fluorescence in buffer. It is highly fluorescent when bound to native AGP, the

Table 1. Binding of acenocoumarol, phenprocoumon and warfarin enantiomers to AGP fractions and native AGP

Ligand	Chirality	Free fraction of the enantiomers (%)		
		ORM 1	ORM 2	Native AGP
Acenocoumarol	*S*	46.3 ± 1.1	79.3 ± 2.5	53.7 ± 1.9
	R	69.2 ± 2.3	77.8 ± 1.2	72.4 ± 3.2
	K_S/K_R	2.61 ± 0.5	0.91 ± 0.11	2.26 ± 0.09
Phenprocoumon	*S*	44.4 ± 1.5	74.3 ± 1.7	40.8 ± 7.1
	R	41.6 ± 2.2	62.4 ± 3.1	45.5 ± 4.9
	K_S/K_R	0.89 ± 0.14	0.57 ± 0.27	1.21 ± 0.21
Warfarin	*S*	44.2 ± 4.6	75.0 ± 4.1	38.9 ± 0.3
	R	59.5 ± 2.8	79.2 ± 5.1	53.0 ± 1.1
	K_S/K_R	1.85 ± 0.12	1.27 ± 0.29	1.77 ± 0.03

In all experiments, $c_{AGP} = c_{rac} = 50$ μM. K_S/K_R values were calculated by assuming one common binding site ($K_S/K_R = \alpha_R(1-\alpha_S)/\alpha_S(1-\alpha_R)$).

binding constant being about $4 \times 10^5\ M^{-1}$. Its fluorescence could be quenched by a series of basic drug molecules, and the acidic *rac*-acenocoumarol[31] and *rac*-warfarin[33] were also found to cause significant displacements. We found that ORM 1 variant showed similar fluorescence to the native AGP, while the fluorescence of the QR–ORM 2 complex was about three times smaller, the binding constants, however, appearing to be similar (data not shown).

Figure 2 shows the quenching of fluorescence of QR bound to native AGP and the genetic variants in the presence of coumarin enantiomers. The coumarins strongly displaced QR from native AGP and ORM 1 variant, with the (*S*)-enantiomers being more effective in all cases. The displacements of QR bound to ORM 2 variant were much less effective: warfarin caused about 40%, phenprocoumon and acenocoumarol about 30% maximal quenching, with stereoselectivities being also

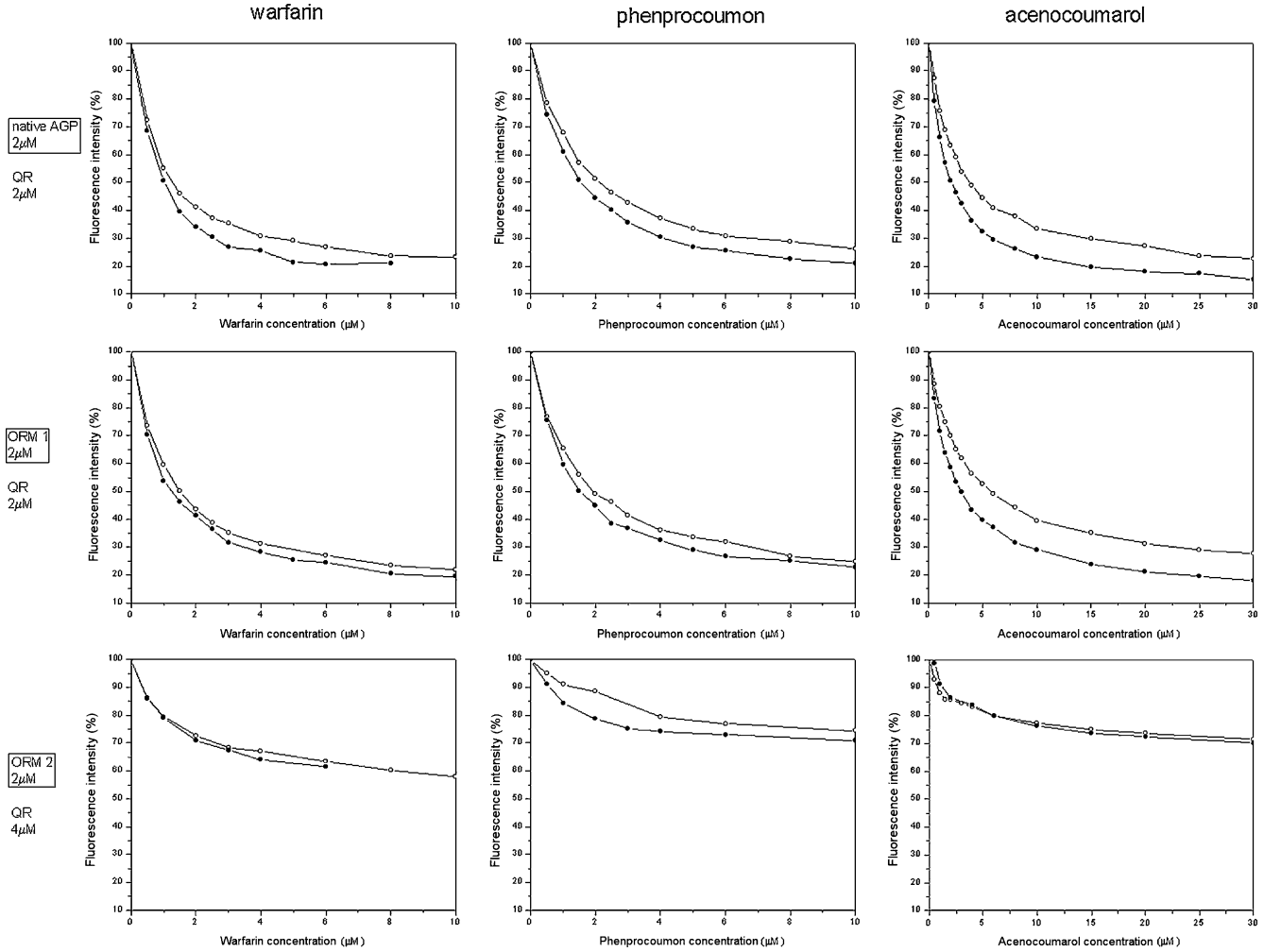

Figure 2. Displacement of QR bound to native AGP, ORM 1 and ORM 2 variants by the enantiomers of warfarin, phenprocoumon and acenocoumarol; (*S*)-coumarin (●), (*R*)-coumarin (○).

Table 2. Stereoselectivity for the binding of coumarins to native AGP (2 μM) and ORM1 variant (2 μM) determined from the displacement of fluorescent label QR (2 μM)

Ligand	Chirality	IC_{50} value (μM)	
		ORM 1	Native AGP
Acenocoumarol	*S*	2.9	2.0
	R	5.8	3.8
S/*R* selectivity		2.0	1.9
Phenprocoumon	*S*	1.5	1.5
	R	1.9	2.1
S/*R* selectivity		1.3	1.4
Warfarin	*S*	1.3	1.1
	R	1.6	1.3
S/*R* selectivity		1.2	1.2

negligible. Thus, competitive displacement can be assumed only on the ORM 1 variant, which is the dominant part of QR fluorescence in native AGP, as well. The concentrations of coumarin enantiomers corresponding to 50% inhibition (IC_{50} values) are given in Table 2. Assuming competition for the same binding site, the ratio of IC_{50} values reflects the ratio of inhibitory dissociation constants.[34] Comparing the IC_{50} values of the investigated coumarins, the following affinity order for both native AGP and ORM 1 was established: (*S*)-warfarin > (*S*)-phenprocoumon > (*S*)-acenocoumarol. The same affinity order was observed in case of (*R*)-enantiomers. The IC_{50} value of (*R*)-acenocoumarol was more than three times higher that of (*R*)-warfarin and (*R*)-phenprocoumon. These results show that the relatively low affinity of (*R*)-enantiomer is responsible for the highest binding stereoselectivity of acenocoumarol.

3.3. Molecular modelling of ligand binding to AGP genetic variants

Molecular modelling calculations were carried out in order to explain the differences in coumarin binding of the genetic variants. Despite the relatively low sequence homology, the three-dimensional structure of the members of the lipocalin protein family is highly conserved. For example, three-dimensional alignment between the major horse allergen (PDB entry: 1EW3) and bovine β-lactoglobulin (PDB entry: 1BSO), two members of the lipocalin family sharing only 18% sequence identity, results in a root mean standard deviation of backbone atoms as low as 1.34 Å. This level of conservation suggests that homology modelling can reliably predict the three-dimensional structure of AGP and the model may be useful for explaining the differences in ligand binding properties of the genetic variants. Homology modelling of both ORM 1 and ORM 2 variants was carried out using major horse allergen (PDB code 1EW3) as a template. Voidoo calculations found one cavity within the protein, where the following residues form the buried part of the cavity in ORM 1: Tyr27, Phe32, Lys39, Ile44, Gln45, Ala46, Phe48, Tyr65, Thr77, Arg90, Val92, Gly93, Glu96, Met111, Asn121, Trp122, Gly123, Leu124 and Tyr127. Moreover, Voidoo calculation found the same positions involved in forming the cavity wall of ORM 2. Two residues differ in the two genetic variants. In case of ORM 2, serine is found at position 77 and glutamate at position 92. Additionally, amino acid residues at positions 98 and 115 forming the entrance of the cavity differ in the two genetic variants, namely Phe98 and Asp115 are found in ORM 1, while Val98 and Tyr115 are found in ORM 2. It should be noted that in position 114 there is a phenylalanine in ORM 1 and serine in ORM 2, but these residues do not point towards the cavity in our model. To summarize, differences in the amino acid sequence in ORM 1 and ORM 2 at the binding cavity substantially alter both the size and hydrophobicity producing a smaller, more hydrophobic cavity in case of ORM 2 as compared to ORM 1.

Docking calculations were performed for both enantiomers of acenocoumarol, phenprocoumon and warfarin to homology models of ORM 1 and ORM 2. All docking results yielded negative docking energies, suggesting that both enantiomers of each ligand can bind to both genetic variants in our model. However, dockings to ORM 1 variant resulted in a much lower intermolecular energy than dockings to ORM 2 (data not shown), suggesting that although binding to both variants is possible, ORM 1 variant binding is more favourable. Energy differences between (*R*)- and (*S*)-enantiomers are not significant and show a slight preference for (*S*)-enantiomers in the case of both ORM 1 and ORM 2. Figure 3 illustrates the binding of the three ligands to ORM 1. The ligands were docked in a very similar position and conformation producing similar intermolecular contacts. However, bound position of the same ligand in ORM 2 markedly differed from the ones bound to ORM 1 (Fig. 4).

The same amino acid residues are involved in the polar interactions with different coumarin-type ligands to ORM 1. Carbonyl oxygen of the coumarin ring accepts

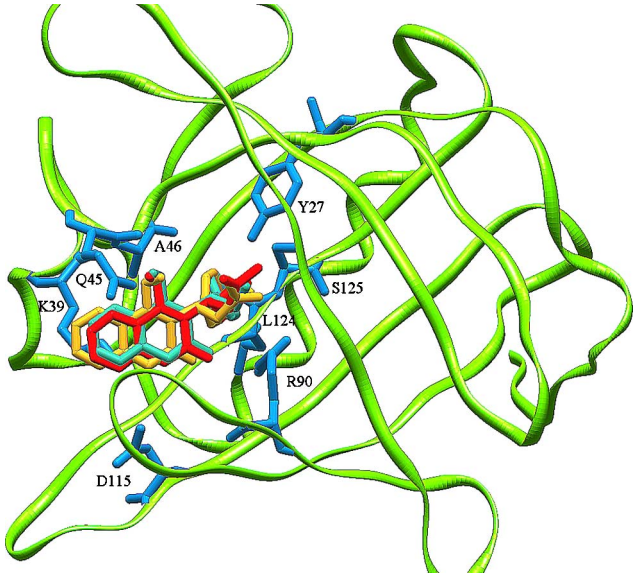

Figure 3. Docking results of (*S*)-warfarin, (*S*)-phenprocoumon and (*S*)-acenocoumarol on the model of ORM 1. The interacting polar residues in the model are indicated.

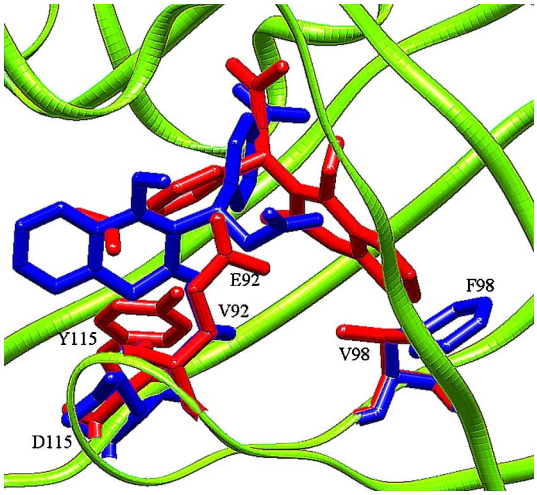

Figure 4. Comparison of dockings to ORM 1 and ORM 2 models. ORM 1 side chains and (*S*)-acenocoumarol docked to ORM 1 are indicated with blue colour, ORM 2 side chains and (*S*)-acenocoumarol docked to ORM 2 with red colour.

a hydrogen bond from Arg90. The negatively charged phenolic oxygen can be an acceptor of a hydrogen bond formed with the amide NH of Ala46. Acetonyl groups of warfarin and acenocoumarol have different interactions in the best energy docking clusters. The conformation of the acetonyl group in warfarin allows the carbonyl oxygen to interact with Lys39 and Asp115 residues. In case of acenocoumarol, both the ring carbonyl oxygen and acetonyl carbonyl oxygen form a hydrogen bond with the amino groups of Arg90. The nitro group present in acenocoumarol has additional interactions with Tyr27, Leu124 and Ser125. Moreover, hydrophobic residues Phe32 and Phe48 are also in interacting distance.

The analysis of the docking results of (*S*)-acenocoumarol to ORM 1 and ORM 2 reveals that the few differences between the two AGP variants lead to different interactions with the ligands (Fig. 4). ORM 2 has a markedly higher number of interacting tyrosine residues (Tyr127, Tyr65, Tyr27 and Tyr115) as compared to ORM 1.

4. Discussion

The binding of drugs to plasma proteins, especially to albumin and AGP, is an important factor in drug distribution and disposition, especially since the relative concentrations of each genetic variant of AGP may change under various physiological and pathological conditions.[4] The genetic variants of AGP were shown to have altered ligand binding properties.[9] The purpose of this study was to characterize the binding affinity and selectivity of coumarins to AGP genetic variants and explain the observed differences at a molecular level.

The crystal structure of AGP has not been reported yet. Therefore, homology models for the genetic variants of AGP were constructed based on the experimentally available structure of the major horse allergen belonging to the lipocalin protein family. There are several differences in the amino acid sequence at the binding cavity of ORM 1 and ORM 2 in our model resulting in a smaller cavity of ORM 2 than that of ORM 1. This indicates steric hindrance of ORM2 for binding of larger ligands. This model is in accordance with earlier results, since ORM 2 was shown to preferably accommodate ligands with limited intramolecular distances.[9] In contrast, ORM 1 binding cavity might contain a broad hydrophobic area or flexible pocket with H-bonding abilities.[36]

To a crude approximation, phenprocoumon and acenocoumarol are structural variants of warfarin. Therefore, comparing warfarin and phenprocoumon, the role of acetonyl group in interactions with AGP can be examined. Binding studies showed no significant difference in binding affinity of phenprocoumon enantiomers to ORM 1, while the *S* enantiomer of warfarin binds stronger to ORM 1 than (*R*)-warfarin. These results demonstrate the role of the side-chain carbonyl group in stereoselectivity. Docking results were in agreement with this experimental finding, since the carbonyl group of (*S*)-warfarin forms a hydrogen bond with Lys39 in our molecular model.

Comparing warfarin and acenocoumarol, the role of nitro group in AGP interactions can be examined. The significantly higher IC_{50} values in QR displacement suggest that the NO_2 group present in acenocoumarol weakens the binding of both enantiomers. The stereoselectivity of acenocoumarol binding is higher than that of warfarin, due to the very weak binding of (*R*)-acenocoumarol to ORM 1. The nitro groups of (*S*)- and (*R*)-acenocoumarol form numerous (attractive and repulsive) van der Waals contacts with the protein and seem to destabilize the binding due to steric hindrance. The reason for the lower affinity of the (*R*)-enantiomer to ORM 1 might be due to the weaker hydrogen bonding between the acetonyl group and the protein side chains in case of (*R*)-acenocoumarol.

The enantiomers of the coumarins were docked to the protein in essentially the same position and orientation, similar to what was observed in the crystal structure of human serum albumin bound warfarin enantiomers.[35] This could be the reason for the relatively poor stereoselectivity of AGP in the case of phenprocoumon and warfarin binding. The stereoselectivities of warfarin, acenocoumarol and phenprocoumon bindings to ORM 1 were very similar to that of native AGP, indicating that binding to ORM 2 variant plays only a minor role.

In conclusion, our binding experiments—in agreement with the displacement studies—demonstrated that all investigated coumarins bound stronger to ORM 1 than to ORM 2 variant. This difference in binding may be due to steric hindrance: ORM 2 had a smaller binding cavity than ORM 1 in our AGP model. Human native AGP preferred the binding of (*S*)-enantiomers of warfarin and acenocoumarol. Acenocoumarol possessed the

highest enantioselectivity in AGP binding due to the weak binding of its (*R*)-enantiomer.

Acknowledgments

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