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Mechanism of inhibition of human secretory phospholipase A2 by flavonoids: rationale for lead design

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Abstract The human secretory phospholipase A2 group IIA (PLA2-IIA) is a lipolytic enzyme. Its inhibition leads to a decrease in eicosanoids levels and, thereby, to reduced inflammation. Therefore, PLA2-IIA is of high pharmacological interest in treatment of chronic diseases such as asthma and rheumatoid arthritis. Quercetin and naringenin, amongst other flavonoids, are known for their anti-inflammatory activity by modulation of enzymes of the arachidonic acid cascade. However, the mechanism by which flavonoids inhibit Phospholipase A2 (PLA2) remained unclear so far. Flavonoids are widely produced in plant tissues and, thereby, suitable targets for pharmaceutical extractions and chemical syntheses. Our work focuses on understanding the binding modes of flavonoids to PLA2, their inhibition mechanism and the rationale to modify them to obtain potent and specific inhibitors. Our computational and experimental studies focused on a set of 24 compounds including natural flavonoids and naringenin-based derivatives. Experimental results on PLA2-inhibition showed good inhibitory activity for quercetin, kaempferol, and galangin, but relatively poor for naringenin. Several naringenin derivatives were synthesized and tested for affinity and inhibitory activity improvement. 6-(1,1-dimethylallyl)naringenin revealed comparable PLA2 inhibition to quercetin-like compounds. We characterized the binding mode of these compounds and the determinants for their affinity, selectivity, and inhibitory potency. Based on our results, we suggest C(6) as the most promising position of the flavonoid scaffold to introduce chemical modifications to improve affinity, selectivity, and inhibition of PLA2-IIA by flavonoids.

Keywords Phospholipase A2 · PLA2 · Inhibitor · Rational design · Flavonoid · Quercetin · Naringenin

Abbreviations

PLA2	Phospholipase A2
PLA2-IB	Phospholipase A2 group IB
PLA2-IIA	Phospholipase A2 group IIA
3D	Three-dimensional
MD	Molecular dynamics

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Introduction

Phospholipase A2 (PLA2, EC 3.1.1.4) catalyzes in a calcium-dependent manner the hydrolysis of the sn-2 position of phosphatidylcholine, phosphatidylethanolamine, and phosphatides at membranes. The mechanism of action of PLA2 includes interfacial binding to the cytoplasmic side of the membrane mainly based on interaction of complementary charges. PLA2 is involved in the generation of

eicosanoids, which play an important role in pathophysiological processes such as inflammation, platelet aggregation, and acute hypersensitivity reactions [1]. Modulation of PLA2 activity is currently a very important pharmacological goal, and there is a great interest in developing specific inhibitors for this enzyme in order to treat certain chronic inflammatory conditions such as rheumatoid arthritis and asthma. To this end, a clear understanding of the interaction of PLA2 enzymes with their ligands is required. During the last two decades computer-assisted methods have been proven to be very powerful and successful in improving our understanding of structure-activity relationships of ligands and their receptors, in particular enzyme–inhibitor complexes, and using this information to rationally modify their interaction [2–4].

Flavonoids are widely produced polyphenolic plant secondary metabolites. Although most often recognized as pigments in flowering plants, flavonoids are distributed to all parts in plants. Some of these flavonoids have demonstrated selective inhibition of PLA2 at micromolar range (i.e., quercetin [5]). This, together with the good accessibility for chemical modifications (Fig. 1) of some of these molecules (i.e., naringenin) makes them suitable chemical scaffolds for development of leads for anti-allergic, anti-cancer, anti-HIV, and anti-inflammatory treatment. However, the binding mode of flavonoids to PLA2 as well as their inhibitory mechanism is still not understood. In our studies we used a combined approach of computational and experimental methods to investigate the binding mode of several naturally occurring flavonoids and flavonoid-based molecules in PLA2 and to characterize their inhibitory mechanism and activity. Utilizing existing three-dimensional (3D) high-resolution data of PLA2 enzymes and their complexes with different ligands, detailed structural models were developed to understand and rationalize the molecular interaction of PLA2 with flavonoids. Molecular Dynamics simulations (MD) showed distinct binding modes in agreement with experimental binding and inhibition data. Both, PLA2 binding and inhibition were measured separately using an improved protocol (M. Böhl et al. 2007, submitted data). Our results allowed us to characterize the determinants of binding affinity and specificity of the flavonoid scaffold toward PLA2, and to propose a rationale to modify these compounds to obtain leads for inhibition of PLA2.

Materials and methods

Set of compounds

The set of compounds used in this study consists of several flavone- and flavanone-like molecules (Sigma, Steinheim, Germany) and five synthesized naringenin-derivatives (7-*O*-allylnaringenin, 8-prenylnaringenin, 6-propylnaringenin, 6-allylnaringenin, and 6-(1,1-dimethylallyl)naringenin) (Fig. 1). All flavonoid 3D structures were generated with the Molecular Builder module of the Molecular Operating Environment (MOE) [6]. AMBER nomenclature and atom types were used. Missing parameters for bond length, angles, and dihedrals were derived from known structures from the Cambridge Structural Database (CSD) [7]. Charges were calculated with MOPAC using semi-empirical Hamiltonian AM1 [8, 9].

Chemical synthesis of naringenin-based compounds

All naringenin derivatives were generated starting from naringenin (**1**) (Fig. 2) by applying an efficient four-step protocol for the synthesis of 8-prenylnaringenin (**2**). This reaction sequence consists of acetylation, *Mitsunobu O*-prenylation, domino *Claisen-Cope* rearrangement, and deacetylation [10]. 6-(1,1-Dimethylallyl)naringenin (**3**) was isolated after deacetylation of the byproduct of an incomplete domino rearrangement. The 7-*O*-allylated naringenin derivative **5** was formed as a byproduct of a *Mitsunobu* reaction with allyl alcohol. After *Claisen* rearrangement of the major product **4** [11] and subsequent deprotection 6-allylnaringenin (**6**) resulted. 6-Propylnaringenin (**7**) was generated by hydrogenation of compound **6**.

Computational characterization of flavonoid interactions with PLA2

For our computational investigations we used available high-resolution structural data of proteins and protein–ligand complexes from the PDB [12]. First of all, structures of Phospholipase A2 group IIA (PLA2-IIA) enzymes with ligands were used to characterize key interactions in the binding crevice of these enzymes. Likewise, structures of PLA2 group IB (PLA2-IB) were analyzed and compared to PLA2-IIA to account for selectivity. Second, structures of flavonoids in complex with other proteins were analyzed to understand the interaction modes of these ligands. We searched for experimentally known protein complexes including flavonoids using the Ligand Search interface of the PDB. We analyzed quercetin in complex with phosphoinositide 3-kinase (1E8W) and with 2,3-dioxygenase (1H1I), as well as naringenin in chalcone synthase (1CGK), chalcone isomerase (1EYQ), and anthocyanidin synthase (2BRT) [13–17].

The structures of PLA2-IIA and PLA2-IB were taken from PDB entries 1KQU and 1L8S, respectively [18, 19]. The ligands in both structures were removed. The two

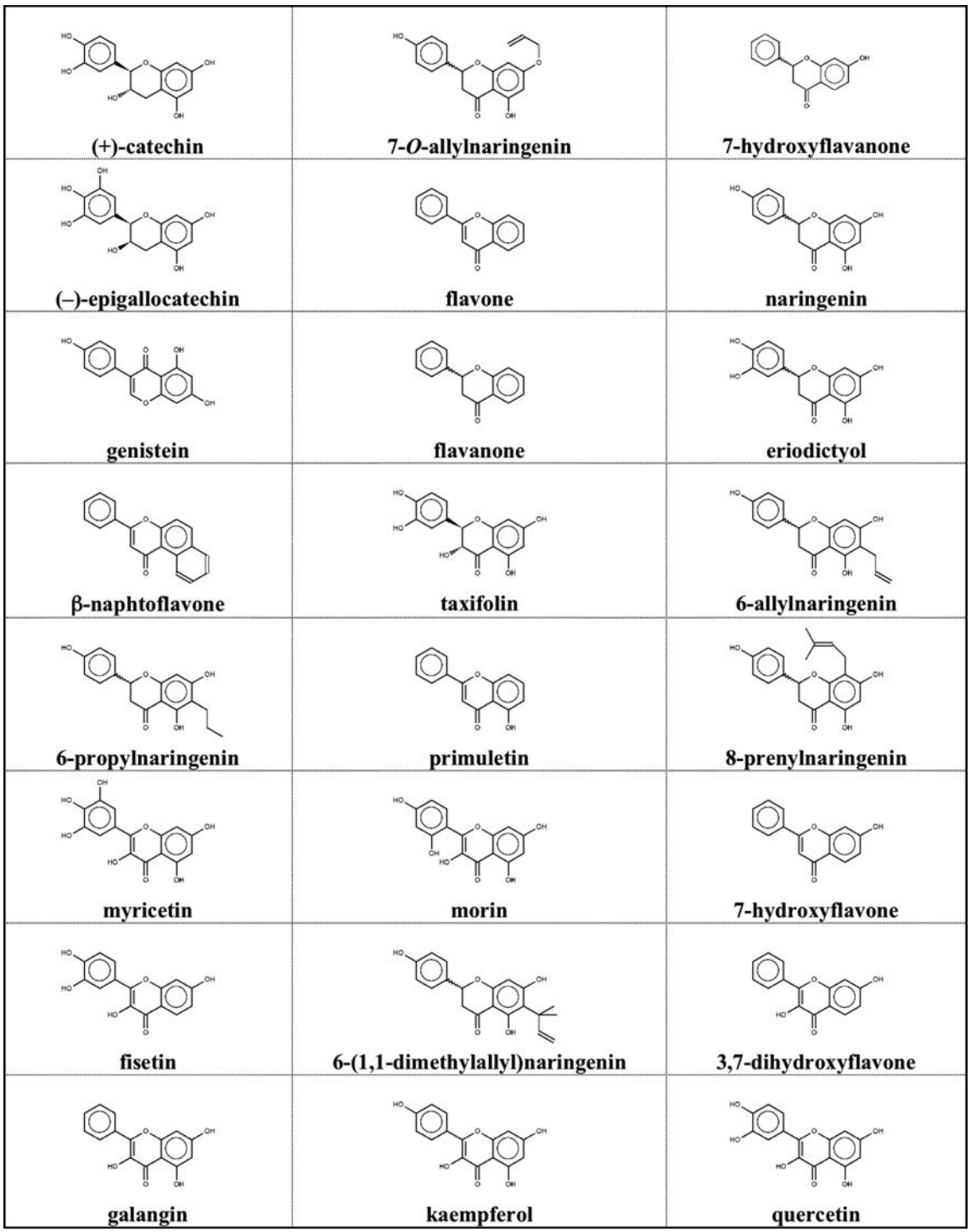

Fig. 1 Set of compounds used in our studies

subunits of 1L8S were superimposed and, as no significant differences were observed, chain A was chosen for our studies.

We established a protocol of automated flexible docking followed by molecular dynamics (MD) simulations that allowed us to reproduce experimentally determined PLA2-ligand interactions. Different ligands were reassembled to PLA2-IIA using flexible docking approaches with FlexX [20] and compared with the original protein–ligand complexes (PDB entries 1DB4, 1DB5, 1DCY, and 1KQU [18, 21]). In order to refine the docking outcomes (i.e., dependent on the rigidity of the ligands) 10 ns MD

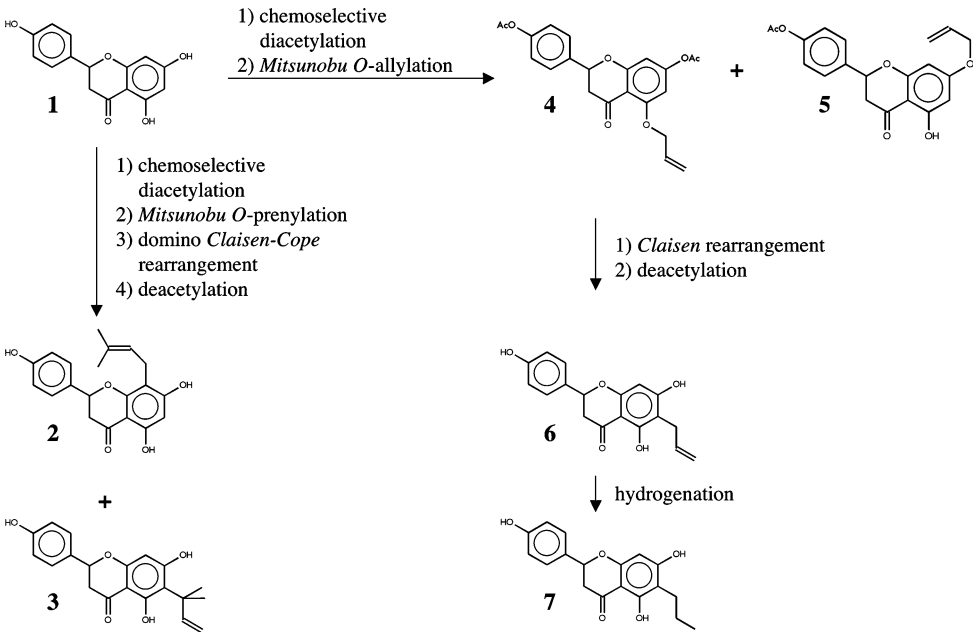

Fig. 2 Scheme of synthetic transformations used to obtain naringenin-based derivatives

simulations using AMBER8 [22] were applied to the FlexX-reassembled ligand–protein complexes. In our MD simulations we used explicit solvent, unit charges for $C\alpha^{2+}$, explicit counter ions, harmonic potentials on protein Cα atoms, and periodic boundary conditions. *PTRAJ* as implemented in AMBER8 was used to analyze water molecules in the binding crevice. Visualization of trajectories and image processing was done using VMD [23].

PLA2 activity assay

Phospholipase A2 activity was determined by an assay based on pyrene self-quenching as long as sn-2 pyrene-modified phospholipids are present in liposomes. After hydrolysis of the sn-2 ester bond by PLA2 action, the pyrene-modified fatty acid is liberated from the liposome and bound by BSA that is also present in the assay mixture. In that way, the self-quenching effect of the liposomal organization is abolished and an increase in pyrene fluorescence can be determined. Besides the phospholipid hydrolysis, the interfacial binding of PLA2 to the substrate liposomes is a crucial step in this reaction as well as the liberation and binding to BSA of the free fatty acid after hydrolyzes from the substrate liposomes. A shortcoming of the assay is that we cannot ascertain if the compounds tested affect exclusively the hydrolysis step of this reaction and our compounds may also affect pyrene fluorescence depending on their spectral properties and binding affinity to BSA. In brief, the assay was performed as follows [24, 25]: Liposomal substrate was prepared by dropwise addition of a 100 μM ethanol stock solution of pyrene phospholipid 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphogylcerol (Invitrogen, Karlsruhe, Germany) in assay buffer (50 mM Tris–HCl, 100 mM NaCl, 1 mM EGTA, 1 mg/ml essentially fatty acid free BSA at pH 7.4) under continuous agitation to reach 1 μM substrate concentration. An appropriate volume of the respective inhibitor stock solution (1 or 10 mM) in DMSO was added to achieve the respective inhibitor concentrations in the range from 0.1 to 50 μM. The assay was performed in a total volume of 1 ml, and the reaction was started by addition of 10 μl 1 M $CaCl_2$ to 990 μl assay mixture. Inhibitors, solvents and buffer constituents were supplied by Sigma. The initial reaction velocity of 1-pyrenedecanoic acid liberation by PLA2 activity from the substrate liposomes was continuously measured at Ex342/Em395 with a Jasco FP 6300 spectrofluorometer, Groß-Umstadt, Germany. The mixture was continuously agitated with a magnetic stirrer in the cuvette holder of the spectrofluorometer and the temperature was maintained at 37°C. In this way the inhibitory effect of molecules on porcine pancreatic secretory PLA2-IB (Sigma) and human secretory PLA2-IIA expressed by stimulated Hep-G2 cells [26] was determined.

Results and discussion

Characterization of the binding sites of PLA2-IIA and -IB

Secretory phospholipase A2-IIA and -IB are structurally very conserved, and only vary in some loop regions. The most conserved region in both enzymes is the binding

crevice, which is composed of a "hydrophobic site" where the fatty acid tails of substrates bind, and the "catalytic site" for substrate cleavage. The "hydrophobic site" in PLA2-IIA and -IB consists of aliphatic and aromatic residues within or close to the N-terminal helix (in PLA2-IIA: Leu2, Phe5, Ile9, Ala17, Ala18, Tyr21, Cys28, Cys44, and Phe98). In contrast, the "catalytic site" in PLA2-IIA and -IB is formed by the hydrophilic residues His47 and Asp48, which together with the catalytic Ca^{2+} are needed for the enzymes' cleavage mechanism consisting on the nucleophilic attack of a water molecule at the sn-2 acyl ester bond of diacylglycerol. Due to the fact that PLA2 substrates interact with the catalytic Ca^{2+}, this interaction has been included in the design of inhibitors as a *hot spot* for improving affinity.

The most striking difference between both enzymes is the six-residue prolonged C-terminus of PLA2-IIA tethered to the core of the protein by disulfide-linkage of the last cysteine residue (Cys124). This provides an additional "hydrophobic cage" close to the binding site of PLA2-IIA, which may be used to introduce selectivity in our design as it has been previously shown [27]. Besides this, amino acid substitutions amongst both PLA2s vary the electronic properties around each binding crevice, which might also be targeted to improve the binding selectivity of ligand molecules.

Analysis of interactions of flavonoids with other proteins

Several experimentally known complexes were studied to delineate the characteristics of flavonoid-protein interactions. In general, flavonoids interact mainly by their hydrophobic functionalities—mainly the A- and B-rings (Fig. 3)—at hydrophobic/aromatic pockets of fitting sizes in their respective proteins. Hydrogen bonds between flavonoid and protein were rarely found in our analysis. In addition, coordination of metal ions was only observed for the complex of quercetin with quercetin-2,3-dioxygenase (1H1I), where the C(3)-hydroxyl group of quercetin coordinates copper. Based on these observations and on the hydrophobic nature of native PLA2 ligands (phosphatidylcholine, phosphatidylethanolamine, and phosphatides), we hypothesized that the interaction of natural occurring flavonoids with PLA2 might be mainly based on their hydrophobic properties (i.e., aromatic rings A and/or B), and that hydrophilicity might play a less important role.

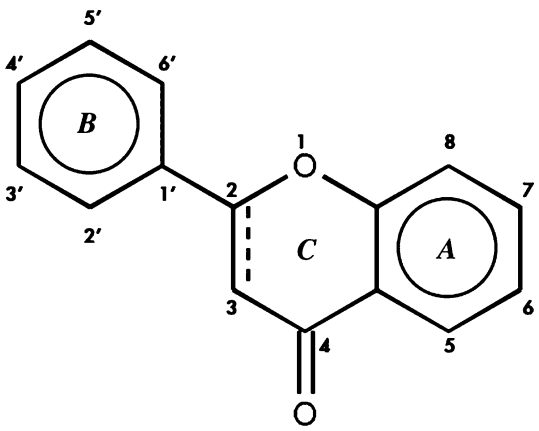

Fig. 3 IUPAC-Nomenclature of the flavonoid scaffold

Binding modes of natural flavonoids to PLA2

- *Quercetin* was positioned by FlexX in a similar position to [3-(1-benzyl-3-carbamoylmethyl-2-methyl-1H-indol-5-yloxy)-propyl-]-phosphonic acid from 1DB4 [21]. After the first 500 ps of MD simulation (see Methods) quercetin turned by about 140° from its initial docked position. In this final position, quercetin interacted mainly within the "hydrophobic site". Here it was stabilized by π-stacking interactions of its A-ring with Phe5 and Phe98, as well as by a hydrogen bond of the C(7)-hydroxyl group with a water molecule that was interacting with the δ-nitrogen of His47 and the backbone carbonyl oxygen of Cys44 (Fig. 4a). As a result, quercetin bound to PLA2-IIA in a Ca^{2+}-independent manner as we have also recently shown for PLA2-IB (M. Böhl et al. 2007, submitted data). The B-ring of quercetin in PLA2-IIA was oriented toward Lys62 establishing hydrogen bonds through its hydroxyl groups (Fig. 4a), whereas in PLA2-IB the corresponding residue (Tyr69) was able to establish π-stacking interactions with the B-ring. Additionally, the resulting binding pose of quercetin in PLA2-IIA and -IB revealed that C(5)-hydroxyl group (A-ring) interacted intramolecular with the C(4)-carbonyl oxygen (C-ring) in most of the conformations obtained in the MD simulations. This intramolecular hydrogen bond satisfied the hydrogen bond donor-/acceptor properties of the C(5)-hydroxyl group and the C(4)-carbonyl oxygen, respectively, allowing them to be positioned into the "hydrophobic site" without paying an enthalpy penalty. Furthermore, comparisons with structural data of flavonoids from the CSD [7] (entries DOLRIF: (*R,S*)-naringenin, KAMJUD: 5-hydroxy-7-methoxyflavone, FEFBEX: quercetin-dihydrate, SATDAS: 5-hydroxy-7,4′-dimethoxy-flavanone, among others) revealed this intramolecular hydrogen bond as the experimentally preferred. Based on our observations, we suggest that for flavonoid-like compounds the C(4)-carbonyl oxygen and C(5)-hydroxyl groups are not directly

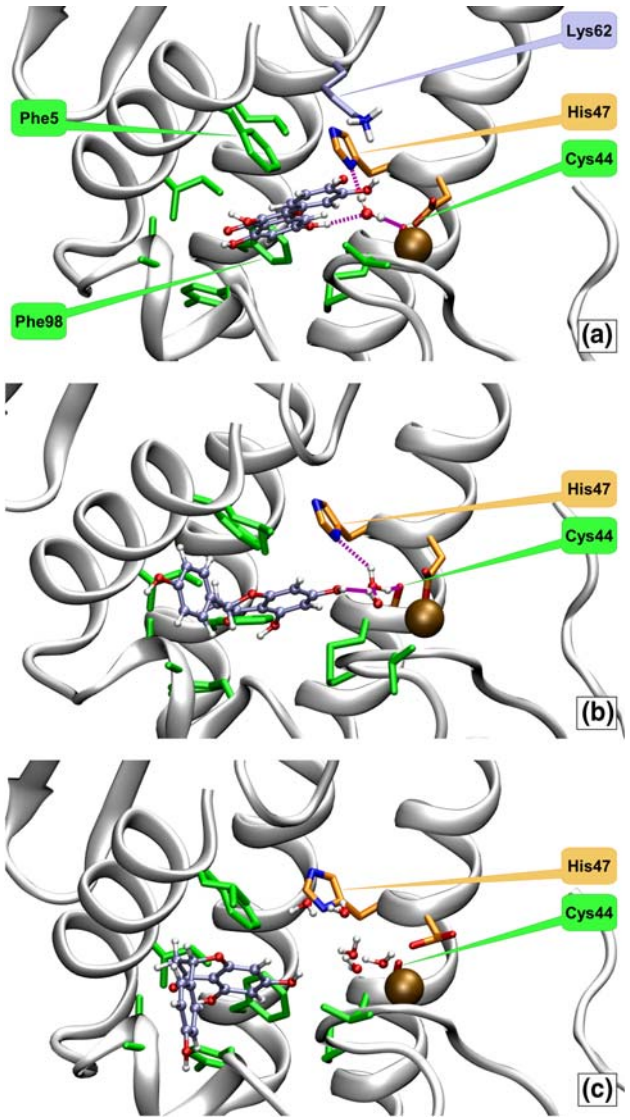

Fig. 4 Binding modes of native flavonoids. White ribbons represent the PLA2 backbone. Sidechains of residues forming the "hydrophobic site" are shown in *green* and residues of the "catalytic site" are colored in *orange*. A sphere represents the catalytic Ca^{2+}. Ligands and water molecules involved in binding are represented as *balls* and *sticks*. (**a**) Binding of quercetin: the A-ring interacts by π-stacking with Phe5 and Phe98. In addition, C(7) interacts by water-mediated (1) hydrogen bonds (*magenta lines*) with His47 and the carbonyl oxygen of Cys44. Lys62 interacts with the B-ring hydroxyl group at (C3′). (**b**) Binding of (*R*)-naringenin: hydrophobic contacts of A- and C-rings within the "hydrophobic site" and water-mediated (2) hydrogen bonds (*magenta lines*) to His47 and the backbone carbonyl oxygen of Cys44. (**c**) Binding of (*S*)-naringenin: it poorly interacts with hydrophobic residues of the hydrophobic region. In addition it finds unspecific interactions out of the binding site due to its bent conformation

interacting with the enzyme but establishing an intramolecular hydrogen bond that compensates their hydrophilic properties resulting in more favorable binding of quercetin into the "hydrophobic site." In this binding mode, position C(8) of the quercetin scaffold represents the most suitable site for introduction of chemical modifications (e.g., aliphatic extensions achieving *van der Waals* contacts with Lys62).

- *Naringenin* (*R*)- and (*S*)-enantiomers were docked by FlexX in a similar position to [3-(1-benzyl-3-carbamoylmethyl-2-methyl-1H-indol-5-yloxy)-propyl-]-phosphonic acid from 1DB4 [21]. Both naringenin enantiomers reoriented their positions often during the MD simulations, which might be due to weaker contacts in the "hydrophobic site" and the increased ligand flexibility induced by the missing coplanarity of the A- and C-rings. In addition (*R*)- and (*S*)-enantiomers demonstrated clear differences in their binding modes due to the shape of the binding site.

During MD simulations (*R*)-naringenin remained in a stretched conformation (with the B-ring in equatorial position to A- and C-ring) finding good interactions within the "hydrophobic site." Similar to quercetin (*R*)-naringenin was binding into the "catalytic site" through its C(7)-hydroxyl group mediated by water. In the case of (*R*)-naringenin, two water molecules mediated the interaction. This could explain the decrease in affinity of this compound compared to quercetin. In addition, the π-stacking interactions observed in quercetin between the A-ring and Phe98 are unfeasible in the case of (*R*)-naringenin because of the different A-ring orientation and the C(4)-carbonyl oxygen and C(5)-hydroxyl group pointing towards this residue (Fig. 4b).

(*S*)-naringenin did not form hydrogen bonds inside the enzyme's binding crevice during the MD simulations, resulting in more unspecific binding orientations. This, together with a poor shape complementarity of (*S*)-naringenin in the binding crevice, resulted in a frequent exchange between a stretched (B-ring equatorial to A- and C-ring) and a bent conformation (B-ring in axial position). The bent conformation of (*S*)-naringenin is the energetically preferred one, and presents interactions within the "hydrophobic site" through its A- and C-rings. Its B-ring points out of the binding site establishing *van der Waals* contacts with Ala17 and Ala18 (Fig. 4c). The unspecific binding mode predicted for both naringenin enantiomers to PLA2 suggests lower affinity and worse inhibitory activity than quercetin. This is in agreement with our experimental studies. Residual PLA2 activity for naringenin was 90% compared to 50% for quercetin (Fig. 6). Weak binding affinity to PLA2-IIA was found for naringenin (IC_{50} >50 μM) compared to a tenfold higher for quercetin (IC_{50} 6.24 μM) (Fig. 7). The value for naringenin corresponds to the sum of the binding inhibitory activities of both e-nantiomers, which were not measured separately.

Based on our computational calculations, we proposed that (*R*)-enantiomers of flavanones might have higher

inhibitory activity than their respective (*S*)-enantiomers. The fact that (*R*)- and (*S*)-enantiomeres exhibited different binding properties makes us hypothesize that a possible "worse" binding of the (*S*)-enantiomer might mask a "better" inhibitory activity of the (*R*)-enantiomer. Independently of these differences in binding modes shown by (*R*)- and (*S*)-naringenin, C(6) and C(7) would be the most suitable positions for modifications in both molecules. Substituents at C(6) and C(7) might be able to establish new *van der Waals* contacts within the binding crevice and, therefore, increase affinity and inhibitory activity towards PLA2. Nevertheless, modifications at C(7) may disrupt the water-mediated hydrogen-bond network to the catalytic site resulting in weak inhibitor compounds.

Binding modes of naringenin-based compounds to PLA2

Our investigations on binding of natural flavonoids to PLA2 suggested C(7), C(8), and in particular C(6) as suitable positions for introduction of substitutions to increase affinity and specificity. Based on our models, the introduction of hydrophobic extensions of increasing bulk at positions C(6), C(7), and C(8) would increase interactions of naringenin-like compounds into the "hydrophobic site", stabilize binding and gain specificity. Five compounds with hydrophobic substitutions (6-allylnaringenin, 6-propylnaringenin, 6-(1,1-dimethylallyl)naringenin, 7-*O*-allylnaringenin, and 8-prenylnaringenin) were synthesized and tested for their binding and inhibition of PLA2, and their binding behavior was computationally compared to naringenin and quercetin.

- *6-(1,1-Dimethylallyl)naringenin* has the most rigid binding mode of all simulated compounds with exception of quercetin. It interacts tightly within the "hydrophobic site" and, as proposed by our models, its bulky C(6)-modification satisfies numerous hydrophobic contacts including π-stacking with Phe5 and Tyr51 in MD simulations (Fig. 5a).

Similar to naringenin, also the enantiomers of 6-(1,1-dimethylallyl)naringenin demonstrated different binding behavior to PLA2. However, in the case of 6-(1,1-dimethylallyl)naringenin the (*R*)-enantiomer presented a bent conformation, whereas the (*S*)-enantiomer remained stretched (contrary to naringenin). As predicted, the (*R*)-enantiomer with its bent conformation is able to adapt better to the shape of the binding crevice and to establish more specific interactions than the (*S*)-enantiomer's stretched conformation in the "hydrophobic site" (Fig. 5a). The resulting good interactions of both enantiomers may explain the good binding and inhibitory activity of

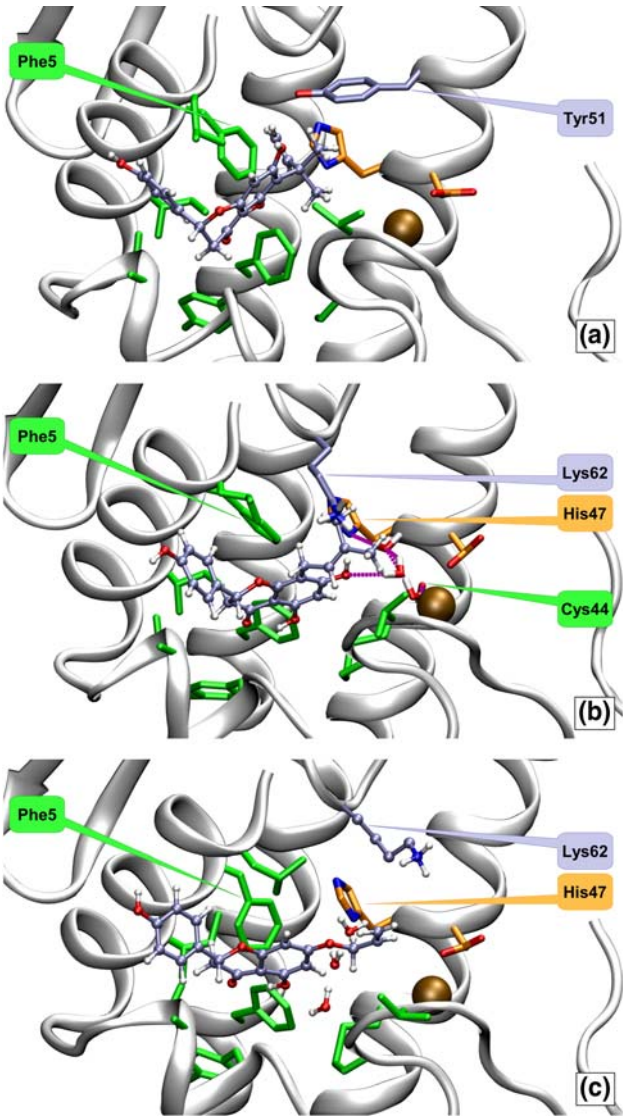

Fig. 5 Binding modes of naringenin-based derivatives. The (*R*)-enantiomers are shown. *White ribbons* represent the PLA2 backbone. Sidechains of residues forming the "hydrophobic site" are shown in *green* and residues of the "catalytic site" are colored in *orange*. A sphere represents the catalytic Ca^{2+}. Ligands and water molecules involved in binding are represented as balls and sticks. (**a**) Binding of (*R*)-6-(1,1-dimethylallyl)naringenin: tight interactions of its bent conformation within the "hydrophobic site" established π-stacking of its dimethylallyl-chain in C(6)-position to Phe5 and Tyr51. (**b**) Binding of (*R*)-8-prenylnaringenin: many hydrophobic contacts within the hydrophobic site as well as interactions between its prenyl-chain and the sidechain of Lys62 and water-mediated (2) hydrogen bonds (*magenta lines*) to His47 and the backbone carbonyl oxygen of Cys44. (**c**) Binding of (*R*)-7-*O*-allylnaringenin: loose interactions within the hydrophobic region. Suboptimal ligand shape results in many unspecific contacts

6-(1,1-dimethylallyl)naringenin. Activity measurements showed that it reduced the activity of PLA2-IIA by 45%, similarly to the best native flavonoids tested (quercetin and kaempferol; Fig. 6). Although these three compounds

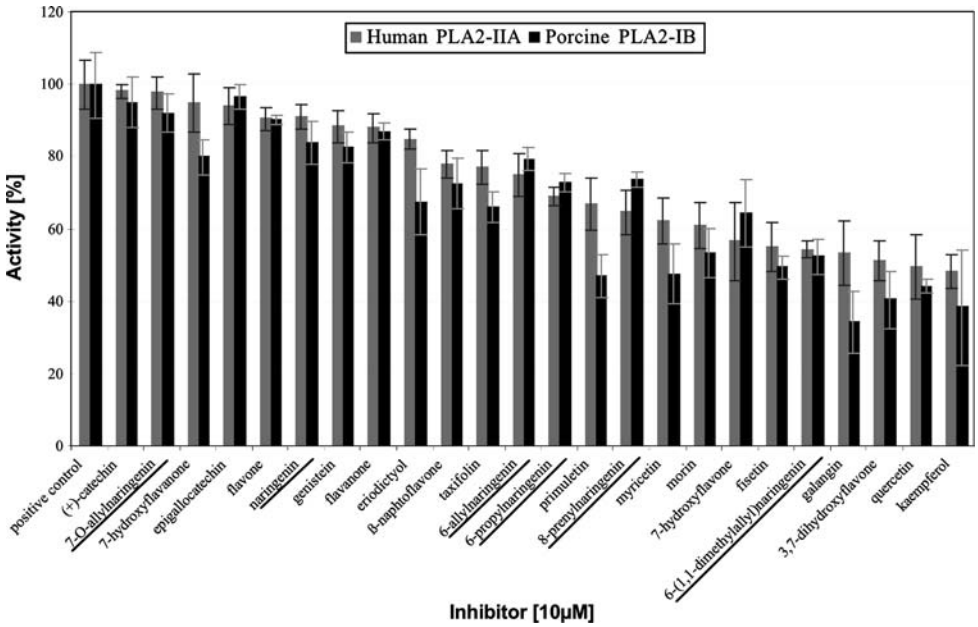

Fig. 6 Residual activity of PLA2-IIA and -IB for all experimentally studied compounds. 10 μM inhibitor concentration was used

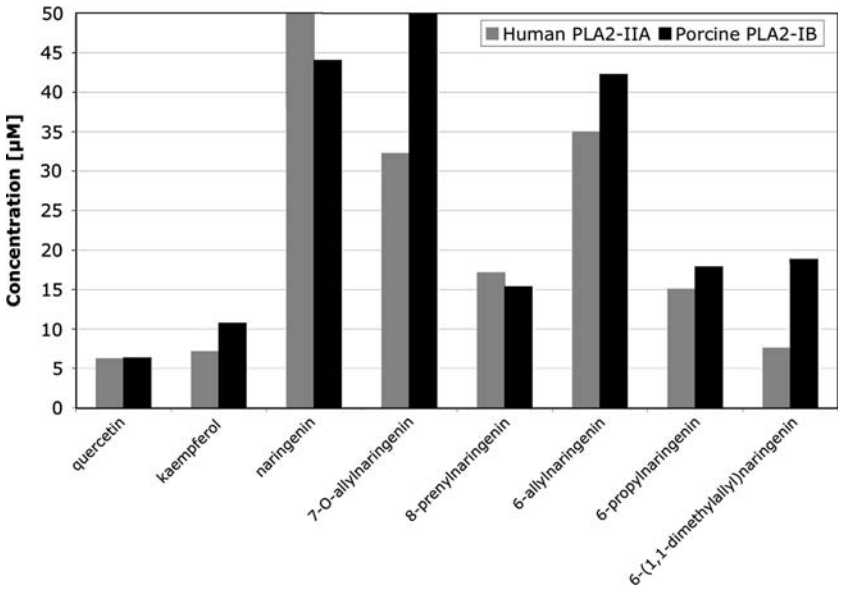

Fig. 7 IC_{50} values of compounds studied computationally and experimentally for inhibition of PLA2-IIA and PLA2-IB

showed similar IC_{50} values for PLA2-IIA (6-(1,1-dimethylallyl)naringenin, 7.6 μM; quercetin, 6.2 μM; kaempferol, 7.1 μM), the naringenin-derivative revealed selectivity for PLA2-IIA versus PLA2-IB (Fig. 7). The PLA2-IB enzyme used in our experiments is of porcine origin but its high sequence and structure similarity to human PLA2-IB, and in particular the conserved binding sites in both enzymes, enable us to assume that the specificity obtained with 6-(1,1-dimethylallyl)naringenin with porcine PLA2-IB might be extrapolated to human PLA2-IB.

- *6-Allylnaringenin and 6-propylnaringenin* binding to PLA2 was comparable to 6-(1,1-dimethylallyl)naringenin and, thereby, similar to known indole-based compounds such as [3-(1-benzyl-3-carbamoylmethyl-2-methyl-1H-indol-5-yloxy)-propyl-]-phosphonic acid from 1DB4 [21] (data not shown). Our models suggest that the inhibitory activity of these compounds increases with the bulk of their modifications: 6-allylnaringenin, IC_{50} 35 μM; 6-propylnaringenin, IC_{50} 15 μM; 6-(1,1-dimethylallyl)naringenin, IC_{50} 7.6 μM (Figs. 6, 7).
- *8-Prenylnaringenin* revealed a binding mode similar to quercetin in our simulations. Its C(8)-prenylation increased the interactions with hydrophobic moieties close to the "catalytic site" (Lys62), while contacts to the "hydrophobic site" were reduced (Fig. 5b). In addition, and similar to quercetin, 8-prenylnaringenin

interacted by its C(7)-hydroxyl group by a water-mediated hydrogen bond with the catalytic His47 (Figs. 4a, 5b). Both enantiomers showed preference for the stretched conformation and interacted similarly with PLA2. This similar behavior of both enantiomers might be an advantage of this molecule for binding. However, their reduced number of interactions within the "hydrophobic site" might represent a disadvantage for binding.

The IC_{50} value of 8-prenylnaringenin (17.1 μM) was in-between those of naringenin (>50 μM) and 6-(1,1-dimethylallyl)naringenin (7.6 μM) (Fig. 6). Substitutions at C(8) position might, therewith, add some unspecific interactions (i.e., with the aliphatic part of Lys62) but not additional affinity gain (Fig. 5b).

- *7-O-Allylnaringenin* behaved similar to naringenin in our MD simulations (Figs. 4b, 5c). Its (*R*)- and (*S*)-enantiomers showed stretched and bent conformations in their binding to PLA2, respectively. The A- and C-rings of both enantiomeres interacted in the "hydrophobic site" in an unspecific fashion. As described before for naringenin, π-stacking interactions between the A-ring and Phe98 were unfeasible in 7-*O*-allylnaringenin because of the individual A-ring orientation and the C(4)-carbonyl oxygen and C(5)-hydroxyl group pointing toward this residue (Fig. 5c). The B-ring of the (*R*)-enantiomer fitted into the binding crevice, while the B-ring of the (*S*)-enantiomer pointed outside due to poor shape complementarity with the binding crevice. There, it established *van der Waals* contacts with Ala17 and Ala18. As proposed by our computational models, the hydrophobic extensions at C(7) of (*R*)- and (*S*)-7-*O*-allylnaringen established additional *van der Waals* contacts with Val30 and Lys62, increasing the binding affinity of 7-*O*-allylnaringenin with respect to naringenin (Fig. 7). However, these additional contacts were not sufficient to increase its inhibitory activity, which remained similar to naringenin (Fig. 6).

Structural features of flavonoids and rationale for lead design

Based on our results and observations, we extract the following conclusions about the determinants of PLA2 binding and inhibition by flavonoids, and propose guidelines for PLA2-inhibitor rational lead design based on these scaffolds.

- *Coplanarity of A- and C-rings*: The most striking feature necessary for good PLA2-inhibition by native flavonoids is the coplanarity of the A- and C-rings of their basic scaffold (Fig. 3). This planarity is based on the aromaticity of the A-ring, a double bond between C(2) and C(3), as well as the carbonyl oxygen at C(4) position.

It has been demonstrated in several cases that the C(2)–C(3) double bond has special importance in binding of flavonoids to enzymes including PLA2 [28, 29]; however, its rationale still remained unclear [5]. Compounds with non-coplanar A- and C-rings missing the C(2)–C(3) double bond (e.g., naringenin) or even the C(4)-carbonyl group (e.g. (+)-catechin), are poor PLA2 inhibitors (Figs. 1, 6). In contrast, we observed that flavonoids including both, the C(2)–C(3) double bond and the C(4)-carbonyl group and, therefore, coplanar A- and C-rings (e.g., quercetin) present good inhibitory activity (Figs. 1, 6). The C(2)–C(3) double bond in the flavonoid scaffold may have two roles: (1) induce coplanarity of A- and C-rings; (2) take part in π-stacking interactions [30]. Because in our simulations the C(2)–C(3) double bond stayed out of reach of possible π-stacking interaction partners, we suggest that the main function of the C(2)–C(3) double bond in this scaffold is the introduction of coplanarity into A- and C-rings.

- *Importance of hydroxyl groups*: Inhibition of PLA2 is very much dependent on a clear substitution pattern of hydroxyl groups at rings A, B, and C. While hydroxyl groups in positions C(5), C(6), and C(7) have been assumed to be necessary for binding to PLA2s, substitutions in positions C(3′) and C(4′) of the B-ring have been demonstrated to be important for selectivity of PLA2-IIA versus other PLA2s [5, 31, 32]. However, the reason why certain hydroxyl groups of flavonoids have special importance in binding to PLA2 is still unclear.
- *C(5)-hydroxyl group* (A-ring): Our computational studies indicated that the C(5)-hydroxyl group interacts with the carbonyl oxygen in position C(4), forming an intramolecular hydrogen bond in all ligands in more than 90% of all conformations of our MD simulations. As a result, their respective hydrogen bond donor-/acceptor properties are satisfied and do not take part in any interaction with the protein or the solvent (Figs. 4, 5). Our experimental data demonstrated that compounds with hydroxylation at this position show generally better inhibition than similar molecules without this hydroxyl group (Figs. 1, 6). Quercetin and primuletin have a hydroxyl group at C(5) and are better inhibitors of PLA2 than their respective C(5)-unhydroxylated derivatives fisetin and flavone.
- *C(7)-hydroxyl group* (A-ring): Our MD simulations of quercetin, kaempferol, and naringenin (representatives of naturally occurring flavonoids and flavanoids)

suggested that C(7) is the most important position for hydroxylation of the flavonoid scaffold, as it interacted—with the exception of (*S*)-naringenin—by a water-mediated hydrogen bond with the "catalytic site" (Fig. 4). The compounds of our series showing best PLA2 inhibition (residual PLA2 activity <60%) were all hydroxylated at C(7), while hydroxyl-substitutions at C(5), C(3), or C(4′) were not conserved. Moreover, the introduction of an aliphatic substituent at C(7) (i.e., 7-*O*-allylnaringenin) resulted in decreased PLA2 inhibitory activity (Fig. 6). This need of C(7)-hydroxyl group is in agreement with the earlier demonstrated importance of that functional group [31]. Introduction of glucuronic acid at C(7) of scutellarein reduced its affinity for *Naja naja* PLA2 and PLA2-IIA from synovial fluid.

- *C(3)-hydroxyl group, C-ring*: Five out of the seven best binding compounds (>40% PLA2 inhibition) are hydroxylated at C3. Our MD simulations revealed that the C(3)-hydroxyl group points out of the binding site. This group may favor the interaction of parts of these compounds with surrounding solvent while binding.
- *B-ring hydroxylation*: Our MD simulations of quercetin resulted in a ligand orientation into the binding site of PLA2-IIA that allowed additional hydrogen bond contacts of hydroxyl groups at C(3′) and C(4′) to Lys62 (nearby the binding crevice). This residue is not conserved in different PLA2s (i.e., Tyr69 in PLA2-IB) and, therefore, could be a good candidate to introduce specificity for certain flavonoids. Trying to analyze how this specificity for flavonoid molecules might be achieved, we simulated the binding of quercetin in both, PLA2-IIA and -IB. Our MD simulations showed that quercetin adopts similar binding modes in PLA2-IIA (Fig. 4a) and -IB (M. Böhl et al. 2007, submitted data) interacting with Lys62 or Tyr69, respectively. Tyr69 in PLA2-IB points inward the binding crevice and interacts with the B-ring through π-stacking, whereas Lys62 in PLA2-IIA points outward the binding crevice and establishes hydrogen bonds with the hydroxyl substituents of the B-ring.

It may appear that the use of natural flavonoids for lead design of PLA2 inhibitors has certain restrictions due to the binding modes found in our studies. First of all, the interaction of the A-ring with Phe5 resulted in the formation of a water-mediated hydrogen bond between the C(7)-hydroxyl group and the catalytic His47. Second, the C(5)-hydroxyl group formed an intramolecular hydrogen bond with the C(4)-carbonyl oxygen, which—as explained before—allowed them to be positioned into the "hydrophobic site" without an enthalpy penalty. In addition, the C(3) position in the C-ring and the substitutions of the B-ring pointed out of the binding site. As a result, a reduced number of positions remained for possible modifications in the general scaffold of natural flavonoids: that is, positions C(6) and C(8) in the A-ring. While substitutions including small hydrophobic groups (i.e., ethyl) in position C(8) might improve affinity for PLA2 by establishing *van der Waals* contacts with the sidechain of Lys62, larger substituents would be disadvantageous. Our simulations indicated that C(8)-prenylation does alter the binding mode of the ligand, resulting in steric clashes in the binding crevice (data not shown). The C(6) position in quercetin was buried in the "hydrophobic site" of the enzyme, and modifications in this position altered the flavonoid's binding mode. Depending on the substitution at this site, modified molecules may achieve PLA2 affinity and inhibition equivalent to the best-known natural flavonoids. As we have shown, C(6)-modified naringenin-derivatives such as 6-allylnaringenin, 6-propylnaringenin, and 6-(1,1-dimethylallyl)naringenin presented binding modes similar to previously described indole-based molecules optimized for PLA2 inhibition [21]. A keystone for the interaction of these indole-based inhibitors was the coordination of the catalytic Ca^{2+}, which was not achieved by natural flavonoids (M. Böhl et al. 2007, submitted data). Using this improved class of naringenin-derivatives, we are able to draw conclusions on how to further improve PLA2 inhibitory activity. Targeting the catalytic Ca^{2+} with C(6)-modified naringenin-derivatives might be useful to increase ligand affinity. Based on our simulations, C(6)-substitutions in flavone- as well as flavanone-based flavonoids would allow similar binding modes to the ones found with C(6)-modified flavonoids. Flavone-based compounds might be more appropriate targets for lead design because of their A- and C-rings coplanarity, which leads to less ligand flexibility. Substitutions in C(6) could be able to target the Ca^{2+} and also interact with residues in the earlier described "hydrophobic cage" close the C-terminus in PLA2-IIA, which has been demonstrated to be useful in obtaining specificity in the development of PLA2 inhibitors [27].

The fact that C(6)-modified compounds bind in different modes opens the possibility to modifications in positions that were prohibited in the non-modified compounds. This means that C(3) and C(8) turn into suitable positions for substitutions in C(6)-modified compounds. Our simulations showed that introduction of methoxy- or ethyl-derivatives at C(3) of C(6)-modified flavonoids might add additional *van der Waals* contacts in the "hydrophobic site," and that a methyl group in position C(8) might establish *van der Waals* interactions with the sidechain of Lys62. However, in C(6)-modified flavonoids C(7) is oriented toward the solvent and, thereby, it is not a suitable target for modification.

Conclusions

Our studies on the so far unknown mechanism of inhibition of human secretory PLA2 by native flavonoids and flavanoids as well as naringenin-based derivatives have revealed interesting binding properties of these compounds, and explained their reported weak enzyme inhibition. In general, the studied compounds interact with PLA2-IIA (and also PLA2-IB) in a different way than other known PLA2 ligands. They present flexible binding modes and interact mainly with residues in a hydrophobic site in the binding crevice close to the N-terminal helix. Also differing from other known PLA2-IIA inhibitors, the molecules we have studied establish few hydrophilic/electrostatic contacts with the enzyme. A remarkable particularity of flavonoids is their binding and inhibition independence from the catalytic Ca^{2+}, which we have shown theoretically and confirmed experimentally (M. Böhl et al. 2007, submitted data). Although it may appear that the flavonoid scaffold is a challenging starting structure for PLA2-IIA inhibition lead optimization, we show that it can be rationally modified to improve affinity and specificity. We propose C(6)-modified flavonoids as molecules with potential for the rational design of selective PLA2-IIA inhibitors.

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