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Computational and experimental approaches assess the interactions between bovine β-lactoglobulin and synthetic compounds of pharmacological interest

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Abstract

Extending a previous investigation, the ability of binding to the model calycin β-lactoglobulin (BLG) was evaluated both *in silico* and *in vitro* for several fluorine-containing (semi-)synthetic molecules of pharmacological and pharmaceutical interest (antibiotics, vastatins, steroid drugs). Simulation procedures included molecular docking according to a Montecarlo-simulated annealing protocol and molecular dynamics; heteronuclear NMR and denaturant gradient gel electrophoresis were the selected experimental techniques. For the tested drugs, ranking of the binding affinity was consistently assessed by computation and by experiment. The affinity for BLG increased in the sequence: 5-fluorosalycilic acid < dexamethasone ≪ sulindac = norfloxacin < fluvastatin. The computed K_i for fluorosalycilate was in the order of 10^{-4} M; accordingly, in a molecular dynamics simulation the chemical diffused out of the BLG calyx in less than 2 ns, and no evidence of binding was found by NMR or electrophoresis. Conversely, the K_i for fluvastatin and norfloxacin were in the order of 10^{-7} and 10^{-6} M, similar to the affinity for BLG by natural ligands, such as retinoids and long-chain fatty acids. Moreover fluvastatin was found still bound to the protein after 5 ns of molecular dynamics simulation. Interaction of fluvastatin and norfloxacin with BLG was made evident by changes in chemical shift and dynamic parameters in the ^{19}F NMR spectra and in effective urea concentration and cooperativity features in denaturant gradient gel electrophoresis. Such findings prove BLG may act as a drug carrier accepting in its cavity molecules of different bulk, rigidity and hydrophobicity.

Keywords: Molecular docking; Monte Carlo; Fluorine nuclear magnetic resonance; Denaturant gradient gel electrophoresis; Statins; Steroids; Fluoroquinolones

1. Introduction

Bovine β-lactoglobulin (BLG) is an 18-kDa protein belonging to the lipocalin superfamily [1]. Lipocalins are extracellular proteins with the ability to bind small hydrophobic molecules [1–3]. BLG was reported to bind palmitic acid, retinol, retinoic acid, 12-bromodecanoic acid and several other hydrophobic compounds [4–15]. At physiological pH, BLG is a dimer with a molecular weight of 36 kDa [16]. Each monomer adopts a β-barrel fold, characterized by a central calyx with an internal hydrophobic surface. Its secondary structure consists of eight subsequent antiparallel β-strands, an α-helix and a short C-terminal β-strand [17–21]. At low pH, BLG is monomeric, with most structural features similar to the dimer [22–25]. This protein shows a peculiar resistance to proteases contained in the gastric milieu and to its acidic pH [26,27], but is sensitive to proteases in a basic environment [26,28]. The access to the BLG calyx is tuned by proton concentration. Our group has

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assessed, by MD simulation, the role of Glu89 protonation state in controlling the conformational orientation of the EF loop that acts either as a closed or as an open lid above the protein calyx [29]. These features, associated to its fair stability and easy purification, make BLG a potential carrier for various oral drugs [30]: complexes between BLG and acid-sensitive, gastrolesive or hydrophobic drugs could pass through the stomach and ultimately release the ligand further down the gut.

To test this hypothesis, we screened ligands with different therapeutic use and chemical structure, all containing fluorine atoms, and evaluated their affinity with respect to BLG through a combined computational and experimental approach. For the computational aspect, we used a molecular docking protocol [31], based on Monte Carlo and simulated annealing (MC/SA), in which the side chains drawing the binding site can freely move [32,33]. The findings for both drugs predicted to bind and not to bind BLG were independently validated through NMR spectroscopy and denaturant gradient gel electrophoresis (DGGE). With the former, the spectroscopic properties of the fluorine atom in the ligands were exploited for the characterization of binding. The longitudinal relaxation time (T_1) of ^{19}F and the heteronuclear Overhauser effect (heteronuclear n.O.e.) are extremely sensitive to the motional regime of the ligands and the comparison between the values measured before and after the interactions is being used as probe of the relative affinity [34–36]. With DGGE, a ranking of the stabilization effects after binding various ligands was assessed through the evaluation of the differences in ΔG_{folded} between *apo* and *holo* forms [37,38]. The stability of the best and the worst scoring complexes was tested in molecular dynamics sessions of 5 ns. The relevance of K60 and K69 in binding was also checked by docking the same ligands to BLG after its *in silico* site selective mutagenesis, or to equine lactoglobulin (ELG), whose structure, not available on PDB, was built through homology modeling.

2. Methodology

2.1. Receptor and ligands

For BLG, three crystallographic structures, obtained from the Protein Data Bank, were used in comparison, an *apo* form (2BLG [20] corresponding to BLG isoform A) and two *holo* forms (1B0O, a complex of isoform B with palmitic acid [13] and 1GX9, a complex of isoform A with retinoic acid [39]). The X-ray crystal structures of BLG were energy minimized with the Discover3 module of the InsightII suite and the CVFF force field (Accelrys, San Diego, CA) according to a multistep protocol [40].

A model for equine lactoglobulin (ELG) was built with the Homology module of Insight II using 1GX9 structure as a template. Due to the high homology of the sequences of BLG and ELG, no loop generation was required. The model was energy minimized with Discover3. The quality of the model was checked with the ProteinHealth module of Quanta (Accelrys, San Diego, CA). Its features were duplicated by a model built in parallel with Homology, a module of the Molecular Operating Environment (MOE) suite (Chemical Computing Group, Montreal, Quebec, Canada).

Table 1
Thermodynamic parameters of BLG–ligand complexes from docking simulations

Ligand	BLG form	K_i (μM)
Fluvastatin	2BLG	2.1
	1B0O	0.39
	1GX9	0.059
Norfloxacin	2BLG	0.45
	1B0O	1.2
	1GX9	2.9
Sulindac	2BLG	10.0
	1B0O	39.0
	1GX9	0.60
Dexamethasone	2BLG	13.0
	1B0O	21.0
	1GX9	100.0
5-Fluorosalycilic acid	2BLG	120.0
	1B0O	420.0
	1GX9	160.0

Several synthetic molecules, listed in Table 1, were docked to BLG. Some of them are pharmacologically active compounds, belonging to different therapeutic classes, but all containing fluorine atoms and acting as NMR active tags; their structures are shown in Fig. 1. Palmitic [9,10,13,14] and retinoic acids [7,10] are well-known ligands of BLG and served as reference; their K_i for BLG are 0.60 [10] and 0.04 μM [41], respectively. The ligands were built and energy minimized with the Builder module of InsightII, then submitted to an optimization with the MOPAC (CAChe Group, Fujitzu, Tokyo, Japan) PM3 semi-empiric method.

2.2. Flexible docking

The flexible docking procedure consisted of two different steps: Monte Carlo (MC) for the generation of 20 starting structures, and simulated annealing (SA)/energy minimization (EM) for the refinement of the best 10 MC-generated complexes. All the described procedures were carried out using the Docking/Affinity modules of the InsightII suite and the CVFF force field. After the MC step, the selected complexes were subjected to a geometric EM protocol. The further step was a SA, that is, an MD simulation starting from 500 K down to 300 K with fifty 100-ps steps. During the MD simulation, the residues located within 6 Å from the protein cavity were left free to move and the ligand was confined into the cavity, according the following relation:

$$E_{\text{LigandConfine}} = k(\max(d - d_0, 0.0))^2$$

in which d is the distance between the center of mass of the ligand and the mass center of the protein cavity, d_0 is a value related to the extension of the protein cavity (5 Å) and k is a force constant with a default value of 100. The value of this function is 0 when $d \leq d_0$.

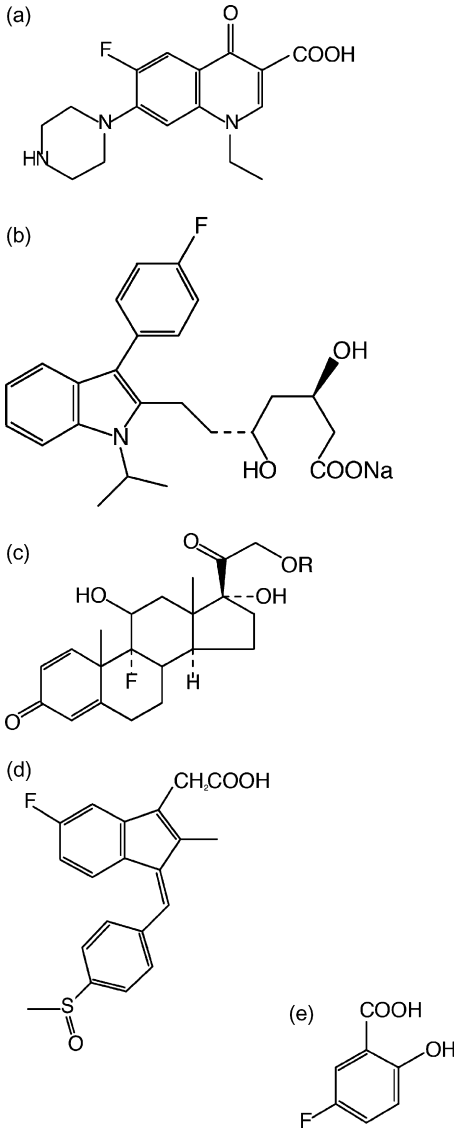

Fig. 1. Chemical structure of the test compounds: (a) norfloxacin, (b) fluvastatin, (c) R = H, dexamethasone; R = PO_3Na_2, dexamethasone 21-phosphate, sodium salt, (d) sulindac and (e) 5-fluorosalicylic acid.

All the complexes generated by this protocol were finally energy minimized. Potential binding energy was evaluated for all the considered complexes as follows:

$$\Delta E_{BE} = \Delta E(P + L) - \Delta E(P) - \Delta E(L)$$

where $E(P+L)$, $E(L)$ and $E(P)$ are the total energies of the complex, of the ligand and of the protein, respectively. ΔE_{BE} has just a comparative meaning and is useful to relatively score the complexes. On the best-scoring complex for each docking series, the K_i were calculated using the empirical scoring functions [42] implemented in the LUDI module. This scoring function has been well validated and widely used (in more than 400 papers since its first proposal). For each ligand, the scoring function with appropriate parameters, optimized according to the specific types of possible interactions, was selected. K_i were evaluated through scoring function 2 in the case of palmitic acid and dexamethasone, whereas scoring function 3, accounting also for the stacking interaction between aromatic rings, was used for the other ligands. This selection differs from the pilot investigation in ref. [31], in which the same scoring function was used for all ligands. A short appendix on the calculation of the free energy of binding through the Böhm and Klebe approach can be found in ref. [31].

2.3. Molecular dynamics

To evaluate the stability of the selected poses, two MD runs (canonical ensemble) of the best solutions (on the basis of the computed binding free energy) for 5-fluorosalycilic acid– and fluvastatin–BLG complexes were performed for 5 ns at 300 K, using the MOE software package (2006.08) with the MMFF94x force field, and a generalized Born implicit solvent model. The non-bonded interactions were evaluated with 'on' and 'off' cutoffs of 8 and 10 Å, respectively. The dielectric constant for electrostatic interactions was 1 for the interior and 80 for the exterior of the protein. The time step was set to 2 fs, the temperature was controlled by a Nosé–Poincaré–Anderson algorithm [43] with a temperature relaxation time of 1 ps. All the bond lengths involving hydrogen atoms were constrained with the LINCS algorithm.

2.4. DGGE across transverse urea gradients

Electrophoresis across transverse urea gradients [37,44] was carried out as described in ref. [31]. The $-\Delta G_{folded}$ was estimated by extrapolating from the transition region to the position of zero urea concentration along the direction of the slanted portion of the curve, while taking equal to $|4RT|$ the distance between the bands of fully folded and fully unfolded protein [45].

2.5. NMR experiments

Isoform B of BLG was purified from cow milk according to ref. [18]. Small aliquots (ca. 20 mg) were defatted by treatment on Lipidex-1000 (Perkin-Elmer) column, washed in an Amicon ultrafiltration cell and eventually concentrated using a Centricon YM10 membrane (Amicon). The concentration was determined from UV absorbance data (ε_{280} = 17,469 M^{-1} cm^{-1}).

Ligand stock solutions were prepared by dissolving weighted amounts in freshly distilled organic solvents: CD_2Cl_2 for norfloxacin (1-ethyl-6-fluoro-4-oxo-7-piperazin-1-yl-1H-quinoline-3-carboxylic acid) (ca. 10 mM), sulindac (2-[6-fluoro-2-methyl-3-[(4-methylsulfinylphenyl)methylidene]inden-1-yl]acetic acid) (ca. 10 mM), 5-fluorosalicylic acid (5-fluoro-2-hydroxybenzoic acid) (ca. 27 mM); MeOH for dexamethasone (9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydrocyclopenta[*a*]phenanthren-3-one) (ca. 10 mM). Appropriate volumes of these solutions were transferred in 5 mm NMR tubes and the solvent was slowly evaporated in a dry nitrogen stream. Stock solutions of fluvastatin sodium salt (7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1H-indol-2-yl]-3,5-dihydroxy-hept-6-enoic acid

monosodium salt) (ca. 10 mM) and dexamethsone-21-phosphate disodium salt (ca. 35 mM) were prepared in 10 mM phosphate buffer pH 7.4. The final concentration of the solutions used for NMR measurements was typically ca. 0.4–1 mM.

For all the ligands, control experiments were performed in the absence of BLG. First the dissolution process was followed through the acquisition of an overnight series of ^{19}F spectra aimed at checking whether it was affected or not by the presence of the protein. For all other experiments, BLG solutions were previously incubated with the drug for at least 12 h at 298 K in a thermostatic bath. Typically $\{^1H\}^{19}F$ spectra were acquired at 298 K on a Bruker DRX300 equipped with a QNP probe collecting 240–400 transients of 8 K data points over a spectral width of 14,000 Hz. The standard inversion recovery pulse sequence was used for the longitudinal relaxation time (T_1) measurements employing 10–12 variable delays (ranging from 1 ms to 10.0 s) for each experiment and allowing for complete relaxation a delay at least five times the estimated relaxation time. T_1 values were obtained from a non-linear three parameters fit of the experimental data. The heteronuclear Overhauser enhancement (heteronuclear n.O.e.) was estimated according to the following relationship: $\eta = (I_d - I_0)/I_0$, where I_d and I_0 are the integrated intensities of ^{19}F signals in spectra acquired with and without proton decoupling during the relaxation time.

3. Results and discussion

3.1. In silico simulations

Using the procedure detailed under Section 2, we docked compounds, listed in Table 1, to the three selected structures of BLG (the *apo* and the two *holo* forms above from which the ligand had been removed *in silico*). The K_i computed for each best complex is reported in Table 1. Data for norfloxacin, from ref. [31], are also included for comparison and further discussion.

The K_i vary from values in the same range as for the natural ligands (fluvastatin < norfloxacin < sulindac, all < 1 μM) to tens of μmol/L. The chemicals with the latter type of K_i (dexamethasone, 5-fluorosalycilate) may be expected not to bind to BLG.

For each ligand, the best solutions with each BLG target structure differ in K_i from one another, ranges spanning from a minimum of 6 times for norfloxacin and a maximum of 65 times for sulindac. As a detailed example, with fluvastatin, the drug with the highest affinity for BLG, computed binding constants resulted 2.1, 0.39 and 0.059 μM after docking to 2BLG, 1B0O and 1GX9, respectively.

In all the above complexes, the carboxylate groups of the synthetic ligands are oriented towards the mouth of the calyx and are solvent-exposed (not shown).

3.1.1. Amino acids of the BLG calyx relevant for ligand binding

Through *in silico* mutagenesis and conformational analysis, we investigated the functional role of some amino acids facing the inner BLG calyx and the analysis of their interactions with ligands was carried out with LigPlot [46].

At the level of primary structures an alignment carried out with CLUSTALW (http://www.ebi.ac.uk/clustalw [47]) shows that the two basic amino acids K60 and K69 and the hydrophobic amino acid F105 are conserved in most of the known lactoglobulins (http://www.expasy.org/sprot/). At the level of secondary/tertiary structure the orientation of F105 side chain differs by approximately 3 Å between 1B0O (or 2BLG) and 1GX9, the one of M107 is offset by approximately 2 Å between 1B0O and 2BLG, deviation for all other amino acids being slight. In a recent paper, we reported the effect of K69, I71 and M107 arrangement on the cavity volumes and on the interaction energies between norfloxacin/levofloxacin and BLG. For example, when performing a rigid docking simulation, a M107 displacement of only 2 Å varied the binding free energy of the complex norfloxacin–BLG from −5.1 kcal/mol (for 1B0O) to +4.7 kcal/mol (for 2BLG) [31].

The evaluation of the cavity in the two *holo* BLG crystal forms with CASTp [48] demonstrates a volume twice as large (at the calyx mouth) in the complex with palmitic acid (620.3 $Å^3$ in 1B0O) than in the complex with retinoic acid (348.9 $Å^3$ plus 140.4 $Å^3$ at the calyx bottom in 1GX9). One of the differences between the two protein structures is the spatial orientation of F105: we shall define *open conformation* the parallel arrangement of the aromatic ring and of the major axis of the cavity as observed with an extended, linear molecule like palmitic acid, *closed conformation* their orthogonal arrangement as observed with a shorter, bulkier molecule like retinoic acid (Fig. 2).

In the complexes obtained with flexible docking procedures, F105 conformation was found to depend not on the BLG form used in the simulation, but on the shape of the ligand, all planar, not elongated structures being compatible with the *closed conformation* of F105 (Table 2).

LigPlot displays also a regular pattern in which the carboxylate groups present on the natural as well as on the synthetic ligands form ionic pairs with K60 and/or K69. Their relevance in ligand binding has already been suggested by literature data [14,49].

Single and double BLG mutants at the two lysines were built *in silico* through K → G substitutions on the best docking form. Glycine was selected as the amino acid with the shortest side chain, for which no conformational search of the best geometrical arrangement is required. All chemicals included in the present investigation as well as one natural ligand (palmitic acid) were docked to the *in silico* mutants; results are listed in Table 3.

The interaction with the ligands, as expressed by K_i, decreases after all BLG mutations. Upon K60G replacement the affinity usually becomes one order of magnitude smaller but K69G replacement causes an even more significant K_i decrease for norfloxacin, fluvastatin and sulindac, while for dexamethasone both the substitutions have comparable effects. The orientation of the polar/charged head of the ligands towards the mouth of the calyx is usually preserved by single substitutions (exception: 1GX9 (K69G)–sulindac) but often upset by double

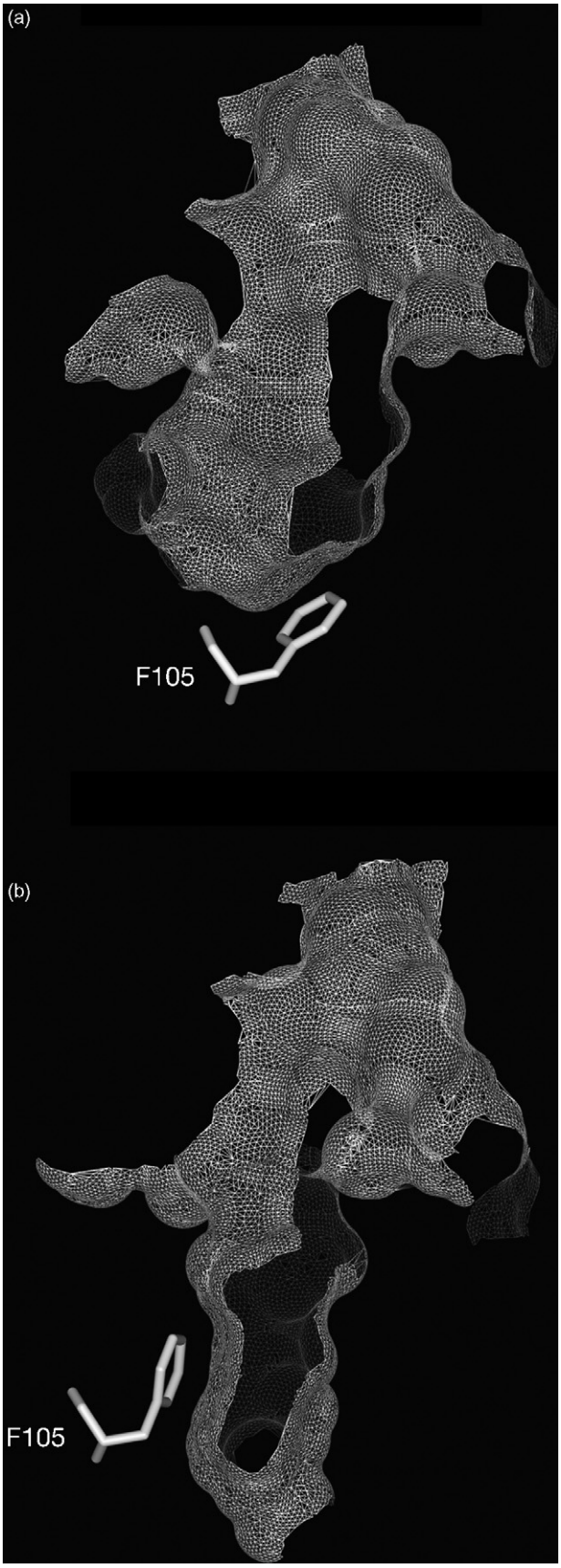

Fig. 2. BLG cavity as modulated by the alternative conformations of F105 in two X-ray structures: (a) closed conformation, PDB code: 1GX9 [13] and (b) open conformation, PDB code: 1B0O [39]. Connolly's surface drawn with Insight II.

Table 2
Ligand–cavity side chains interactions

Ligand	BLG form	F105		M107	K60	K69
Palmitic acid	1B0O	+[a]	Open[b]	+[a]	−[a]	+[a]
	1GX9	+	Open	+	+	+
	2BLG	+	Open	+	+	+
Retinoic acid	1B0O	+	Closed	+	+	−
	1GX9	+	Closed	+	+	+
	2BLG	+	Closed	+	+	−
Fluvastatin	1B0O	+	Closed	+	− (+Hydrophobic)	−
	1GX9	+	Closed	+	−	+
	2BLG	+	Closed	+	−	+
Norfloxacin	1B0O	+[c]/+[d]	Closed	−[c]/+[d]	+[c]/−[d]	+[c]/+[d]
	1GX9	+/+	Closed	+/+	−/+	−/+
	2BLG	+/−	Closed	+/+	+/+	+/+
Sulindac	1B0O	+	Closed	+	−	−
	1GX9	+	Open	+	−	+
	2BLG	+	Closed	+	−	+
Dexamethasone	1B0O	+	Closed	+	−	−
	1GX9	+	Open	−	−	−
	2BLG	+	Open	+	− (+Hydrophobic)	−
5-Fluorosalycilic acid	1B0O	−	Open	+	−	+

[a] (+) Presence and (−) absence of amino acid–ligand interaction, as drawn in LigPlot output.
[b] Conformation of F105 side chain, as defined in the text.
[c] No freedom of torsional exploration for ethyl group and for N_{18}–C_{ARO} bond.
[d] With freedom of torsional exploration for ethyl group and for N_{18}–C_{ARO} bond.

mutation (palmitic acid, norfloxacin, dexamethasone). These results suggest an important role of lysine residues at the calyx mouth in the interactions with the ligands with greater affinity.

In order to support further the *in silico* predictions, a natural LG with a different charge distribution in the calyx than BLG was sought for. Equine LG (ELG) differs at one of the selected positions for a K69E substitution [50]. Since no X-ray or NMR investigation on this protein has been reported, models of its structure were built by homology modeling using two different programs (Homology, in InsightII, and Homology, in Moe). The results of the docking experiments to these ELG models are listed in Table 3; K_i is in the mM range for palmitic acid, and cannot even be computed for all other tested ligands, none being docked by our protocol in ELG calyx. Moreover, in these adducts, an electrostatic interaction leading to a 'salt-bridge' between K60 and E69 is observed, the possible partner for the interaction with docked ligands thus resulting sequestered.

3.1.2. Molecular dynamics simulations

To assess the stability of the proposed complex structures, we performed 5 ns MD simulations for the most strongly interacting complex (fluvastatin–1GX9) as well as for a poorly interacting pair (5-fluorosalycilic acid–2BLG).

Interestingly, after approximately 2 ns, 5-fluorosalycilic acid is found outside the calyx, supporting the concept of an

Table 3
Docking to natural and *in silico* mutants of BLG

Ligand	LG form	K_i (μM)
Palmitic acid	2BLG (K60G)	51
	2BLG (K69G)	20
	2BLG (K60G K69G)[a]	1100
	ELG (Insight)	1200
Fluvastatin	1GX9 (K60G)	1.1
	1GX9 (K69G)	10
	1GX9 (K60G K69G)	34
	ELG (Insight)	–
Norfloxacin	2BLG (K60G)	28
	2BLG (K69G)	132
	2BLG (K60G K69G)[a]	117
	ELG (Insight)	–
Sulindac	1GX9 (K60G)	11
	1GX9 (K69G)[a]	45
	1GX9 (K60G K69G)	26
	ELG (Insight)	–
Dexamethasone	2BLG (K60G)	96
	2BLG (K69G)	89
	2BLG (K60G K69G)[a]	85
	ELG (Insight)	–

[a] In this complex, the polar/charged head is buried in the calyx.

inefficient interaction with BLG; on the contrary, fluvastatin after 5 ns is found still bound to the protein even if with a poorer score (K_i ca. 1 mM). We need, however, to mention that the force field used in the MD sessions (MMFF94x) describes better the ligand than the protein and that we calculated a significant RMSD for the protein between the first and the last time frame, namely ca. 8 and 10 Å for the two simulations. Furthermore, the simulated time could be too short to reveal the effect of different k_{off} values on the dynamics of the two examined complexes.

3.2. Experimental data

3.2.1. From DGGE

Electrophoresis across a transverse urea gradient demonstrates stabilization of the complexes between BLG and several of the tested ligands in comparison with *apo* BLG. The concentration of urea at one-half transition from the folded conformation with high electrophoretic mobility to the unfolded conformation with low electrophoretic mobility increases by 0.2 M (for palmitic acid and fluvastatin), by 0.3 M (for norfloxacin) (Table 4). Also ΔG_{folded} is affected with shifts between ca. 2 and 4 RT (corresponding to 0.09 and 0.18 kcal/mol). A straight band of lower slope than the main curve in the transition region (a spur) pointing toward low urea concentrations (and containing molecules in metastable form, unable to refold through the duration of the electrophoretic run [45]) is observed with all tested ligands, including dexamethasone.

This feature is even more prominent at lower pH (6.1); at alkaline pH (8.7), the complexes give rise to parallel lines, corresponding to poor reversibility of the folding/unfolding transition (half-lives in either state longer than 6 min) (for data at pH other than 7.4, please refer to Table A, Supplementary data).

Table 4
Differences in urea unfolding parameters between *holo* and *apo* forms of BLG

Ligand	$[U]_{50}$ *holo*BLG–$[U]_{50}$ *apo*BLG (mol/L)	ΔG_{folded} *holo*BLG–ΔG_{folded} *apo*BLG (RT)
Palmitic acid	0.2	1.8
Fluvastatin	0.2	0
Norfloxacin	0.3	3.8
Dexamethasone	0	1.3

3.2.2. From ^{19}F-NMR

All the studied ligands have a not negligible solubility in PBS and reference data were taken at concentrations comparable with those of the putative complexes with BLG.

After the interaction with BLG (1:1 ratio), a single ^{19}F signal is observed for all the studied ligands (Table 5). This indicates that, even if the binding constants of the complexes are small, there is fast exchange between the bound and the free ligands. All the experimental spectral parameters are, therefore, the average, weighted according to the respective molar fraction, of the corresponding properties in the bound and the free states. The chemical shifts do not appear very sensitive to the occurrence of the interaction with BLG, showing small variations both upfield and downfield for the various ligands. Instead, more significant changes are observed for the linewidths, the relaxation times and the heteronuclear n.O.e. Different mechanisms can contribute to the relaxation rates. Dipole–dipole (d.d.) and chemical shielding anisotropy (c.s.a.) mechanisms are the most likely for the studied molecules [51]. Heteronuclear n.O.e. is a measure of the relevance of the dipole interaction between fluorine and surrounding protons: when d.d. is the only relaxation mechanism for ^{19}F in small molecules $\eta_{max} = 0.53$ is measured [52]. Both T_1 and n.O.e. depend upon the molecular correlation time τ_c, which increases with the

Table 5
^{19}F NMR data for ligands and their complexes with BLG (PBS, 298 K, 7 T)

	Chemical shift (ppm)	$\Delta\nu_{1/2}$ (Hz)	T_1 (s)	η
Fluvastatin				
Ligand	−116.45	0.77	1.95 (2)	0.31
Complex	−116.47	61.0	0.57 (3)	−0.55
Norfloxacin				
Ligand	−124.71	8.7	0.78 (1)	0.16
Complex	−124.52	16.1	0.45 (1)	0.09
Sulindac				
Ligand	−113.56	1.1	1.17 (2)	0.23
Complex	−113.65	5.7	1.04 (1)	0.0
Dexamethasone				
Ligand	−164.29	5.0	0.81 (3)	0.41
Complex	−164.29	8.5	0.67 (1)	0.0
5-Fluorosalycilic acid				
Ligand	−125.08	1.5	4.9 (2)	0.1
Complex	−125.12	5.4	4.5 (1)	0.1
Dexamethasone-21-phosphate				
Ligand	−164.43	2.7	0.74 (1)	0.34
Complex	−164.40	6.7	0.68 (1)	0.21

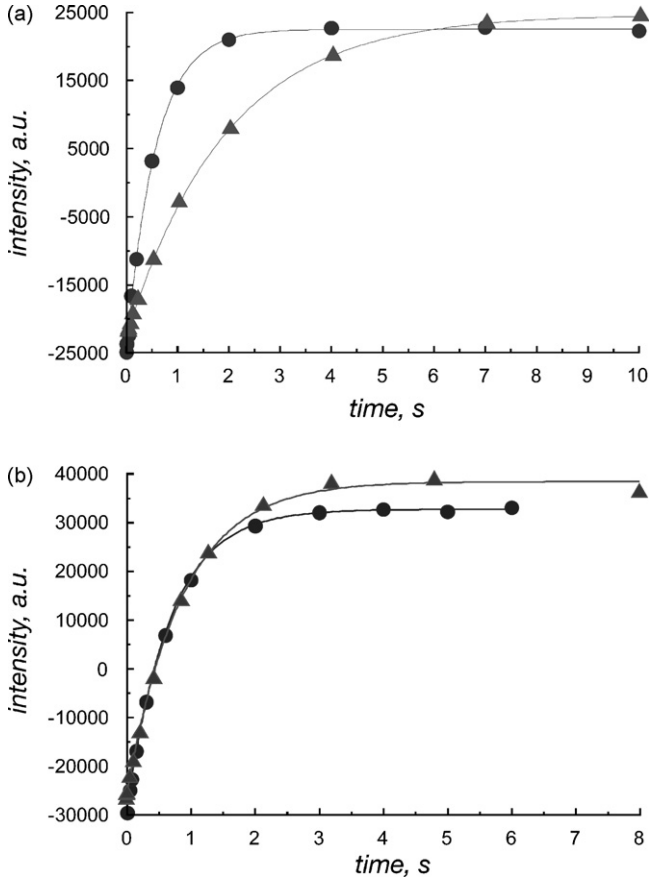

Fig. 3. Time dependence of the intensities of the ^{19}F NMR signals during inversion recovery T_1 measurements for (a) fluvastatin and (b) dexamethasone (7.0 T, 298 K, 10 mM PBS pH 7.4) in the absence (triangles) and in the presence of BLG (circles).

molecular dimensions [51]. When a small molecule, like a drug, is bound to a macromolecule, it assumes the correlation time of the new environment, that is its τ_c becomes longer. Accordingly, we expect a decrease of both the longitudinal relaxation time T_1 and of the heteronuclear n.O.e. [52]. Moreover, this last parameter can become negative and, when τ_c becomes exceedingly long, η = ca. -1, eventually leading to the disappearance of the signal when the spectrum is recorded under full proton decoupling. The comparison of the changes occurring for ^{19}F T_1 and heteronuclear n.O.e. when measured on the drugs in the absence and in the presence of the protein allows, therefore, to rank them according to their affinity toward the receptor, provided the samples have similar concentrations. On the contrary, the increase of the linewidth *per se* cannot be taken as a comparative parameter for these interactions. In fact the linewidth of the signal is expected to increase with the increase of τ_c but further broadening can derive from the exchange between the bound and free states if its rate is small compared to the difference of their chemical shifts.

For 5-fluorosalycilic acid and dexamethasone-21-phosphate the dynamic parameters are not, or only slightly, reduced in comparison with the free ligands, indicating the occurrence of just a weak interaction. On the contrary, there is evidence of increasingly stronger interactions for dexamethasone ≈ sulindac < norfloxacin < fluvastatin. Fig. 3 compares the T_1 data for fluvastatin and dexamethasone either alone or in the presence of BLG. The faster recovery of the former in the presence of BLG indicates that most of the ligand molecules experience a longer τ_c

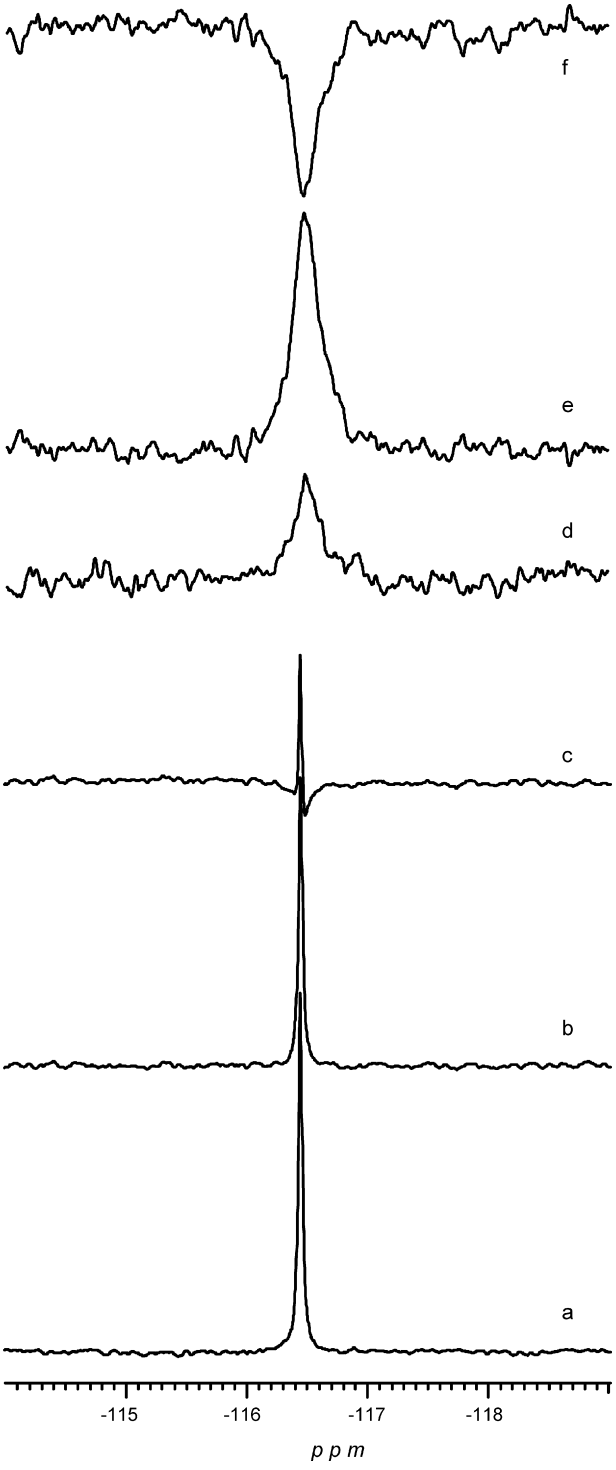

Fig. 4. ^{19}F NMR spectra of fluvastatin (7.0 T, 298 K, 10 mM PBS pH 7.4) in the absence (traces a and b) and in the presence of BLG (traces d and e). The spectra shown in traces a and d were recorded with ^{1}H decoupling during both relaxation delay and acquisition times while for the spectra in traces b and e ^{1}H decoupling was applied only during the acquisition time. Traces c and f were obtained by the Fourier transform of the free induction decay differences (a–b) and (d–e), respectively.

environment, that is, they are bound to BLG. Fig. 4 further supports this view showing the signal of fluvastatin recorded with different heteronuclear decoupling schemes in the absence (traces a and b) and in the presence of BLG (traces d and e): the difference between the two, drawn in traces c and f, is a visual evidence that the heteronuclear $\{^1H\}^{19}F$ n.O.e. becomes negative in the presence of BLG.

The significant increase (more than three times) of the relaxation rate and the very negative value of the heteronuclear n.O.e. measured in the case of fluvastatin support the idea that, among the drugs examined in this study, this ligand has the strongest interaction with BLG. The huge increase of the linewidth does not contrast this view. The evaluation of the dissociation constant for the complex fluvastatin–BLG was attempted both through NMR and fluorescence titrations. In the case of NMR relaxation titrations, the sensitivity limits of our equipment did not allow measurement at concentrations lower than 0.1 mM. At ligand/protein ratios < 1, almost constant values of relaxation rates were measured indicating a quantitative binding. When ligand/protein > 1, the relaxation rates decreased less than expected for a contribution of the free ligand; this suggested the possibility of further interactions with the other binding sites on BLG [31]. Fluorescence measurements could not be performed since the ligand resulted unstable upon irradiation. However, a conservative upper limit for the K_i of the fluvastatin–BLG complex can be set at 0.1 μM, on the basis of the above described evidence of an interaction with BLG stronger for fluvastatin than for norfloxacin and of the experimental K_i (0.089 μM) obtained for the norfloxacin–BLG complex [31].

4. Conclusions

Methods for the *in silico* evaluation of the interactions between ligand and receptor are of great relevance not only in pharmaceutical chemistry, but also in biochemistry, pharmacology and biotechnology. The procedures used in these methods differ extensively, the degree of freedom of the 'receptor' protein being the main variable [53].

Our approach, based on a multistep MC/SA-EM protocol, considers the binding site as a flexible structure, in which the side chains can rearrange and make room for a potential ligand. This flexible molecular docking procedure, because of its significant computational requirements, is not intended for a rapid screening of a ligand database, but can analyze at the molecular level receptor–ligand interactions and provide an approximated value for the dissociation constant. More reliable, but still more computationally expensive, methods for the accurate evaluation of complex binding free energy have been proposed and successfully applied [54], but they are suitable just for the analysis of a very limited number of ligands, especially if belonging to homologue/analogue series. In the present investigation, this MC/SA-EM protocol, previously validated by docking palmitic and retinoic acid to various crystallographic forms of BLG [31], has been used to study the interaction between fluorinated small molecular weight synthetic compounds and three forms of BLG.

The reported results show that the allowance of side chain flexibility for the amino acids leaning into the calyx is able to find 'best results' even starting from an *apo* structure (Table 1), in which usually the internal side chains protrude inside the cavity and can result in a failure of the 'rigid' docking procedures. Moreover, for most ligands, the affinities obtained with the screened crystallographic forms are within one order of magnitude. The information provided by the experimental DGGE and NMR data ranked the drug–BLG complexes in almost the same order (Tables 4 and 5) as the scoring function of the *in silico* procedures (Table 1). This evidence further validates the flexible docking protocol we have devised as suitable for the study of the interactions with small ligands of BLG and possibly of other calycins.

In silico results have proved also the crucial role of some conserved residues. Both the positive charges provided by K60 and, in particular, by K69 are relevant for ligand binding and as well for the orientation of the negatively charged heads of the ligands. These findings are further supported by our results on ELG. Indeed in ELG, the docking of the same ligands fails because K69E seizes K60 in a salt bridge leaving no more positive charge available for the ligands.

The observation of an especially strong interaction of one of the screened chemicals, the cholesterol-lowering drug fluvastatin, in addition to the strong affinity to BLG we already demonstrated for the antibacterial fluoroquinolone norfloxacin, sets the precondition for further *in vitro* (conditional stability to pH) and *in vivo* testing of the selected drug–BLG complexes.

It is well known that the IIAEK peptide, coming from the digestion of BLG, can exert a hypocholesterolemic effect [55]. Moreover, McAlpine and Sawyer suggested that the peculiar properties of resistance to acid and proteolytic environments could make BLG a vehicle for hydrophobic and/or acid-sensitive drugs for oral intake [30]. Complexes between BLG and statins, whose calculated dissociation constant (0.06 μM for fluvastatin) is comparable to palmitic and retinoic acid, could reach the gut undissociated. The degradation of the protein in the intestinal environment should release at the same time the drug and the cholesterol-lowering peptide.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmgm.2007.08.006.

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