514 J. Med. Chem. 2010, 53, 514–518
DOI: 10.1021/jm901309r

Journal of Medicinal Chemistry
Brief Article

The Binding of Synthetic Retinoids to Lipocalin β-Lactoglobulins

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Received August 14, 2009

The binding of therapeutically relevant synthetic retinoid derivatives to bovine and reindeer β-lactoglobulin (βLG) is demonstrated using fluorescence quenching and ultrafiltration/HPLC methods. Furthermore, synthesis of methyl (*E*)-3-[4-[(*E*)-2-(2,6,6-trimethylcyclohex-1-enyl)vinyl]phenyl]-acrylate **4** and (*E*)-3-[4-[(*E*)-2-(2,6,6-trimethylcyclohex-1-enyl)vinyl]phenyl]acrylic acid **5** is described. All studied compounds bind to both βLG homologues with nanomolar K_d values, and the interaction diminishes the pH-dependent aggregation of retinoids. Thus, βLG may show benefits in improving the bioavailability of retinoid derivatives.

Introduction

Retinoids are a class of compounds whose chemical structures are related to the diterpene vitamin A (all-*trans* retinol).[1,2] Vitamin A is physiologically required for normal growth, development, reproduction, immune system function, and vision.[3] Chemically, retinoids consist of a cyclic group connected to a polar functional group through a dimer of isoprenoid (2-methyl-1,3-butadiene) units joined in a head-to-tail manner.[4] The interest toward synthetic retinoid analogues relates not only to the endogenous activities of vitamin A but also their capacity to induce cell differentiation and apoptosis. Retinoids have found use in the treatment of acne and other dermatological diseases as well as in cancer chemotherapy and chemoprevention.[1,2,5]

β-Lactoglobulin (βLG[a]) is a whey protein of the lipocalin family, found in the milk of many mammals such as bovine, reindeer, and other ruminant species.[6,7] The precise biological function of bovine βLG is not known but it, as well as other lipocalins, has been shown to bind lipophilic nutrients, such as retinol and fatty acids, into its central calyx.[8−11] The calyx is a conserved structure within this protein family, in the case of βLG formed by eight antiparallel β strands.[12] Bovine βLG is usually found as a dimer where the weight of one subunit is 18 kDa. The X-ray crystal structure of reindeer βLG is highly similar to the structure of bovine βLG,[13] and structural variations are found only in the residues located on the surface of the protein. The immunological cross-reactivity of the bovine specific anti βLG IgE with reindeer's βLG in patients allergic to cow's milk has been investigated, and the results indicate that reindeer βLG shows less IgE binding to the capturing antigen than the bovine protein.[14] Thus, reindeer βLG could also be suitable for persons suffering from an adverse immunological response to bovine βLG. Despite the variation in surface amino acids, in our previous studies we have proved that bovine and reindeer βLG bind ligands in similar fashion.[11]

In this report, evaluation of the applications of βLG is extended to cover synthetic retinoids (Figure 1). TTNPB **2** and 4-HPR **3**, also known as fenretinide, are synthetic retinoids exhibiting anticancer activity. In addition to these compounds that already are in the pipeline, we synthesized the retinoid ester **4** and carboxylic acid **5** (Scheme 1). Retinoids **2**, **4**, and **5** are aromatic due to the presence of disubstituted phenyl moiety. It has been shown that aromatization of the polyene chain of simple retinoic acid analogues is a facile way to obtain new retinoids.[1] For example, in **2**, the combination of aromatization at C8−C18 and aromatization at C12−C14 gave a stilbene acid that has been found to have activity in the in vivo papilloma assay and to induce differentiation and apoptosis.[1,5] Compound **3** is a typical synthetic retinoid amide derived from all-*trans*-retinoic acid. Like other retinoids, it has been shown to be a potent chemopreventative and therapeutic agent for cancer, showing efficacy, e.g., against human prostate carcinoma cells.[15] In this work, a miniaturized fluorescence-based ligand binding assay[11] and a biofingerprinting chromatogram analysis using ultrafiltration/HPLC[16] are utilized to study the binding of retinoids to βLG.

Results and Discussion

As retinoid-type compounds are known to bind into the central calyx of βLG[8,11] and are also known to have several biological activities,[1] the study was initiated by synthesizing

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[a] Abbreviations: βLG, β-lactoglobulin; DMSO, dimethyl sulfoxide; HPLC, high performance liquid chromatography; Ig immunoglobulin; K_d, apparent dissociation constant; *n*, binding site per monomer; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; THF, tetrahydrofuran; TTNPB 4-[(*E*)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid, 4-HPR, *N*-(4-hydroxyphenyl)retinamide; RP-HPLC, reverse phase high performance liquid chromatography.

 Published on Web 11/25/2009

novel retinoid derivatives. The starting material 4-bromobenzaldehyde (**4a**) was converted to the corresponding methyl acrylate (**4b**) via the microwave-assisted Heck reaction.[17] The resulting aldehyde (**4b**) was treated with sodium cyanoborohydride and chlorotrimethylsilane to yield benzylic alcohol (**4c**).[18] Subsequently, the benzylic alcohol was converted to benzyl bromide (**4d**) in the presence of phosphorus tribromide. Finally, the halide (**4d**) was transformed to the corresponding dimethyl phosphonate (**4e**) by treating it with trimethylphosphite. The Horner−Wadsworth−Emmons reaction was used to prepare the retinal analogue. Phosphonate (**4e**) was treated with *n*-butyllithium in the presence of *β*-cyclocitral to yield the final product **4**. After the aqueous hydrolysis, the corresponding carboxylic acid **5** was obtained in moderate yield.

In the next step, we moved to measure the binding of the retinoids to bovine and reindeer *β*LG utilizing in vitro methods. For the binding studies, we used our previously miniaturized 96-well plate assay which is based on fluorescence quenching[11] and a method of ultrafiltration sampling combined with high performance liquid chromatography (HPLC).[16] The apparent dissociation constants (K_d) and the number of binding sites per monomer (*n*) were determined with the 96-well plate assay. The results, presented in Figure 2, indicate that all retinoids bind to the bovine and reindeer *β*LG. The number of independent binding sites for the ligand (*n*) was close to one for all ligands except **1** and **3** (Figure 2A). Thus, **2**, **4**, and **5** bind to only one binding site, while **1** and **3** may be able to occupy multiple sites. In previous studies, presence of multiple binding sites in *β*LG has been suggested for a range of ligands. The main binding site in the central calyx has been confirmed by X-ray crystallographic studies,[19,20] but according to both experimental and in silico data, additional binding sites may exist between the α-helix and *β*-strand and/or sites on protein's surface.[21−23]

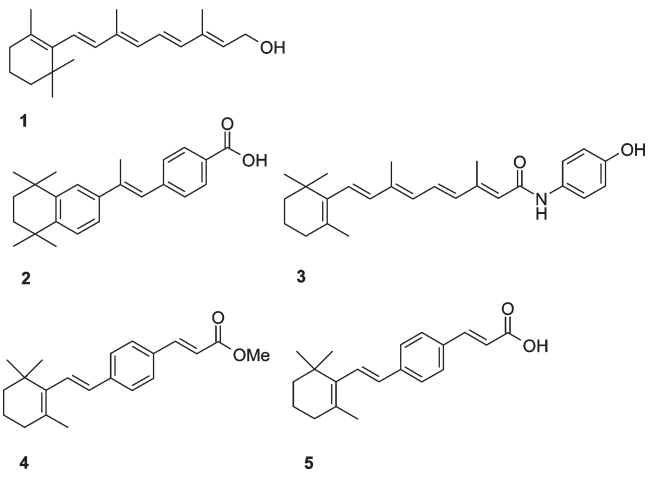


Figure 1. Chemical structures of retinoids **1**−**5**.

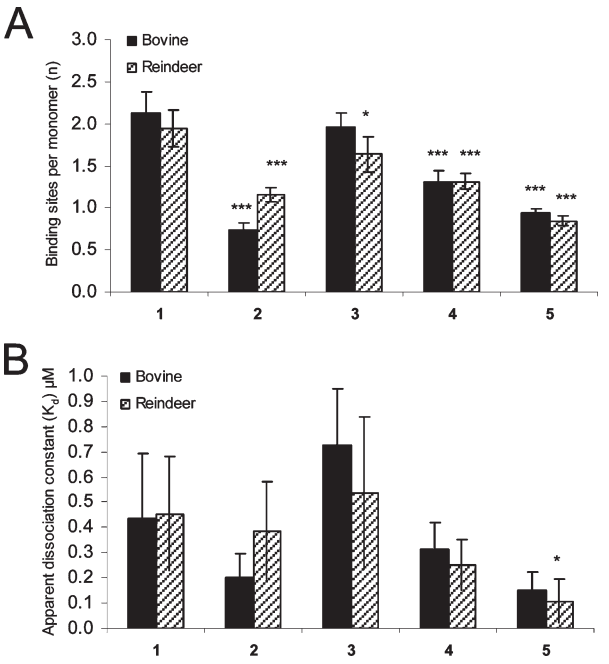
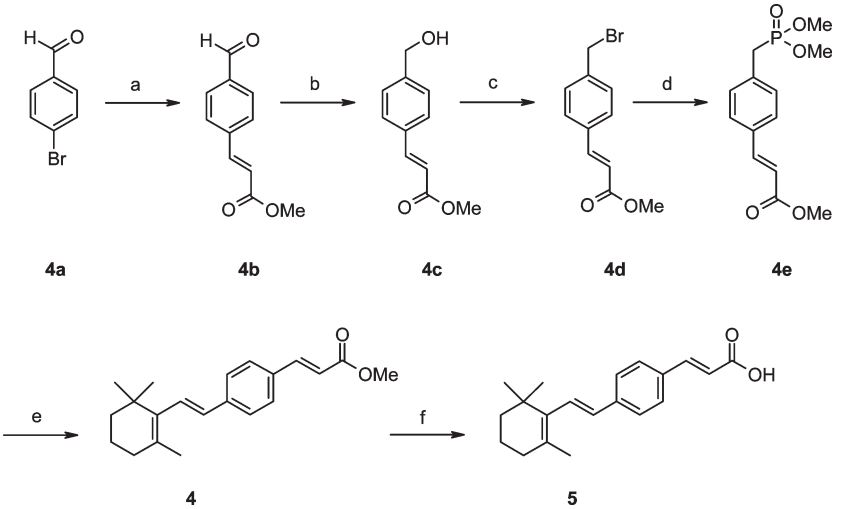


Figure 2. Binding constants of **1**−**5** by bovine and reindeer *β*LG determined by fluorescent quenching: (A) number of binding sites per monomer (*n*); (B) apparent dissociation constants (K_d). *p*-Values were calculated between retinol and its derivatives ($n = 4-8$). $*p < 0.1$, $**p < 0.05$, $***p < 0.01$.

Scheme 1[a]



[a] Reagents and conditions: (a) methyl acrylate, $Pd(OAc)_2$, $(o\text{-MePh})_3P$, DIPEA, NMP, 4 min, 200 °C, microwave, 64%; (b) $NaCNBH_3$, TMSCl, 3 Å sieves, MeCN, 25 min, −5 °C, 90%; (c) PBr_3, Et_2O, 90 min, −5 °C → rt, 80%; (d) $(MeO)_3P$, 30 min, 100 °C → rt, overnight, 50%; (e) *β*-cyclocitral, *n*-BuLi, THF, 2 h, −79 °C → rt, 50%; (f) LiOH, THF/H_2O (4:1), 48 h, 50 °C, 40%.

The apparent dissociation constant (K_d) values were fairly similar for all ligands for bovine βLG (Figure 2B). In the case of reindeer βLG, the K_d value of **5** differed from those of **1**, indicating a higher affinity of **5** to reindeer βLG than **1** has. However, the binding constants of all of the ligands were low and demonstrated that the studied ligands bind with high affinity to both bovine and reindeer βLG. The ranges of the binding constant values (to bovine βLG $K_d = 1.53 \times 10^{-7}$–$7.27 \times 10^{-7}$ M and to reindeer βLG $K_d = 1.07 \times 10^{-7}$–$5.37 \times 10^{-7}$ M) were also comparable to those reported in previous retinol βLG binding studies in which K_d values range between 3.6×10^{-8} and 2.0×10^{-7} M.[9,24–26] Despite the chance of additional binding site in the cases of **1** and **3**, the similarity of K_d values between the two proteins is likely to reflect the role of the evolutionarily conserved central calyx as the primary binding site for these compounds.

The binding of retinoids was further analyzed with ultrafiltration/HPLC, a method previously used to study the binding of peptides and palmitate to βLG.[27,28] Also in this setup, all five retinoids were found to bind bovine and reindeer βLG. Retinoid **5** appears to bind tightest to both βLG homologues (29.1% and 18.8% in bovine and reindeer βLG retentate, respectively, when 100 μM ligand was allowed to interact with 50 μM protein), followed by **1** and **4** (7.9% and 7.6% of **1** and 7.0% and 11.0% of **4** in bovine and reindeer βLG retentates, respectively). Compounds **2** and **3** bound also to both reindeer and bovine βLG but, in contrast to experiments run on **1**, **4** and **5**, **2** and **3** were not found in any samples from filtrate side, neither in the presence nor the absence of βLG. The βLG-retention samples contained 38–52% of **2** and **3**, whereas blank PBS retention samples contained no detectable amounts of retinoids. Finally, unbound **2** and **3** were found with MeOH washing of the filtration sample reservoirs.

As the ultrafiltration data suggested that the studied compounds may not be completely soluble in the conditions used, we conducted dynamic light scattering (DLS) analysis to detect the presence of aggregates or other compound-borne insolubilities. Indeed, 200 μM PBS solution of all five compounds had aggregates detectable by DLS, and in the case of **2**, **3**, and **5**, the critical concentration for formation of DLS-detectable aggregates were 50, 100, and 100 μM, respectively. Aggregate size ranged according to concentration, and at 200 μM, was approximately 600–900 nm, which is well above the pore size used for ultrafiltration experiments. Therefore, it was concluded that the observed differences in the behavior of the compounds in ultrafiltration studies are likely to be due to the differencies in the tendency to form aggregates. In addition to **2** and **3**, also compound **5** gave DLS signal at 100 μM (concentration used in ultrafiltration experiments) and the amount of **5** in LG retentate is in the same range as in **2** and **3**. However, tighter binding of **5** presumably decreased the free fraction of **5** below the aggregate-formation limit and thus allowed the unbound fraction to pass through the filter. On the other hand, the critical concentrations for aggregate formation are at least 2 orders of magnitude higher than K_d values determined by the fluorescence quenching assay, and thus aggregate formation is not likely to affect these data.

Aggregating behavior of organic small molecules is known to cause problems in the form of false positives in biossays,[29] and recently, it has also been suggested that formation of aggregates may affect the oral bioavailability of poorly soluble, lipophilic drugs.[30] To mimic the pH shift occurring during nutrient processing in gastrointestinal tract, the formation of aggregates by 100 μM retinoids **1**–**5** was analyzed in maleic acid buffer over a pH range from 2.0 to 7.0. Here, the compounds **1** and **5** gave DLS signals only in acidic pH (pH 4.0 and below), whereas **2**, **3**, and **4** were more prone to aggregate formation close to neutral pH (pH 5.0 and above). To study whether βLG could affect the aggregate formation, DLS analysis was also carried out by adding 50 μM bovine βLG into retinoid dilutions. Table 1 illustrates the results of these measurements, demonstrating the decline in DLS signals after addition of βLG. In acidic conditions, βLG had no effect on DLS signal (data not shown).

Table 1. Effect of Bovine βLG on Retinoid Borne Aggregate Formation[a]

ligand	200 μM retinoid (kCps/nm)	200 μM retinoid + 50 μM βLG (kCps/nm)
blank	1.6	25.6
1	163.4/972.2	70.4/128.7
2	384.7/848.5	87.7/243.3
3	185.1/818.1	42.4/423.6
4	164.1/791.6	14.4/276.1
5	137.4/668.7	156.6/188.0

[a] The data represents mean intensity and particle diameter values (kCps/nm) in dynamic light scattering experiments, where compounds **1**–**5** were diluted in PBS. After DLS run of each retinoid, 50 μM bovine βLG was added into cuvettes, and the second run was performed after 30 min stabilization phase. See Experimental Section for detailed DLS protocol.

Conclusion

To conclude, we have demonstrated that the studied retinoids bind tightly to both bovine and reindeer βLG, and it seems likely that the binding occurs in the proteins' central calyx. Further indication of the fitting of the synthetic retinoids into the central calyx was also provided by preliminary molecular docking studies with bovine βLG (data not shown). βLG and other similar proteins have been suggested to be useful as carrier proteins for lipophilic small molecules, and in fact, clinical data indicates that the bioavailability of 4-HPR (compound **3** of this study) is improved in coadministration with protein-rich meals.[31] However, the calyx of βLG is gated by the EF loop that is in open conformation in neutral pH, whereas in acidic environment the EF loop turns to closed conformation.[10] This conformational change is reversible, but in the case of palmitic acid it leads to release of the ligand in low pH[10] and it may have implications on binding affinity of retinoids as well.[32] There is evidence that βLG is relatively resistant to cleavage in gastric conditions, but more prone to degradation in small intestine.[33] This makes the delivery of retinoids to the intestine for absorption a plausible aim. On the other hand, the carrier function seems not to cover the actual absorption as βLG had no effect on the transport of retinol across Caco-2 cell monolayers in our previous studies.[34] In a medicinal context, the benefits may include prevention of the early degradation of retinoids and masking their taste in the oral administration of retinoid drugs. Here, we have observed a concentration and pH dependent aggregate formation of the studied retinoids. The aggregating behavior is significantly diminished in the presence of βLG, potentially having positive implications in retinoid solubility and thus also in vivo bioavailability.

Experimental Section

General. The reagents and materials for synthetic purposes were obtained from commercial suppliers and were used without further purification apart from β-cyclocitral, which was distilled

prior use. THF was dried with sodium and distilled before use. Reactions were monitored by thin-layer chromatography on silica gel 60-F_{256} plates acquired from Merck (Darmstadt, Germany). Column chromatography was carried out manually by using silica gel 60 (230−400 mesh) from E. Merck (Darmstadt, Germany) or by Biotage SP1 purification system (Charlottesville, VA) using Flash 25+M or 12+M silica cartridges. ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian Mercury Plus 300 MHz spectrometer (Varian, Palo Alto, CA) using $CDCl_3$ as a solvent. Chemical shifts are reported relative to TMS. *J* values are given in Hz. Purity of the compounds was checked using the GC-MS system that consisted of a HP 5890A gas chromatograph and a 5970 mass selective detector (Hewlett-Packard, Palo Alto, CA). GC-MS spectra were analyzed with the HP Chemstation program on Windows 3.1 workstation. Column: HP-5MS (15 m × 0.254 mm, 0.25 μm). GC-MS parameters: scan range 50−650, solvent delay 3.00 min, injector temperature 250 °C, detector temperature 280 °C, initial temperature 100 °C, final temperature 310 °C, temperature ramp 20 °C/min, runtime 13.50 min, carrier gas He 99.9996%. Purity of the target compounds was >95% demonstrated as stated above.

Synthesis. Methyl (*E*)-3-[4-[(*E*)-2-(2,6,6-trimethylcyclohex-1-enyl)vinyl]phenyl]acrylate (4). Methyl (*E*)-3-[4-(dimethoxyphosphorylmethyl)phenyl]acrylate (**4e**) (156 mg, 0.614 mmol) was dissolved in dry THF (6 mL) under argon atmosphere. Reaction mixture was cooled to −79 °C and 1.6 M solution of *n*-butyllithium in THF (425 μL, 0.676 mmol) was added dropwise. The resulting mixture was stirred at −79 °C for 20 min, and β-cyclocitral (93 mg, 0.61 mmol) was added dropwise in anhydrous THF (3 mL). Reaction mixture was stirred for 90 min and allowed to warm to room temperature. Reaction was quenched with saturated aqueous solution of NH_4Cl (10 mL). Aqueous phase was separated and extracted with diethyl ether (2 × 5 mL). The combined organic phases were washed with brine (5 mL), dried with Na_2SO_4, and evaporated to dryness. The crude product was purified by silica gel column chromatography using toluene as an eluent to give **4** in 50% yield.

(*E*)-3-[4-[(*E*)-2-(2,6,6-Trimethylcyclohex-1-enyl)vinyl]phenyl]-acrylic acid (5). Methyl (*E*)-3-[4-[(*E*)-2-(2,6,6-trimethylcyclohex-1-enyl)vinyl]phenyl]acrylate (**4**) (193 mg, 0.62 mmol) was dissolved in THF (8 mL) and H_2O (2 mL). Lithium hydroxide (150 mg, 3.11 mmol) was added, and the resulting reaction mixture was stirred for 48 h at 50 °C and concentrated in vacuo. Water (2 mL) was added to the residue. Mixture was acidified with 6 M HCl and extracted with ethyl acetate (3 × 10 mL). The combined organic phases were washed with brine (10 mL), dried with Na_2SO_4, and evaporated in vacuo. The crude product was purified by Biotage SP-1 flash chromatography system using hexane−ethyl acetate (3:1) as an eluent to give **5** in 40% yield.

Acknowledgment. The financial support from the Graduate School of Pharmaceutical Research in Finland (L.H.R.), the Finnish Cultural Foundation/Elli Turunen foundation (L.H.R.), the National Graduate School in Informational and Structural Biology (M.J.V.), Sigrid Jusélius Foundation (M.J.V.), Academy of Finland (M.J.V.), National Technology Agency (J.Y.K., P.M.V.), and Tor, Joe and Pentti Borg-fund from Åbo Akademi University (P.M.V.) is greatly acknowledged.

Supporting Information Available: Synthesis of intermediates **4a**−**4e** as well as the experimental protocols for binding assays and DLS analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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