Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 3725–3731
Bioorganic & Medicinal Chemistry Letters

In vitro binding of leukotriene B_4 (LTB_4) to human serum albumin: Evidence from spectroscopic, molecular modeling, and competitive displacement studies

Ferenc Zsila,[a,*] Zsolt Bikádi[a] and Samuel F. Lockwood[b]

[a]*Department of Bioorganic Chemistry, Institute of Biomolecular Chemistry, Chemical Research Center, P.O. Box 17, Budapest H-1525, Hungary*
[b]*Hawaii Biotech, Inc., 99-193 Aiea Heights Drive, Suite 200, Aiea, HI 96701, USA*

Received 23 March 2005; revised 17 May 2005; accepted 18 May 2005
Available online 1 July 2005

Abstract—Circular dichroism (CD) and UV absorption spectroscopy were utilized for the first time to investigate the interaction between leukotriene B_4 (LTB_4) and human serum albumin (HSA) in vitro. The weak intrinsic CD signal of LTB_4 was enhanced fivefold in the presence of HSA. The red-shifted, hypochromic, and reduced vibrational fine structure of the ligand/protein UV absorption spectrum indicated complexation of the two molecules in solution. Results obtained from CD titration experiments were subjected to non-linear regression analysis to estimate the binding parameters ($K_a = 6.7 \times 10^4$ M^{-1}, $n = 1$). Palmitic acid strongly decreased the induced CD signal of the LTB_4/HSA complex, suggesting the role of a high-affinity fatty acid HSA binding site in the leukotriene complexation. Molecular modeling calculations based on the crystal structure of HSA predicted that the long-chain fatty acid site that overlaps with drug binding site II in subdomain IIIA was the most likely binding location for LTB_4. Using the drug site II-specific marker ligand *rac*-ibuprofen, this prediction was confirmed with induced-CD displacement measurements. To the authors' knowledge, the current study represents the first demonstration of binding of LTB_4 to HSA in vitro and has implications for the biological transport of this important pro-inflammatory mediator in vivo.

1. Introduction

The metabolism of free arachidonic acid by the 5-lipoxygenase pathway results in the formation of several chemically related leukotriene molecules.[1,2] Leukotriene B_4 (LTB_4, Fig. 1) is an extremely potent lipid inflammatory mediator derived from membrane phospholipids by the sequential actions of cytosolic phospholipase A_2, 5-lipoxygenase, and leukotriene A_4 (LTA_4) hydrolase.[3] LTB_4 has been shown to exert a wide range of biological activities. Its binding to specific G-protein-linked cell surface receptors stimulates a number of leukocyte functions, including: (1) adhesion to vascular endothelial cells; (2) transendothelial migration; (3) chemotaxis; (4) release of lysosomal enzymes; and (5) production of reactive oxygen species (ROS). These properties have implicated LTB_4 as a pro-inflammatory mediator in the pathogenesis of several important inflammatory diseases, including arthritis, psoriasis, inflammatory bowel disease, asthma, and most recently, atherosclerosis.[4,5]

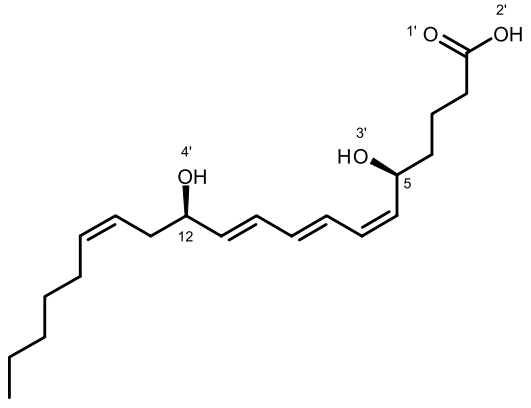


Figure 1. Chemical structure of leukotriene B_4 (LTB_4; 5*S*,12*R*-dihydroxy-6*Z*,8*E*,10*E*,14*Z*-eicosatetraenoic acid).

Abbreviations: CD, circular dichroism; CE, cotton effect; FA, fatty acid; HSA, human serum albumin; IBP, ibuprofen; LCFA, long-chain fatty acid; LTB_4, leukotriene B_4; L/P, ligand/protein molar ratio; m-deg, millidegree; NSAID, non-steroidal anti-inflammatory drug; UV, ultraviolet.

Keywords: Leukotriene B_4; Human serum albumin; Circular dichroism spectroscopy; Palmitic acid; Ibuprofen; Induced chirality.

* Corresponding author. Tel.: +36 1 438 4141; fax: +36 1 325 7750; e-mail: zsferi@chemres.hu

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.05.125

Human serum albumin (HSA), the most abundant protein in human plasma, is responsible for the reversible binding and transport of a large variety of endogenous and exogenous substances.[6,7] Forty percent (40%) of the total body store of HSA circulates in whole blood; 60% is located extravascularly, and therefore is a major protein in the extracellular compartment where leukotrienes may be synthesized and exert their actions in autocrine and paracrine functions.[6,8] Limited treatment of the potential interactions of leukotrienes with HSA is found in the literature; in previous reports 14,15-leukotriene A_4 (14,15-oxido-5,8,10,12-eicosatetraenoic acid),[9] thromboxane A_2,[10] and leukotriene A_4 (5,6-oxido-7,9,11,14-eicosatetraenoic acid)[11] have been reported to bind to albumin.

In the current study, we have capitalized on the advantageous UV and CD spectroscopic properties of the triene chromophore of the leukotrienes to investigate the binding interactions between LTB_4 and HSA. We report, for the first time, spectroscopic evidence of the complexation between this important pro-inflammatory mediator and HSA. We subsequently utilized the available X-ray crystallographic information on HSA to model the probable high-affinity binding site for the LTB_4 molecule. In a series of induced-CD competitive binding experiments, we used the HSA drug-site II-specific ligand *rac*-ibuprofen (IBP) to competitively displace LTB_4 from HSA, confirming the probable location of the complexation between LTB_4 and HSA predicted from the molecular modeling studies. These seminal results will likely provide support for in vivo studies of the interaction of plasma proteins and pro-inflammatory mediators generated by important lipoxygenase activity in disease.

2. Materials and methods

2.1. Materials

Fatty acid-free human serum albumin (Sigma), leukotriene B_4 (Cayman Chemical), *rac*-ibuprofen (Sigma), palmitic acid (Sigma), buffer constituents (Reanal, Hungary), and organic solvents (HPLC grade) were obtained commercially and used as supplied.

2.2. Preparation of HSA and LTB_4 solutions

For spectroscopic measurements, solid HSA was weighted and placed in a rectangular cuvette with 1 cm pathlength, and then dissolved in 2 ml of a 0.1 M, pH 7.4, phosphate-buffered solution. The protein concentration was determined spectrophotometrically from the UV spectrum using an absorbance value of 0.531 (279 nm, 1 g/L, optical pathlength 1 cm).[6] The molecular weight (M_w) of HSA utilized for these calculations was 66,500 Daltons (Da).

The LTB_4 stock solution was prepared in ethanol (EtOH; 0.1 mg/0.29 ml, M_w = 336.5). The concentration was verified by measuring the ethanolic UV spectrum (ε_{max} = 50,000 at 270 nm).

2.3. CD and UV spectroscopic measurements

Circular dichroism (CD) and UV spectra were recorded on a Jasco J-715 spectropolarimeter at 25 ± 0.2 °C, in a rectangular cell with 1.0 cm pathlength equipped with magnetic stirring. The spectra were accumulated three times with a bandwidth of 1.0 nm. Induced CD spectra were obtained as the difference between the spectra of the LTB_4–HSA mixture and those of the HSA protein solution alone. Ellipticities (Θ) were expressed in millidegrees (mdeg). Ellipticity values are converted to '$\Delta\epsilon$' values using the equation $\Delta\epsilon = \Theta/(33982cl)$, where $\Delta\epsilon$ is the molar circular dichroic absorption coefficient expressed in M^{-1} cm^{-1}, c is the concentration of the sample expressed in mol/L, and l is the path length through the cell expressed in cm. The ligand (L) was added in small aliquots of stock solution in ethanol to the HSA solutions (P), and the L/P ratio was recorded.

2.4. Evaluation of the binding stoichiometry and association constant of the LTB_4–HSA complex

The stereospecific interaction between a ligand (L) and its primary site on the protein (P) may be quantified by the association constant (K_a):

$$L + P \rightleftharpoons LP; \quad K_a = \frac{[LP]}{[L][P]}. \tag{1}$$

From Eq. 1, then:

$$[L] = c_L - [LP], \tag{2}$$

and

$$[P] = c_P - [LP], \tag{3}$$

where c_L and c_P equal the total concentrations of the ligand and protein, respectively.

Because the formation of ligand–protein complexes is responsible for the induced CD activity, it follows that:

$$CD\ (mdeg) = k[LP], \tag{4}$$

where k is a constant.

Using Eqs. (1)–(4), we obtain the following equation:

$$CD\ (mdeg) = \frac{k}{2}\left(c_P + c_L + K_a^{-1} - \sqrt{\left(c_P + c_L + K_a^{-1}\right)^2 - 4c_P c_L}\right). \tag{5}$$

To calculate the optimal value of K_a from the experimental data, non-linear regression analysis (using the CD titration data between 260 and 285 nm) was performed with the NLREG® statistical analysis program (version 6.3).

2.5. Molecular modeling calculations

All computer modeling procedures were carried out using the Sybyl 6.6 program (Tripos Inc., St. Louis, MO) on a Silicon Graphics Octane workstation utilizing

the Irix 6.5 operating system. The three-dimensional coordinates of HSA complexed with palmitic acid were obtained from the Protein Data Bank (entry PDB code 1E7H). Gasteiger–Huckel partial charges were applied both for ligands and proteins. LTB_4 was energy-minimized using the MMFF94 force field by the Powell Conjugate Gradient method until the convergence was less than 0.01 kcal/(molA). The multifit command was used to perform a flexible fit between the six molecules of palmitic acid bound to the long-chain fatty acid sites 1–6 and LTB_4 molecules. The resulting complex was energy-minimized using the 'dock' command of the Sybyl program.

3. Results and discussion

3.1. CD and UV spectral properties of LTB_4 in buffer solution

The conjugated double-bond system of LTB_4 constitutes the light-absorbing chromophore that gives the strong absorption band associated with an electronic dipole allowed π–π* transition polarized along the long axis of the triene system (Fig. 2). The UV absorption peak exhibits the resolved vibronic structure characteristic of polyene compounds. When free in organic or aqueous solution, LTB_4 displays a weak, positive π–π* CD band between 230 and 300 nm (Fig. 2). The conjugated triene moiety is planar without structural features that result in helical distortion. Thus, chiral perturbation of the intense π–π* transition by the adjacent chiral centers (5*S* and 12*R*) is responsible for the measured intrinsic CD activity. At room temperature, free rotation of the chiral centers around the C5–C6 and C11–C12 single bonds is not restricted; a significant number of distinct conformer populations therefore exist in solution.[12] Conformers exist in solution whose CD contributions are both positive and negative; an overall weak net CD signal due to mutual cancellation effects is therefore observed.

Significant changes in both the CD and UV spectra of LTB_4 were observed after addition of the ligand to the pH 7.4 buffer solution containing HSA. To visualize these spectral changes, the intrinsic CD and absorption spectra of the HSA were subtracted from those of the mixture (Fig. 3). In comparison with the data obtained

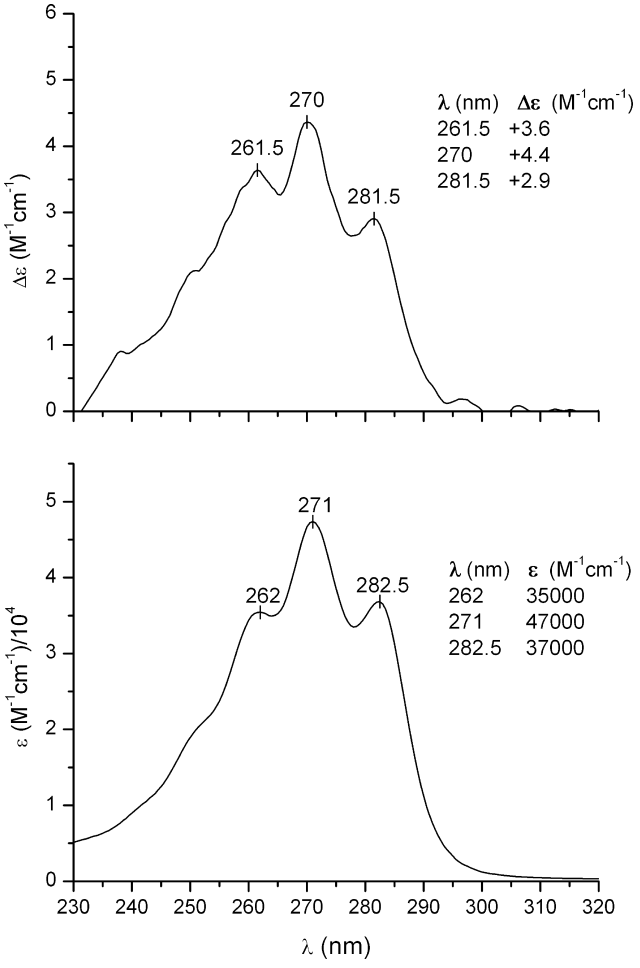


Figure 2. CD and UV spectra of leukotriene B_4 in 0.1 M, pH 7.4, phosphate buffer ($c = 2.0 \times 10^{-5}$ M, $t = 25$ °C). Maximum values of the molar absorption (ε) and circular dichroic absorption coefficients ($\Delta\varepsilon$) are shown.

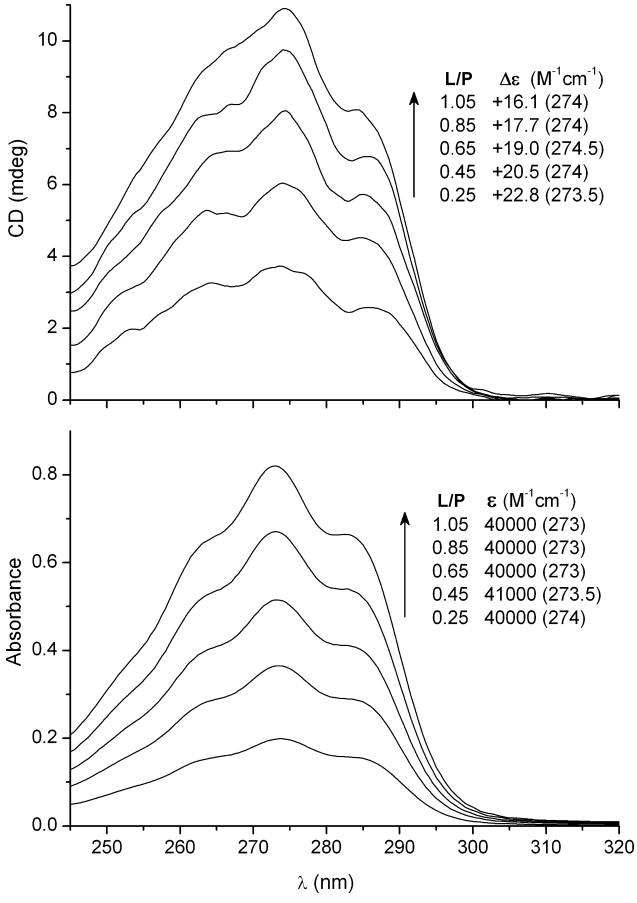


Figure 3. Representative CD and UV spectra obtained following the titration of the buffer solution of HSA with LTB_4. Spectral contributions of the protein alone were subtracted from the spectra of the ligand–protein mixture ([HSA] = 2.0×10^{-5} M, $t = 25$ °C). ε_{max} and $\Delta\varepsilon_{max}$ values calculated on the basis of the total ligand concentrations are displayed at the different ligand/protein molar ratios (L/P). Arrows denote increasing concentration of LTB_4.

for LTB_4 in buffer alone (Fig. 2), the main features of the complexed spectra were as follows:

(1) The molar CD intensity of the LTB_4 Cotton effect was greatly increased; in buffer alone, the $\Delta\varepsilon_{max}$ was $+4.4\ M^{-1}\ cm^{-1}$, while in the presence of HSA, this value was amplified fivefold (+20–$22\ M^{-1}\ cm^{-1}$).
(2) The UV band exhibited significant hypochromism ($47{,}000_{buffer} \rightarrow 40{,}000_{buffer\ +\ HSA}$).
(3) The vibrational fine structure of the absorption peak was less pronounced and the UV maximum exhibited a slight bathochromic (red) shift.
(4) The spectral position and shape of the CD peak matched well with that of the absorption band, and did not change upon increasing the concentration of the ligand.

The above results strongly suggested binding of LTB_4 to HSA under these experimental conditions. It appears that the protein environment strongly reduced the conformational freedom of the ligand and restricted rotation of the 5*S* and 12*R* chiral centers relative that of triene chromophore. The observation that the HSA-bound LTB_4 molecules also gave rise to a positive CD signal suggested that the binding site preferred (and therefore enriched) the chiral conformation likely responsible for the weak positive CE noted in ligand-only organic and buffer solutions. The hypochromism of the UV band indicated that the triene chromophore was completely encompassed by the protein environment. This situation favored dispersion interactions between the conjugated leukotriene and individual HSA protein residues, leading to the observed hypochromism and bathochromic shift of the UV peak. Association of LTB_4 with the solvent-exposed HSA protein surface should not result in a decrease of the measured absorption.

The spectral characteristics of the CD bands were found to be stable through time. It was subsequently utilized to estimate the binding affinity (K_a) and the probable number of binding sites (n) for the LTB_4/HSA interaction. A CD titration series was performed by adding LTB_4 stock solution consecutively to a buffer solution containing HSA at constant concentration. Non-linear regression analysis applied to the induced CD values obtained from the titration series yielded a K_a of $6.7 \times 10^4\ M^{-1}$ (Fig. 4). A 'best curve' fit was then obtained for an $n = 1$. The results of this analysis suggested that LTB_4 bound to a single site of HSA with moderate affinity.

Based on the physicochemical and structural similarities between LTB_4 and long-chain fatty acids (LCFA), further spectroscopic experiments were undertaken to obtain information on the potential location of the HSA binding site of LTB_4. X-ray crystallographic studies have previously revealed seven LCFA binding sites distributed across the three domains of HSA.[13] The highest affinity sites are associated with domains I and III. Palmitic acid, one of the most physiologically important fatty acid ligands of HSA, was used to study the effects of fatty-acid binding on the optical properties of the

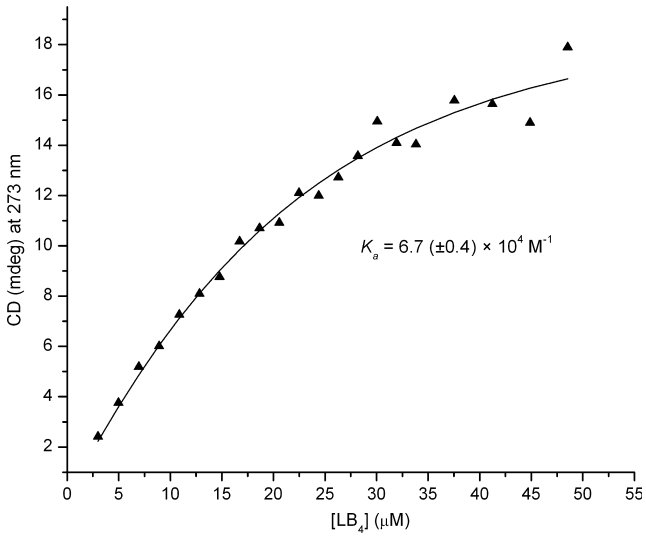


Figure 4. Maximum CD band intensities measured during titration of HSA with LTB_4 plotted against the concentration of the ligand ([HSA] = 2.0×10^{-5} M, t = 25 °C). Solid line: non-linear regression analysis (best-fit) performed on experimentally derived CD data points. The derived value of the association constant (K_a) is indicated (inset).

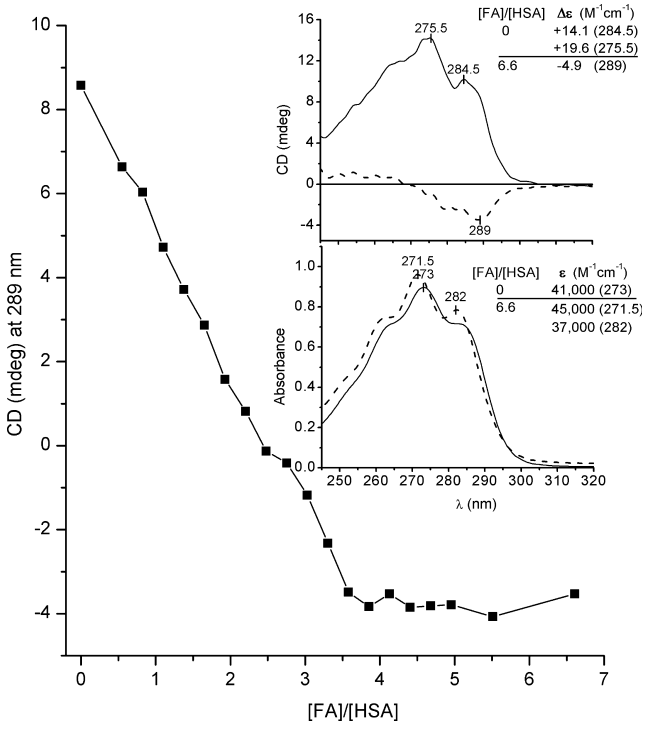


Figure 5. Titration of LTB_4–HSA (21 and 22 μM) solution with palmitic acid (FA). Inset: effect of palmitic acid on the CD and UV spectra of the LTB_4–HSA complex (pH 7.4 phosphate buffer at 25 °C). Solid line: [FA] = 0; dotted line: [FA] = 144 μM.

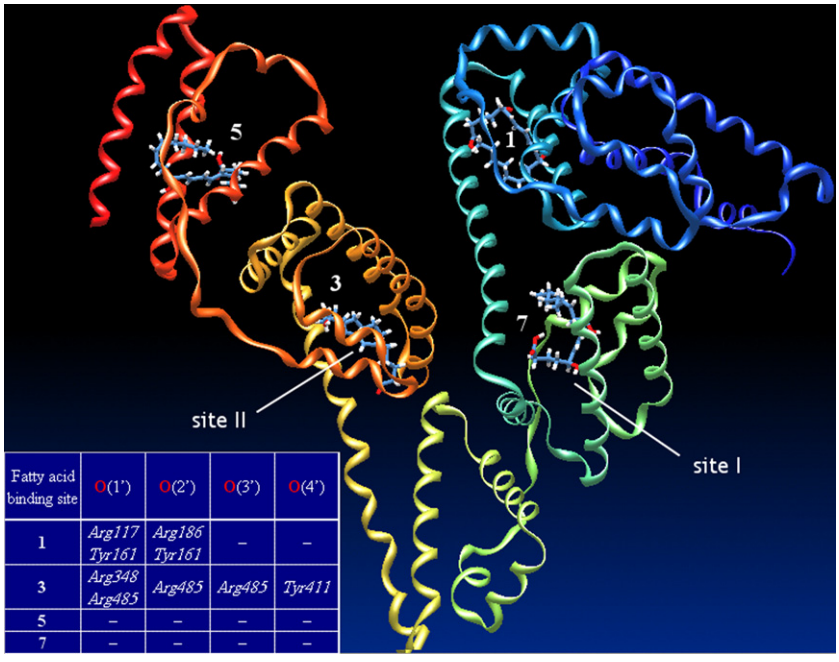
LTB_4–HSA complex. As shown in Fig. 5, palmitic acid titration rapidly reduced the induced positive CD band of LTB_4–HSA complex to zero. In addition, titration with palmitic acid shifted the UV peak of the complex to shorter wavelengths, with a concomittant increase of the observed vibrational fine structure. These spectral

changes strongly suggested the direct competition between palmitic acid and LTB_4 for the binding site, with displacement of the leukotriene ligand from the probable common binding site. Notably, nearly complete extinction of the positive CD signal was achieved at a [FA]/[HSA] ratio of 2, further suggesting that LTB_4 did in fact bind to one of the high-affinity fatty acid sites. Interestingly, a weak negative CE appeared after the positive CE disappeared; the spectral shapes were similar, with the exception that the negative CE appeared at slightly longer wavelengths (Fig. 5). It is possible that after displacement from their primary binding site, a portion of LTB_4 molecules might have been associated with a secondary binding site yielding an overall negative CD contribution.

The results of the previous fatty-acid CD displacement experiments prompted additional investigations employing molecular modeling methodology. The high resolution X-ray structure of the palmitic acid–HSA complex is now available.[13] We utilized the reported crystal structure for docking calculations; the palmitic acid molecules were replaced by LTB_4 molecules using flexible fit. The ligand–protein complex obtained after energy minimization is displayed in Figure 6. Only the best energy results are shown, numbered according to the LCFA binding sites of HSA. Theoretical projections suggested that all LCFA protein domains were suitable binding environments for LTB_4. Two high-affinity fatty acid binding sites (3 and 5) were identified, with sites 1 and 7 classified as secondary, lower-affinity sites.[13] The X-ray crystallography and modeling studies revealed that electrostatic interactions between the fatty acid carboxylate group(s) and the binding site residues crucially determine the binding affinity.[13] The table in Fig. 6 summarizes the ionic and hydrogen (H)-bond interactions between LTB_4 molecules and the critical protein residues. The resulting data show that the site 3 ligand (subdomain IIIA) established the largest number of such interactions. In addition to the electrostatic interactions of the carboxylate moiety, both the hydroxyl groups were also involved in intermolecular H-bonds with arginine and tyrosine residues, respectively (Fig. 7). The computational modeling results therefore suggested that the primary binding site of LTB_4 was located within subdomain IIIA of HSA.

The main drug binding sites I and II of HSA[6,7] can each be occupied by fatty acids (sites 7 and 3 in Fig. 6). Drug site II (subdomain IIIA) has been suggested to have high affinity for long-chain fatty acids, in contrast to site I, where no fixed carboxylate interaction has been found with these lipid ligands.[13] Several exogenous and endogenous compounds are well known which bind selectively to either site I or II.[6,7] Using such marker ligands, the site specificity of a given experimental compound can be determined. Ibuprofen (IBP; see Fig. 8), a non-steroidal anti-inflammatory drug (NSAID), is a site II marker with a high affinity association constant ($3.3 \times 10^6\ M^{-1}$).[6,14] Its chiroptical properties make it ideal for CD competition measurements of the LTB_4–HSA complex: (1) the benzene ring is a very weak chromophore, and therefore its UV contribution is negligible; and (2) the racemic (‘*rac*’) sample is CD inactive.

The CD and UV spectra of the LTB_4–HSA complex were recorded during a sequential increase of the *rac*-ibuprofen concentration in the sample solution (Fig.



Fatty acid binding site	O(1')	O(2')	O(3')	O(4')
1	*Arg117 Tyr161*	*Arg186 Tyr161*	–	–
3	*Arg348 Arg485*	*Arg485*	*Arg485*	*Tyr411*
5	–	–	–	–
7	–	–	–	–

Figure 6. Energy-minimized molecular model of HSA obtained by removing the palmitic acid ligands and by the subsequent docking of LTB_4 ligand molecules. According to Ref. 13, the numbers refer to the long-chain fatty acid (LCFA) sites of HSA. The primary drug-binding sites I and II are indicated. The protein secondary structure is shown schematically with the domains color-coded as follows: I, blue; II, green; III, red. The table inset lists the ionic as well as hydrogen (H)-bond interactions found between the LTB_4 molecules and HSA protein residues (see the oxygen atom numbering in Fig. 1).

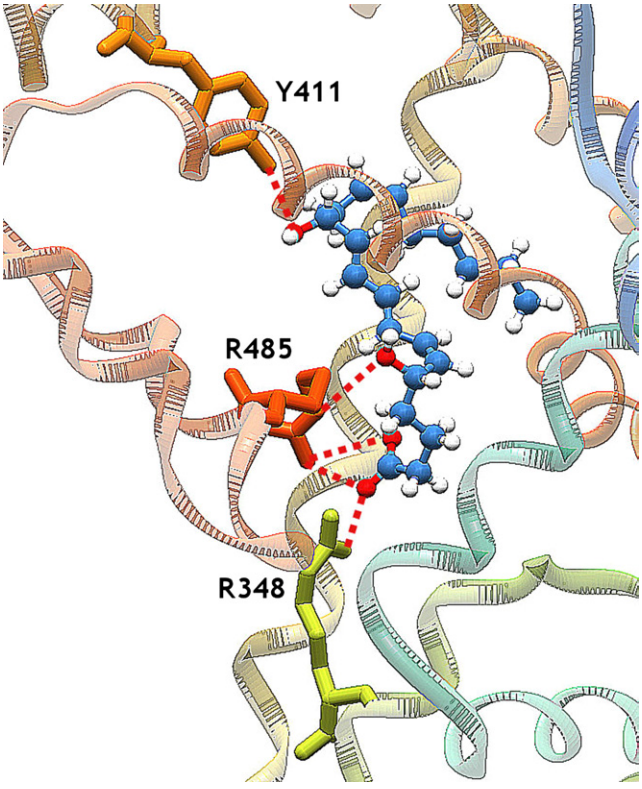


Figure 7. Expanded view of the HSA FA site 3–LTB_4 molecule interaction (ball-and-stick representation) identifying the participating HSA protein residues (denoted by their one-letter amino acid codes).

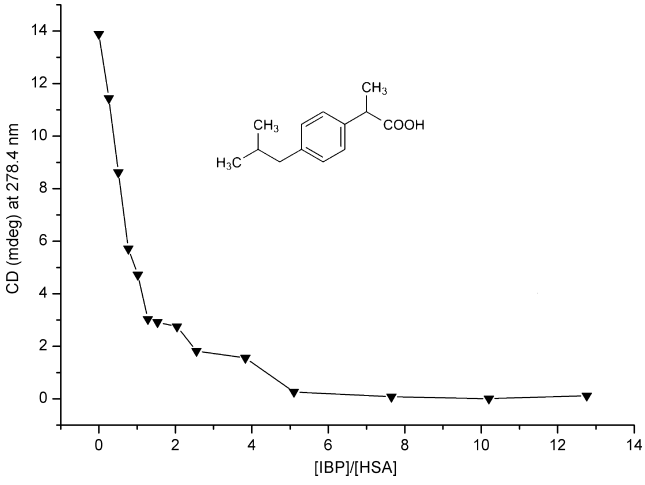


Figure 8. Effect of *rac*-ibuprofen (IBP) on the CD spectrum of 20 μM LTB_4 in 26 μM HSA solution (0.1 M, pH 7.4, phosphate buffer at 25 °C).

8). The positive CD signal decreased rapidly between [IBP]/[HSA] ratios of 0 and 1, where the *rac*-IBP drug bound to its high-affinity site in subdomain IIIA. A small negative CD peak around 287 nm again appeared at higher concentrations of *rac*-IBP (data not shown). During the titration, the UV absorption intensity and vibronic fine structure of LTB_4 increased and exhibited a hypsochromic shift (spectra not shown). Taken together, these spectral changes induced by the addition of *rac*-IBP provided clear experimental evidence of the displacement of the bound LTB_4 at drug site II (=fatty acid site 3). The results are also in full agreement with the results obtained above with palmitic acid as well as predictions obtained with the molecular docking calculations.

4. Conclusions

The nature and dynamics of small molecule interactions with biomacromolecules represents an active area of scientific research. The current study explored the potential in vitro non-covalent binding of LTB_4 to human serum albumin, the major protein in the human intra- and extravascular space. Detailed analysis of the CD and UV spectroscopic properties of the leukotriene–albumin complex and molecular modeling calculations provided essential data on the observed binding interaction. LTB_4 exhibited a single, moderate-affinity binding site on HSA with an experimentally determined association constant, $K_a = 6.7 \times 10^4\ M^{-1}$. CD displacement experiments with two well-characterized HSA ligands (palmitic acid, *rac*-ibuprofen) strongly suggested that LTB_4 bound to a long-chain fatty acid site on the protein in close proximity or equivalent to drug binding site II in subdomain IIIA of HSA. These results complement earlier studies with eicosanoid compounds that had been found to bind at the HSA drug site I (subdomain IIA),[10,11] and expand the potential binding capability of HSA for leukotrienes to include drug binding site II. The observed differential binding preferences for these molecules may be due in part to structural differences among these arachidonic acid metabolites. Competitive binding by other biologicaly important ligands (e.g., NSAIDS and fatty acids) may influence the carrying capacity of albumin for these highly potent pro-inflammatory mediators. Leukotrienes closely related to LTB_4 (such as its 6-*trans*- and 12-epi isomers, its 20-carboxy and 20-hydroxy derivatives, as well as leukotriene B_3) may also reasonably be expected to show similar binding characteristics.

Acknowledgment

Funding for the current study was provided through a scientific collaborative development grant from Hawaii Biotech, Inc, to one of the authors (F.Z.)

References and notes

1. Samuelsson, B.; Dahlen, S. E.; Lindgren, J. A.; Rouzer, C. A.; Serhan, C. N. *Science* **1987**, *237*, 1171.
2. Hedqvist, P.; Raud, J.; Palmertz, U.; Kumlin, M.; Dahlen, S. E. *Adv. Prostaglandin Thromboxane Leukot. Res.* **1991**, *21B*, 537.
3. Crooks, S. W.; Stockley, R. A. *Int. J. Biochem. Cell Biol.* **1998**, *30*, 173.
4. Silverman, E. S.; Drazen, J. M. *Proc. Assoc. Am. Physicians* **1999**, *111*, 525.
5. Spanbroek, R.; Grabner, R.; Lotzer, K.; Hildner, M.; Urbach, A.; Ruhling, K.; Moos, M. P.; Kaiser, B.;

Cohnert, T. U.; Wahlers, T.; Zieske, A.; Plenz, G.; Robenek, H.; Salbach, P.; Kuhn, H.; Radmark, O.; Samuelsson, B.; Habenicht, A. J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1238.
6. Peters, T. *All about albumin*; Academic Press: San Diego, 1996.
7. Carter, D. C.; Ho, J. X. *Adv. Protein Chem.* **1994**, *45*, 153.
8. Benz, M.; Werz, O.; Jacob, R.; Steinhilber, D. *Inflamm. Res.* **1997**, *46*, 366.
9. Haeggstrom, J.; Fitzpatrick, F.; Radmark, O.; Samuelsson, B. *FEBS Lett.* **1983**, *164*, 181.
10. Folco, G.; Granstrom, E.; Kindahl, H. *FEBS Lett.* **1977**, *82*, 321.
11. Fitzpatrick, F. A.; Morton, D. R.; Wynalda, M. A. *J. Biol. Chem.* **1982**, *257*, 4680.
12. Sugiura, M.; Beierbeck, H.; Belanger, P. C.; Kotovych, G. *J. Am. Chem. Soc.* **1984**, *106*, 4021.
13. Bhattacharya, A. A.; Grune, T.; Curry, S. *J. Mol. Biol.* **2000**, *303*, 721.
14. Yamasaki, K.; Rahman, M. H.; Tsutsumi, Y.; Maruyama, T.; Ahmed, S.; Kragh-Hansen, U.; Otagiri, M. *AAPS PharmSciTech.* **2000**, *1*, E12.