Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic Chemistry 31 (2003) 378–388
BIOORGANIC CHEMISTRY
www.elsevier.com/locate/bioorg

An NMR spectroscopy study of bendaline–albumin interactions

M. Delfini,[a,*] C. Bianchetti,[a] M.E. Di Cocco,[a] N. Pescosolido,[b] F. Porcelli,[c] R. Rosa,[d] and G. Rugo[a]

[a] *Dipartimento di Chimica, Università degli Studi di Roma "La Sapienza", Piazzale Aldo Moro 5, 00185 Rome, Italy*
[b] *Dipartimento di Scienze dell'Invecchiamento, Policlinico Umberto I, Viale del Policlinico, 00185 Rome, Italy*
[c] *Dipartimento di Scienze Ambientali, INFM sez. B Università della Tuscia, L.go dell'Università, 01100 Viterbo, Italy*
[d] *Dipartimento di Scienze Oftalmologiche, Policlinico Umberto I, Viale del Policlinico, 00185 Rome, Italy*

Received 7 February 2003

Abstract

The complete assignment of the ^{1}H and ^{13}C NMR spectra of bendaline (BNDL) was performed by mono-dimensional and homo- and hetero-correlated two-dimensional NMR experiments. The interaction between bendaline and albumin was also studied by the analysis of the motional parameters spin–lattice relaxation times, allowing the motional state of the BNDL free and bound with albumin to be defined. In absence of albumin the indazolacetic and benzylic moieties are characterized by roughly the same mobility and by positive σ (cross-relaxation rates) values. In the presence of the macromolecule, the indazolacetic and benzylic moieties and the lysine change their motional behaviour to different extents, as indicated by correlation times. Data obtained in absence and in presence of the protein show that the molecular moiety of the bendaline most involved in the binding with albumin is the fragment H-4 H-5. The binding constant was evaluated at $2.4 \times 10^{3}\ M^{-1}$.

Keywords: Bendaline; Albumin; NMR spectroscopy

* Corresponding author. Fax: +39-06-490631.
E-mail address: maurizio.delfini@uniroma1.it (M. Delfini).

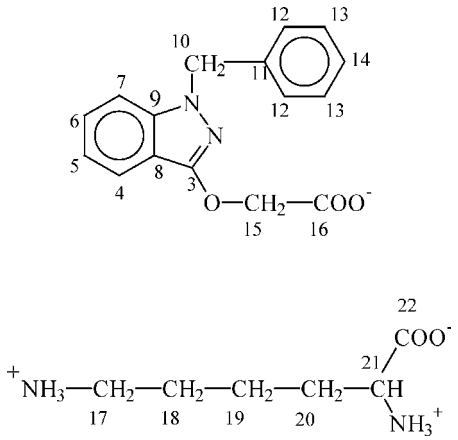
0045-2068/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0045-2068(03)00078-6

1. Introduction

Bendaline (BNDL) (Scheme 1) is the lysine salt of the [[1-(phenylmethyl)-1H indazol-3-yl]oxy]acetic acid (BDZC). The main properties of BDZC are its anti-denaturant and mild immunomodulation effects on proteins, although the drug also exhibits anti-inflammatory, anti-necrotic, choleretic, and anti-lipidaemic properties [1]. BNDL is largely absorbed orally as the lysine salt, and can be administered in order to prevent cataract formation [2]. BDZC and BNDL seem to exert their anti-cataract action by inhibiting the denaturation of lens proteins, although the exact mechanism of action is not yet clear. BNDL has been shown to inhibit the in vitro denaturation of various proteins, including albumin, by heat, UV radiation, free radicals, and various chemical denaturants [1,3,4]. This action is also present in vivo; in particular, crystalline lens proteins are protected by BNDL against the changes induced by temperature denaturation, as detected by electrophoretic experiments [5,6]. Moreover, BNDL prevents the deposition and aggregation of lachrymal proteins on the contact lens surfaces [7,8].

BNDL can be administered per os or by ocular instillation. In both cases, a study of its interaction with albumin is extremely important, because the degree of protein-binding is a critical parameter in the evaluation of the pharmacological and pharmacokinetic properties of drugs [9]. By oral administration, the binding of albumin to drugs governs their transport and tissue distribution and is important in determining their activity, toxicity, excretion, and metabolism in the body [10]. Albumin is also ubiquitous in the eye, so that an understanding of the binding interactions between it and BNDL is essential for predicting the drug's distribution [11–13].

BNDL has aroused new interest because of its peroxy radical scavenging properties [14], most notably it prevents the inactivation of natural radical scavengers like albumin. Moreover, anti-denaturant agents, such as BNDL, are considered to be appropriate drugs for the treatment of "condensation disease," a heterogeneous



Scheme 1.

pathological condition in which the primary pathogenic step is loss of solubility of specific substances, resulting in the formation of a condensed phase. This will manifest itself in cataract, nephrolithiasis, and gallstone disease [15].

In this paper, the interactions between BNDL and albumin were studied by NMR spectroscopy. The motional parameters, mono-selective and non-selective proton spin–lattice relaxation rates were the primary means for the detection of the relatively weak binding interactions [16–19].

2. Materials and methods

Bendaline was provided by Angelini Industries. The albumin was obtained from ICN Biomedical Research Products (Costa Mesa CA USA). The molecular weight was 65 kDa. The albumin was used without further purification.

2.1. NMR experiments

NMR experiments were carried out at 300 K on Varian XL-300 (7.05 T) spectrometer (i) operating at 299.99 MHz for proton and 75.43 MHz for ^{13}C and on a Bruker AM 500 (11.47 T) spectrometer and (ii) operating at 500.13 MHz for proton and 125.37 for ^{13}C. The chemical shift values are expressed in ppm relative to dioxane in deuterium oxide (external reference) and reported with respect to tetramethylsilane. BNDL solutions have been split into two different portions. In one, 20 μl of dioxane were added and chemical shifts were first measured with respect to internal dioxane. In order to avoid any interference of internal dioxane in the interaction studies, the internal dioxane-free solutions were measured (using NMR spectroscopy) by introducing the same amount of dioxane as the external reference in a capillary tube. The chemical shifts reported in the Tables have been duly corrected. *Measurements conditions*: (i) ^{1}H pulse width 7.5 μs (30°), acquisition time 2.0 s, repetition time 1.0 s, spectral width 4000 Hz, number of data points 32K, numbers of scans 64; ^{13}C pulse width 6.0 μs (30°), acquisition time 0.9 s, repetition time 1.1 s, spectral width 16,000 Hz, number of data points 16K, numbers of scans 720; and (ii) ^{1}H pulse width 3.0 μs (30°), acquisition time 2.0 s, repetition time 1.0 s, spectral width 8064 Hz, number of data points 64K, numbers of scans 32.

Longitudinal relaxation times were measured on a Varian XL-300 spectrometer and on a Bruker AM 500 spectrometer. Proton spin–lattice relaxation times were measured with inversion-recovery pulse sequences (180°–τ–90°) and calculated from three-parameters exponential regression analysis of the recovery curves of longitudinal magnetization components. Single selective proton spin–lattice relaxation times were measured with inversion-recovery pulse sequence implemented with DANTE sequence in order to obtain single inversion of the desired proton resonances, as reported elsewhere [20,21]. *Measurements conditions*: Varian XL-300: 90° pulse length, 22.5 μs; 12 different τ values from 1 ms to 35 s; repetition rate, 35 s; Bruker AM 500 90° pulse length, 9.0 μs; 12 different τ values from 1 ms to 40 s; repetition rate, 40 s.

2D-COSY spectra were obtained on a Varian XL-300 spectrometer by using the pulse sequence available in the routine of the instrument. *Measurements conditions*: spectral width 2350 Hz in both dimensions; data matrix 2048×2048 points. 512 FIDS were collected for each value of t_1. HETCOR: F1 spectral width 2350 Hz; td1 256; F2 spectral width 16,500 Hz; td2 1024. Data matrix 1024×256.

BNDL samples were prepared in deuterium oxide (D_2O) 99.95% phosphate buffer 0.13 M, pH 7.24, solutions. No correction has been made for the isotope effect.

On the basis of the BNDL ^1H chemical shifts variation as a function of concentration, it has been determined that there is no significant self-association processes occurring below concentrations of 6 mM. The concentrations used in the interaction studies were lower than this value. The study was performed on bendaline alone and on bendaline and albumin at different molar ratios. Both were carried out in phosphate buffer.

3. Results and discussion

3.1. Peaks assignments

In Table 1 are reported the ^1H NMR chemical shifts of BNDL and their assignments. The latter were made on the basis of chemical shift values, comparison with model compounds, spectral multiplicities, signal area values and experiments at different concentrations. The assignments of lysine signals were made on the basis of literature data [22]. For both moieties the unequivocal assignments were performed by 2D NMR COSY experiments. With regard to BDZC moiety, the doublet at 7.63 ppm was assigned to H-4 because it shifted the furthest downfield. The doublet of doublets at 7.26 ppm, exhibiting different coupling constants, were assigned to H-6; the doublet at 7.16 ppm was assigned to H-7. The assignment was confirmed

Table 1
^1H chemical shifts and assignments of BNDL in phosphate buffer (0.13 M, pH 7.24, $T = 300 \pm 1$ K)

Position	δ (ppm)
4	7.63
6	7.26
7	7.16
14	7.14
12 a,b	7.11
5	6.99
13 a,b	6.96
10	5.20
15	4.59
21	3.70
17	2.95
20	1.92
18	1.55
19	1.40

by 2D-NMR COSY experiments, showing a correlation between the two protons. Correlations between H-13 and H-12 and between H-13 and H-14 allow peaks at 6.96 ppm (H-13), 7.11 ppm (H-12), and 7.14 ppm (H-14) to be unequivocally assigned, in agreement with the integration values. Correlations between H-5 and H-6 and between H-5 and H-4 allow H-13 and H-5 to be unequivocally determined.

In Table 2 are reported the ^{13}C chemical shifts assignments. The assignments of the lysine signals have been obtained by the comparison with those in literature [22]. The unequivocal assignments of ^{13}C resonances and of proton peaks not unequivocally assigned from proton experiments were obtained by heterocorrelated ^{13}C-^{1}H 2D NMR experiments.

The short-range proton–carbon spectrum shows those carbons which are directly attached to protons. In our case, H-6 absorbing at 7.26 ppm is one-bond coupled to the carbon peak at 131.15 ppm, giving us C-6 directly. Similarly, we can assign C-12 a,b at 131.50 ppm, C-14 at 130.50 ppm, C-13 a,b at 129.75 ppm, C-4 at 123.01 ppm, C-5 at 122.73 ppm, C-7 at 112.09 ppm, C-15 at 70.31 ppm, and C-10 at 54.05 ppm.

The long-range proton–carbon spectrum (optimized for two- and three-bond couplings) shows correlations between protons and carbons that are separated by two or three bonds. Correlations between the protons at 4.59 ppm with the carbon signal at 157.95 ppm and the carboxyl group allow assignments at the same time of protons H-15, of carboxyl C-16 and C-3 by using the J coupling constants at two and three bonds, respectively. The correlation between the protons at 5.20 ppm and quaternary carbons at 140.04 and 144.35 ppm allows assignment of H-10 and C-11 and C-9, respectively. Thus the signal of the quaternary carbon at 114.77 ppm can be assigned to C-8.

Table 2
^{13}C chemical shifts and assignments of BNDL in phosphate buffer (0.13 M, pH 7.24, $T = 300 \pm 1$ K)

Position	δ (ppm)
16	178.58
22	177.40
3	157.95
9	144.35
11	140.04
12 a,b	131.50
6	131.15
14	130.50
13 a,b	129.75
4	123.01
5	122.73
8	114.77
7	112.09
15	70.31
21	57.34
10	54.05
17	41.89
20	32.73
18	29.23
19	24.27

3.2. Motional parameters

The study of the interaction of bendaline with human serum albumin in phosphate buffer was carried out by first considering the structural parameter chemical shift. Ligand solutions at different concentrations and ligand–albumin solutions at different molar ratios were studied.

By examining resonances ascribed to the indazolacetic and benzylic moieties of BNDL in the absence and presence of albumin, very small chemical shift differences were observed (Table 3). Such a behaviour is expected in ligand–macromolecules diamagnetic complexes, where the effects on chemical shift are very small under fast exchange conditions even in the presence of a high binding constant.

The data obtained by the motional parameters, such as the spin–lattice relaxation rates ($R_{ij} = 1/T_1$), give more information, with the relaxation mechanism for any proton H_i being mainly provided by the dipole–dipole interactions with protons nearby, such that

$$R_i^{\mathrm{NS}} = \sum_{j \neq i} \rho_{ij} + \sum_{j \neq i} \sigma_{ij} + \rho_i^*,$$

where ρ_{ij} and σ_{ij} are the direct- and cross-relaxation rates relative to the H_i–H_j dipolar interaction and ρ_i^* accounts for eventual contributions from other relaxation mechanisms. Such a rate is labeled NS (non-selective) in order to emphasize that all proton resonances are excited by the same irradiation field, so that all spin populations are perturbed from their equilibrium values. It is also possible to excite only one proton resonance while leaving the rest of the spin system at the equilibrium. It has been shown [20] that the spin–lattice relaxation rate measured upon these conditions is given by (all σ_{ij}'s are obviously zero) the equation

$$R_i^{\mathrm{sel}} = \sum_{i \neq j} \rho_{ij} + \rho_i^*,$$

where the sel label means selective. It has been, therefore, suggested that measuring the ratio

$$F = \frac{R_i^{\mathrm{NS}}}{R_i^{\mathrm{sel}}}$$

for any proton H_i yields the following:

Table 3
^{1}H chemical shift variations of BNDL protons in presence of different molar ratios BNDL/albumin (0.13 M phosphate buffer, pH 7.24, $T = 300 \pm 1$ K)

Proton position	δ (ppm) BNDL	δ (ppm) ratio 100/1	δ (ppm) ratio 20/1
4	7.63	7.72	7.72
7	7.16	7.34	7.35
14	7.14	7.21	7.21
12 a,b	7.11	7.09	7.09
10	5.20	5.35	5.36

(i) whenever $\omega\tau_c \ll 1$ and $\rho_i^* = 0$, $F = 1.5$;
(ii) in the same region of molecular motions, $F < 1.5$ is determined by the occurrence of relaxation mechanisms other than the dipolar;
(iii) if and only if $\rho_i^* = 0$, $F < 1.5$ is determined by molecular motions having relatively slow reorientational correlation times, $\tau_c^{ij} > 0.2/\omega$.

The motional correlation time can be evaluated by the dipolar interaction energy between protons at fixed distances, σ^{ij}, as measured by double-selective relaxation rates according to the equation [21]

$$\sigma^{ij} = R_1^{ij} - R_i^{\mathrm{sel}} = \frac{1}{10}\frac{\gamma^4\hbar^2}{r_{ij}^6}\left\{\frac{6\tau_c^{ij}}{1+\omega^2\left(\tau_c^{ij}\right)^2} - \tau_c^{ij}\right\},$$

where R_1^{ij} (s^{-1}) is the double-selective relaxation rate measured for H_i upon selective excitation of H_i and H_j, R_i^{sel} is the selective relaxation rate measured for H_i, γ is the proton magnetogyric ratio ($= 26{,}753\,\mathrm{rad\,s^{-1}\,G^{-1}}$), ω is the proton Larmor frequency, $\hbar$ is the reduced Planck's constant ($\equiv h/2\pi = 1.0545887 \times 10^{-27}\,\mathrm{erg\,s\,rad^{-1}}$), and r_{ij} is the H_i–H_j vector.

In the fast exchange conditions the equation

$$\sigma_{if}^{\mathrm{obs}} = p_f\sigma_{ij}^{f} + p_b\sigma_{ij}^{b}$$

made it possible to evaluate σ_{ij} in the bound state (σ_{ij}^{b}).

Even in the presence of the protein at low molar ratios, BNDL is expected to exchange between the free and the bound environments. Such exchange has been shown to yield a certain enhancement of selective proton spin–lattice relaxation rates, while leaving the non-selective ones almost unaffected, especially at high proton Larmor frequencies [23]. This occurs because R_i^{sel}, and not R_i^{NS}, is contributed by W_0 (zero-quantum probability) which becomes progressively larger as the motion slows down, as in the case of the bound environment. It is, therefore, evident that the larger the enhancement of R_i^{sel}, the more the corresponding proton is involved in intermolecular interactions with the protein.

The motional properties can be properly described by the correlation time measured on the basis of the F values, σ values or alternatively by spin–lattice relaxation times (T_1) ratio at different magnetic fields [24], using the equation

$$\frac{T_1^{\mathrm{NS}}(\omega_1)}{T_1^{\mathrm{NS}}(\omega_2)} = \frac{\left(5+8\omega_2^2\tau_c^2\right)\left(1+5\omega_1^2\tau_c^2+4\omega_1^4\tau_c^4\right)}{\left(5+8\omega_1^2\tau_c^2\right)\left(1+5\omega_2^2\tau_c^2+4\omega_2^4\tau_c^4\right)},$$

where γ is the proton magnetogyric ratio, ω_1 and ω_2 are the proton Larmor frequencies at certain magnetic fields and τ_c is the correlation time for molecular reorientation. Hence, τ_c for any proton can be evaluated when knowing the T_1^{NS} values at two different frequencies.

The experimental spin–lattice relaxation ratios and τ_c values as evaluated using this equation for bendaline alone and in interaction with albumin (albumin:bendaline ratio 1:100), both in phosphate buffer, are reported in Table 4. The analysis of relaxation rates allows the motional state of the bendaline free and bound with albumin to be defined [16].

Table 4
Bendaline protons correlation times (τ_c) in absence (a) and in presence (p) of albumin (albumin:bendaline ratio 1:100), measured on the basis of the equation in the text[a]

Position	$\frac{T_1^{NS}(\omega_1)}{T_1^{NS}(\omega_2)}$ (a)	τ_c[b] (a) (s)	$\frac{T_1^{NS}(\omega_1)}{T_1^{NS}(\omega_2)}$ (p)	τ_c[b] (p) (s)
H-4	1.83	2.2×10^{-9}	2.66	9.3×10^{-9}
H-5	2.13	3.1×10^{-9}	2.63	8.4×10^{-9}
H-10	2.31	4.3×10^{-9}	2.52	6.1×10^{-9}
H-15	2.44	5.1×10^{-9}	2.56	7.4×10^{-9}
H-17	2.44	5.1×10^{-9}	1.83	2.2×10^{-9}
H-18	2.52	6.1×10^{-9}	2.13	3.1×10^{-9}

[a] The bendaline concentration is 3×10^{-3} M and the albumin concentration is 3×10^{-5} M.
[b] Errors on τ_c values are less than 0.2×10^{-9}.

The measured spin–lattice relaxation rates of BNDZ moiety in absence of albumin lead to consider that the indazolacetic moiety is characterized by roughly the same mobility and the σ values are positive.

Upon binding with the macromolecule, the indazolacetic and lysine moieties change their motional behaviour to a different extent as shown by the τ_c values reported in Table 4. The lysine moiety exhibits higher mobility and the indazolacetic moiety shows different internal mobility, with the fragment H-4 H-5 being the most affected.

The cross relaxation rate becomes strongly negative ($\sigma_f^{4,5} = 0.11\,s^{-1}$; $\sigma_{obs}^{4,5} = -0.49\,s^{-1}$), which indicates that the molecular motion is quite different in the absence and presence of albumin. The negative value of $\sigma^{4,5}$ suggests a very low mobility of the H-4 H-5 fragment with respect to the values obtained for the other groups. The $\sigma^{4,5}$ becomes larger and negative as a consequence of the binding, which indicates that the corresponding internuclear vector is modulated by relatively slow reorientational motions. Consequently the data demonstrate that fragment C4–H C5–H is the molecular moiety most involved in the binding with albumin.

Measurements of the enhancement of selective relaxation rates, as BNDL concentrations vary in the presence of a fixed protein concentration, allowed the evaluation of the interaction constant in the limit of 1:1 interaction (Table 5). As demonstrated, at low [protein]/[ligand] ratios the following equation can be obtained [25]

Table 5
Selective relaxation rates of H-4 proton of BNDL in absence (a) and in presence (p) of albumin

[BNDL] (mM)	R_{sel}[a] (a) (s^{-1}) (free)	R_{sel}[a] (p) (s^{-1}) (observed)	$R_{sel}^{obs} - R_{sel}^{f}$ (s^{-1}) (ΔR)	$1/\Delta R$ (s)
1	0.29	7.43	7.14	0.14
2	0.32	4.02	3.70	0.27
3	0.25	2.95	2.70	0.37
4	0.27	2.37	2.10	0.48
5	0.29	2.04	1.75	0.57

[a] Errors on the relaxation rates are less than 3%.

$$\frac{1}{\Delta R_i^{\mathrm{sel}}} = \left\{ \frac{1}{K_{\mathrm{int}}} + [\mathrm{L}] \right\} \frac{1}{R_{ib}^{\mathrm{sel}} P_0},$$

where [L] is the ligand concentration, P_0 is the total concentration of the protein and R_{ib}^{sel} is the selective rate in the bound environment. As a consequence, a plot of $1/\Delta R_i^{\mathrm{sel}}$ vs the ligand concentration extrapolates at zero at $[\mathrm{L}] = -1/K_{\mathrm{int}}$. The plot relative to the behaviour of $1/\Delta R_i^{\mathrm{sel}}$ of H-4 proton of BNDL as a function of concentration is shown in Fig. 1 and leads to calculate $K_{\mathrm{int}} = 2.4 \times 10^3\ \mathrm{M}^{-1}$. Such a behaviour is independent of viscosity (as showed by measurements as a function of concentration) and it only depends on the interaction between BNDL and albumin. The protons of the lysine are not influenced by the presence of protein (Table 4).

Bendaline behaviour is quite similar to that exhibited by indolic compounds [18,26]. In fact, selective spin–lattice relaxation rates indicate that the C4–H C5–H fragment is the molecular moiety most involved in the binding to albumin. This is in good agreement with similar findings obtained for the corresponding proton resonances of the aromatic moiety of indolic compounds such tryptophan and indomethacin interacting with human serum albumin (HSA) and bovine serum albumin (BSA), respectively [18,26]. Moreover, as observed by NMR spin–lattice relaxation rates, the protons of the aromatic moiety of β-carbolines, these being pyrido-indoles [19], are the most involved in the interaction with HSA, which is also dependent on the type of substituents on the pyrrolic ring. It has also been observed by NMR spectroscopy that β-carbolines interact at the same site of HSA, in agreement with binding studies of radiolabeled ligands [27].

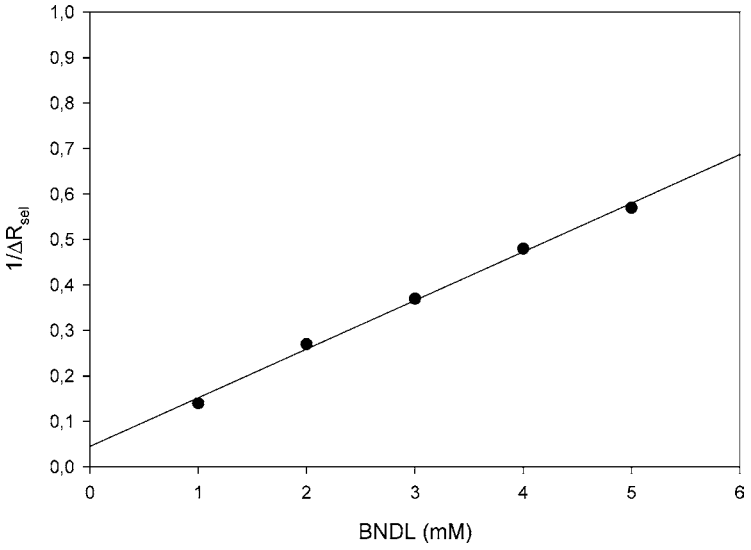


Fig. 1. Behaviour of $1/\Delta R_{\mathrm{sel}}$ of BNDL H-4 proton as a function of concentration. The albumin concentration is 3×10^{-5} M.

These observations implicate the same binding site in the subdomains IIA and IIIA of the albumin where other aromatic carboxylic acid bind [28]. Those subdomains are predominantly hydrophobic, but a polar contribution of the lysine of the IIA subdomain interacting with carboxylate group cannot be excluded. This is in agreement with the affinity exhibited by albumin with negatively charged small molecules [29]. On the other hand, binding does not require a negative charge provided that the molecule has a strongly electronegative center. This is the case for diazepam, which mainly exists in the un-ionized form at neutral pH and binds to human serum albumin with high affinity to site II. Short-chain fatty acids, 3-carbon to 5-carbon, did not displace marker drugs or fluorescent probes from site I or II when added at equimolar ratios with albumin. In addition, arylpropionic acid non-steroidal anti-inflammatory drugs bind at site II, suggesting that bulkier aromatic molecules form more effective interactions at the binding site [30].

Structural binding studies on the complex between HSA and diflunisal have been recently performed and provide support for these results. They indicate that the aromatic rings of diflunisal are involved in specific interactions with hydrophobic residues of HSA. In addition to structural studies, NMR-derived binding constants were obtained for diflunisal and its analogues enabling the development of structure–affinity relationship for binding [31].

As a result of our study, we were able to evaluate the BNDL–albumin binding constant and determine the BNDL moiety most involved in albumin binding. The results are consistent with those observed for other indolic compounds as well as spectrophotometry data indicating interactions [29]. These findings are significant because absorbance measurements of albumin solutions in the presence of different concentrations of BNDL have demonstrated that BNDL is able to prevent albumin heat-induced aggregation [4].

Because the main action of bendaline may be the protection of the lens proteins, the present study can be extended to account for the mechanism of action of the bendaline with crystalline lens proteins to prevent cataract formation.

References

[1] J. Balfour, S.P. Clissold, Drugs 39 (1990) 575–596.
[2] P. Valeri, M. Palmery, B. Martinelli, B. Catanese, Pharmacol. Res. Commun. 19 (8) (1987) 517–525.
[3] B. Silvestrini, B. Catanese, R. Lisciani, A. Alessandroni, Arzneimittel-Forschung 20 (1970) 250–253.
[4] L. Saso, E. Grippa, M.G. Leone, B. Silvestrini, Res. Commun. Mol. Pathol. Pharmacol. 102 (1) (1998) 15–28.
[5] B. Silvestrini, B. Catanese, R. Lisciani, Excerta Medica International Congress Series No 188, in: Proceedings of an International Symposium, Como (Italy), October 11–13, 1968.
[6] B. Silvestrini, B. Catanese, G. Barillari, E. Iorio, P. Valeri, Int. J. Tissue React. 5 (1983) 217–225.
[7] A. Kidane, J.M. Sxabocsik, K. Park, Biomaterials 19 (22) (1998) 2051–2055, doi:10.1016/S0142-9612(98) 00111-2.
[8] C.R. Arciola, L. Montanaro, R. Caramazza, V. Sassoli, D. Cavedagna, J. Biomed. Mater. Res. 42 (1) (1998) 1–5.
[9] T. Hanai, A. Koseki, R. Yoshikawa, M. Ueno, T. Kinoshita, H. Homma, Anal. Chim. Acta 454 (2002) 101–108.

[10] H.S. Kim, J. Austin, D.S. Hage, Electrophoresis 23 (2002) 956–963.
[11] H. Gong, N. Johnson, W. Ye, R.D. Kamm, T.F. Freddo, Exp. Eye Res. 65 (1997) 747–756.
[12] Y.A. Gryzunov, A.I. Deev, N.I. Kurysheva, M.N. Komarova, Bull. Exp. Biol. Med. 128 (1999) 1179–1181.
[13] J. Bours, Exp. Eye Res. 16 (1973) 487–499.
[14] L. Saso, M.L. Casini, E. Mattei, G. Valentini, G. Mazzanti, Phytother. Res. 10 (Suppl. 1) (1996) S53–S55.
[15] L. Saso, B. Silvestrini, Med. Hypotheses 56 (1) (2001) 114–120.
[16] G. Valensin, M. Delfini, E. Gaggelli, Biophys. Chem. 24 (1986) 25–31.
[17] E. Gaggelli, N. Gaggelli, A. Maccotta, G. Valensin, J. Magn. Reson. B 104 (1994) 89–94.
[18] J.P. Monti, M. Sarrazin, C. Briand, A. Crevat, J. Chem. Phys. 74 (1977) 942–946.
[19] G. Veglia, M. Delfini, M.R. Del Giudice, E. Gaggelli, G. Valensin, J. Magn. Reson. 130 (1998) 281–286.
[20] R. Freeman, H.D.W. Hill, B.L. Tomlinson, L.D. Hall, J. Chem. Phys. 61 (11) (1974) 4466–4473.
[21] L.D. Hall, H.D.W. Hill, J. Am. Chem. Soc. 98 (1976) 1269–1270.
[22] T.W.M. Fan, Prog. NMR Spectrosc. 28 (1996) 161–219.
[23] G. Valensin, T. Kushnir, G. Navon, J. Magn. Reson. 46 (1) (1982) 23–29.
[24] T. Sai, N. Takao, M. Sugiura, Magn. Reson. Chem. 30 (1992) 1041–1046.
[25] E. Gaggelli, G. Valensin, Concepts Magn. Reson. 5 (1993) 19–42.
[26] M. Tanaka, Y. Asahi, S. Masuda, Polymer 35 (7) (1994) 1512–1517.
[27] C.A. Fenerty, E.W. Lindup, J. Neurochem. 53 (2) (1989) 416–422.
[28] X. Min He, D.C. Carter, Nature 358 (1992) 209–215.
[29] L. Zolla, G. Amiconi, B. Catanese, Biochem. Pharmacol. 25 (7) (1977) 659–662.
[30] S. Wanwimolruk, D.J. Birkett, P.M. Brooks, Mol. Pharmacol. 24 (3) (1983) 458–463.
[31] H. Mao, P.J. Hajduk, R. Craig, R. Bell, T. Borre, S.W. Fesik, J. Am. Chem. Soc. 123 (43) (2001) 10429–10435.