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Original Scientific Paper

Epitope Mapping of Macrolide Antibiotics to Bovine Serum Albumin by Saturation Transfer Difference NMR Spectroscopy*

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Saturation transfer difference NMR spectroscopy was employed to characterize epitopes of macrolide antibiotics, azithromycin, oleandomycin and telithromycin binding to bovine serum albumin. The structural parts of azithromycin and oleandomycin in intimate contact with bovine serum albumin were found to be similar while those of telithromycin showed similarities but also some differences. The latter were mostly due to different structural elements of antibiotics that interact with the protein, especially the alkyl-heteroaryl side chain in telithromycin and cladinose and desosamine sugars in azithromycin and oleandomycin. The epitope maps as determined in this study can contribute to better understanding of the overall bioactivity of macrolides.

Keywords:
STD NMR spectroscopy
macrolide antibiotics
albumin
epitope mapping

INTRODUCTION

NMR techniques based on a magnetization transfer such as saturation transfer difference (STD) and transferred NOE spectroscopy (trNOESY) have become an indispensable tool for characterization of ligand binding to their receptors.[1] A combined use of the two techniques can provide a wealth of information regarding binding epitopes and bound ligand conformations and can also be exploited for screening purposes and ligand design. STD is a sensitive technique that can identify binding epitopes very quickly and use only a small amount of a macromolecule, providing a fast exchange equilibrium between free and bound states with dissociation constants in the range 10^{-3}–10^{-8} mol dm^{-3}.[2–5] Selective saturation of a target (usually macromolecule) proton resonance can result in a rapid spread of saturation over the entire molecule *via* an efficient spin diffusion mechanism. The ligand protons become saturated too if they are in a close proximity to the macromolecule, *i.e.,* if binding occurs. Saturation is most efficient for those protons that are closest to the macromolecular surface. This can be further exploited to map the binding epitopes of ligand-receptor interactions in general.

In our previous paper, we reported on a strategy to elucidate and better understand macrolide interactions with their target ribosome by combining NMR and molecular modeling methods.[6] Macrolides exert their activity by

* Dedicated to Professor Haruo Hosoya in happy celebration of his 70th birthday.

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binding to the 50S ribosomal subunit in the peptidyl transferase region and thus sterically blocking the growth of the nascent bacterial polypeptide chain.[7–9] The knowledge of free[10–15] and bound state macrolide structures[6,15] and determination of the key structural elements responsible for binding is a crucial prerequisite for designing novel inhibitors possessing better activity. Furthermore, the overall effect of drug molecules depends also on their physicochemical properties such as, for example, solubility, permeability and bioavailability. The latter is greatly influenced by binding to plasma proteins. Albumins are common plasma proteins that reversibly interact with drug molecules and can thus serve as a drug delivery system. Hence, in order to asses the overall biological profile of macrolides, it is of importance to characterize also these interactions. In this study, we have used bovine serum albimine (BSA) as a model system to study its interactions with macrolide antibiotics azithromycin (**1**), oleandomycin (**2**) and telithromycin (**3**) (Figure 1) and to determine their binding epitopes. In that respect, STD NMR experiments have been performed with samples containing macrolides and BSA in a ratio 100:1 and STD spectra have been analyzed and discussed.

EXPERIMENTAL

Materials

Bovine serum albumin (>99 %) was purchased from Sigma-Aldrich Co. as lyophilized powder. D_2O and Tris-d_{11} were obtained from Cambridge Isotope Laboratories.

For STD experiments, 4×10^{-6} mol dm^{-3} solutions of azithromycin, oleandomycin and telithromycin samples were prepared by dissolving the samples in aqueous 20×10^{-3} mol dm^{-3} deuterated Tris buffer with 60×10^{-3} mol dm^{-3} KCl at a physiological apparent pH 7.4. Bovine serum albumin was titrated into the solution until the final concentration 280×10^{-6} mol dm^{-3} of protein was achieved with a protein/ligand ratio of 1/100 in a total volume of 700 μL.

NMR Measurements

NMR spectra were recorded on Bruker Avance DRX500 and DPX300 spectrometers equipped with *z*-gradient accessories. Samples were measured at 298 K in 5 mm NMR tubes with TSP-d_{11} as an internal reference.

Prior to STD NMR measurements, complete proton assignments in Tris D_2O buffer have been performed by using two-dimensional homo- and heteronuclear correlation experiments (COSY, HSQC and HMBC).

Proton spectra with spectral width of 6200 Hz and a digital resolution of 0.09 Hz per point were measured with 8-16 scans. WATERGATE pulse train was used to suppress the HDO signal.

In the COSY experiment, 2046 points in the *f*2 dimension and 512 increments in the *f*1 dimension were used. For each increment, 8 scans and the spectral width of 4007 Hz were applied. Digital resolution was 1.97 and 7.82 Hz per point in *f*2 and *f*1 dimensions, respectively. Typical spectral conditions for HSQC and HMBC spectra were as follows. Spectral width was 3906 Hz in *f*2 and 18870 Hz in *f*1 dimension for both experiments. 2K data points were applied in the time domains and for each data set 157 and 246 increments were collected for HSQC and HMBC spectra, respectively. The resulting digital resolution was 3.81 Hz per point in the *f*2 dimension and 34.3 and 36.9 Hz per point in the *f*1 dimension in HSQC and HMBC spectra, respectively.

1D STD NMR spectra were collected with 16k data points and a spectral width of 15 ppm by using a sequence provided by Bruker. Selective saturation of BSA was performed using a 50 ms E-BURP pulse. The saturation time was 2s. The number of scans was 16-64. Saturated and reference spectra were acquired and processed simultaneously by creating a pseudo 2D experiment. The saturation frequency was switched from on--resonance (–1 ppm) to off-resonance (35 ppm) after each scan. WATERGATE pulse train was used to suppress the HDO signal. To eliminate the residual protein resonance signals, $T_{1\rho}$ filter was applied.

Molecular Modelling

Molecular modelling calculations were obtained using an SGI Origin 3400 platform running software programs InsightII, modules: Builder, Discover and Analysis version 2000.1 (Accelrys Software Inc.) Dynamics calculations were done with the Discover® program version 2000.1 (Accelrys Software Inc.) using the cvff force--field. The solution conformation of **2** was taken from Ref 6. The solution conformations of **1** and **3** were obtained by applying the same protocol as that described in Ref. 6.

RESULTS AND DISCUSSION

NMR Assignments and Structure Elucidation

Chemical shift assignments in Tris D_2O buffered solutions were made by the combined use of standard one- and two-dimensional NMR experiments (COSY, HSQC and HMBC). The proton chemical shifts of **1** are given in Table I. The chemical shifts of **2** and **3** were similar to those previously reported.[13,15]

Binding to BSA

All three macrolide antibiotics gave STD NMR spectra as shown in Figure 2, thus confirming their binding to BSA. When creating an epitope map, STD intensity can be expressed as:

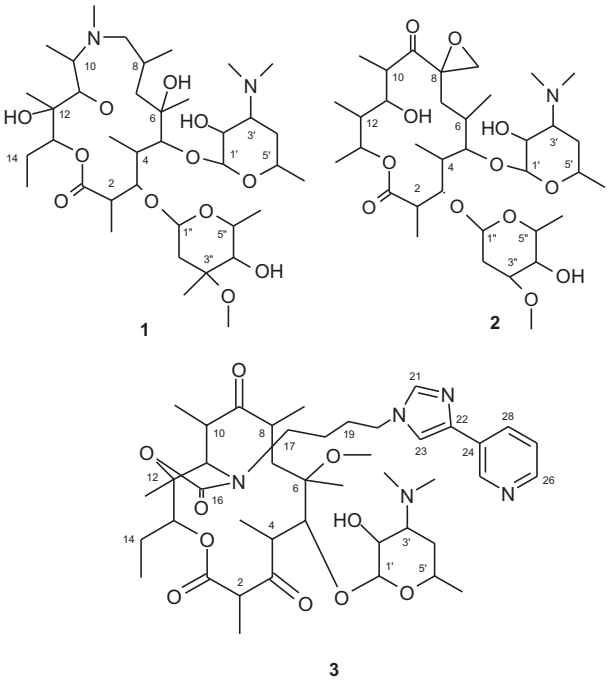

Figure 1. Chemical structures of azithromycin (**1**), oleandomycin (**2**), and telithromycin (**3**), and the atom numbering.

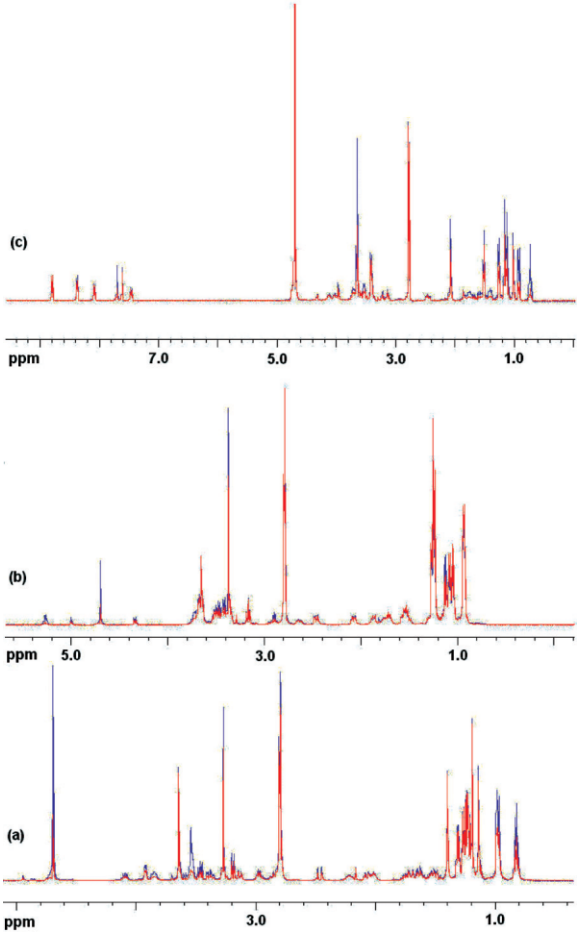

Figure 2. Off-resonance (blue) and on-resonance (red) STD NMR spectra of a) azithromycin, b) oleandomycin and c) telithromycin.

TABLE I. Proton chemical shifts (δ/ppm) of **1** in D_2O buffer

Atom	δ_H /ppm
1	–
2	2.97
2-CH_3	1.23
3	3.92
4	2.01
4-CH_3	0.97
5	3.65
6	–
6-CH_3	1.40
7a	1.65
7b	1.49
8	2.22
8-CH_3	0.99
9a	3.12
9b	2.85
9-NCH_3	2.81
10	3.65
10-CH_3	1.30
11	3.65
12	–
12-CH_3	1.14
13	4.85
14a	1.75
14b	1.54
14-CH_3	0.82
1''	4.94
2''a	2.45
2''b	1.62
3''	–
3''-CH_3	1.19
3''-OCH_3	3.27
4''	3.18
5''	4.09
5''-CH_3	1.26
1'	4.53
2'	3.46
3'	3.37
3'-$N(CH_3)_2$	2.79
4'a	2.09
4'b	1.51
5'	3.85
5'-CH_3	1.25

$$A_{STD} = (I_0 - I_S)/I_0 \quad (1)$$

where I_0 is the signal intensity in the off-resonance or reference spectrum while I_S is the signal intensity in the on-resonance spectrum.

TABLE II. STD signal intensities expressed as A_{STD}[a] of azithromycin (**1**), oleandomycin (**2**) and telithromycin (**3**)

Atom	A_{STD}/ % **1**	A_{STD}/ % **2**	A_{STD}/ % **3**	Atom	A_{STD}/ % **1**	A_{STD}/ % **2**	A_{STD}/ % **3**
2-H	31	97	32	18a-H	–	–	76
2-CH_3	3	50	49	18b-H	–	–	76
3-H	44	28	-	19a-H	–	–	23
4-H	15	4	32	19b-H	–	–	56
4-CH_3	53	3	53	20a-H	–	–	32
5-H	6	58	40	20b-H	–	–	37
6-H	–	77	–	21-H	–	–	82
6-CH_3	4	2	33	23-H	–	–	29
6-OCH_3	–	–	53	25-H	–	–	–8
7a-H	44	30	45	26-H	–	–	34
7b-H	31	13	44	27-H	–	–	25
8-H	24	–	28	28-H	–	–	26
8a-CH	–	28	–	1"-H	64	79	–
8b-CH	–	34	–	2"a-H	44	28	–
8-CH_3	52	–	63	2"b-H	39	13	–
9a-H	17	–	–	3"-H	–	32	–
9b-H	29	–	–	3"-CH_3	5	–	–
9-NCH_3	12	–	–	3"-OCH_3	29	53	–
10-H	6	18	51	4"-H	36	22	–
10-CH_3	15	4	84	5"-H	56	28	–
11-H	6	72	*	5"-CH_3	3	0	–
12-H	–	8	–	1'-H	84	52	34
12-CH_3	16	4	71	2'-H	60	52	32
13-H	**100**	**100**	11	3'-H	60	52	28
13-CH_3	–	2	–	3'-$N(CH_3)_2$	22	21	15
14a-H	38	–	74	4'a-H	28	36	29
14b-H	26	–	60	4'b-H	28	13	35
15-CH_3	54	–	**100**	5'-H	66	71	44
17a-H	–	–	43	5'-CH_3	3	0	55
17b-H	–	–	43				

[a] $A_{STD} = (I_0 - I_S) / I_0$.
* signal overlapping.

For all antibiotics, the proton or protons that exhibited the highest STD intensity were set to 100 % and all the others were normalized to that value (Table II).

The saturation transfer in **1** was the most efficient for proton H-13 (Table II), which was saturated to the highest degree. A strong enhancement was observed for desosamine proton H-1'. Other desosamine protons H-5', H-3' and H-2' were also enhanced, but to a much smaller extent. The cladinose protons H-1" and H-5" showed moderate degrees of saturation. Similarly, also in **2** saturation produced the largest enhancement of the H-13 proton and H-2 being only slightly less enhanced (Table II). Protons H-1", H-11 and H-6 gave the STD signal of moderate intensities. Somewhat smaller enhancements of desosamine protons were also observed. All the other protons showed small STD effects. The STD spectrum of **3** (Figure 2c) exhibited the highest intensity of methyl protons at position 15. The Me-10 protons and imidazole proton H-21 had similar STD intensities, giving relative saturation degrees of 84 % and 82 %, respectively. The methylene-14 and methyl-12 protons showed somewhat lower STD intensities. It is interesting to note that unlike **1** and **2**, **3** did not exhibit appreciable enhancement of desosamine sugar protons.

According to the STD epitope mapping of the studied macrolides, as shown in Figure 3, proton H-13 in **1** and **2** and proton H-15 in **3** are found to be the closest to BSA surface. The epitopes determined for the former two antibiotics are similar while that for the latter showed some differences. For example, in **3** the desosamine pro-

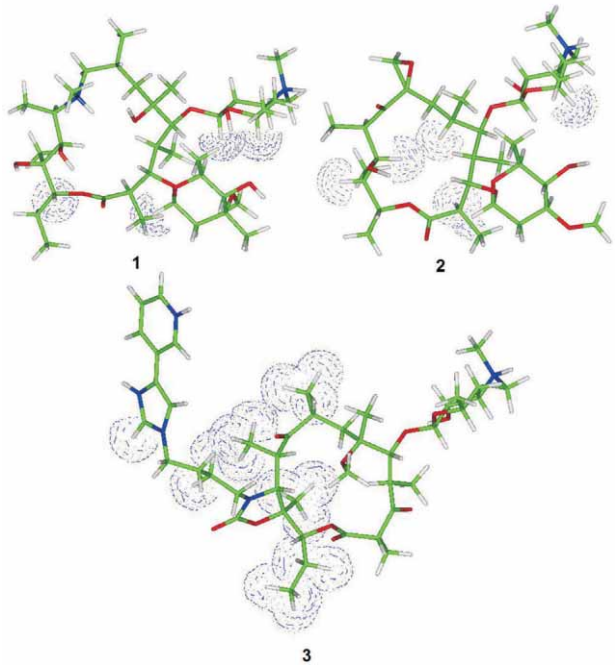

Figure 3. Binding epitopes as determined by STD NMR spectroscopy of azithromycin (**1**), oleandomycin (**2**) and telithromycin (**3**) mapped onto the solution state structures.

tons are not in intimate contact to BSA, while in **1** and **2** it is likely that desosamine interacts with BSA. On the other hand, **3** forms additional interactions with its alkyl--heteroaryl side chain at position 11,12. These findings point towards somewhat different binding modes for the three macrolide antibiotics. The differences are likely due to different structural elements of macrolides responsible for binding to BSA, such as cladinose sugar in **1** and **2** and alkyl-heteroaryl group in **3** that can form additional hydrophobic interactions with BSA. Similar was found for macrolide-ribosome complexes as determined by crystallography.[8,9] Further structural studies are needed to delineate the nature of macrolide-BSA interactions in more detail, followed by kinetic and biochemical measurements.

In conclusion, STD NMR spectroscopy has proven useful to detect and characterize interactions between BSA and macrolide antibiotics. The information gained from this investigation can be further exploited to help assess the overall biological profile of macrolides.

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SAŽETAK

Primjena NMR spektroskopije prijenosa razlike zasićenja za određivanje veznih epitopa makrolidnih antibiotika u interakciji s albuminom iz goveđeg seruma

Predrag Novak, Predrag Tepeš i Vedrana Lazić

Opisana je primjena spektroskopske NMR metode razlike prijenosa zasićenja za karakterizaciju veznih epitopa makrolidnih antibiotika azitromicina, oleandomicina i telitromicina u interakciji s albuminom iz goveđeg seruma. Strukturni dijelovi koji su u neposrednom dodiru sa albuminom sličniji su za azitromicin i oleandomicin dok kod telitromicina postoje neke razlike. To se može objasniti različitim strukturnim elementima koji stvaraju interakcije s proteinom, posebice alkilni-arilni lanac kod telitromicina te šećeri kladinoza i desozamin kod azitromicina i oleandomicina. Mape epitopa koje su određene i opisane u ovome radu mogu pomoći boljem razumijevanju ukupne biološke aktivnosti makrolidnih spojeva.