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Communication

Application of NMR Screening Methods with ^{19}F Detection to Fluorinated Compounds Bound to Proteins

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Abstract: The combinational use of one-dimensional (1D) NMR-based screening techniques with ^{1}H and ^{19}F detections were applied to a human serum albumin–diflunisal complex. Since most NMR screening methods observe ^{1}H spectra, the overlapped ^{1}H signals were unavailable in the binding epitope mapping. However, the NMR experiments with ^{19}F detection can be used as an effective complementary method. For the purpose of identifying the ^{1}H and ^{19}F binding epitopes of diflunisal, this paper carries out a combinatorial analysis using $^{1}H\{^{1}H\}$ and $^{19}F\{^{1}H\}$ saturation transfer difference experiments. The differences of the ^{1}H-inversion recovery rates with and without target irradiation are also analyzed for a comprehensive interpretation of binding epitope mapping.

Keywords: NMR-based screening; fluorinated compound; diflunisal; ^{19}F NMR

1. Introduction

Protein–ligand interactions can provide useful insights for understanding the molecular recognition system. However, arriving at such understandings requires the developments of useful methods for selectively observing the ligand. Although X-ray analyses can determine such interactions of the complex at the atomic level, difficulties in crystallization often interfere with the process of X-ray studies. In some cases, NMR spectroscopy can be a useful alternative for analyzing macromolecular complexes and screening compounds with an affinity to target proteins. Various NMR-based screening methods to observe the ligand signals have been proposed. It has been shown that NOE-pumping [1], saturation transfer difference (STD) [2], water–ligand observed via gradient spectroscopy (WaterLOGSY) [3,4], and reverse NOE-pumping [5] experiments could directly detect ^{1}H of the bound ligands. Recently, the NMR-based methods have been extended to fluorine detection [6–8]. Since the spectral elucidation in the aforementioned experiments [1–5] depends on the dispersion of ^{1}H signals, its signal degeneracy leads to a lack of information for the target molecules. Considering these difficulties, the NMR-based screening methods with ^{19}F-detection were applied to the human serum albumin (HSA)-diflunisal complex. HSA is an abundant plasma protein that binds to a wide range of drugs. Diflunisal contains two fluorine atoms in a molecule, and is a nonsteroidal anti-inflammatory drug that is effective in treating fever, pain, and inflammation. Since the X-ray crystal structure of a diflunisal-HSA complex has been determined (pdb: 2BXE), this complex could be a suitable model system for studying the molecular interactions of ^{1}H and ^{19}F using NMR spectroscopy. Information of the binding epitopes can be obtained for ^{19}F as well as ^{1}H of the fluorinated compound.

2. Results and Discussion

To investigate the ^{1}H binding epitopes of ligands, two representative methods—the ^{1}H{^{1}H} STD method acquired with various saturation times [9], or the difference of inversion recovery rates with and without target irradiation (DIRECTION) [10] method—were generally used. In the present study, the binding epitopes of diflunisal (Figure 1) were investigated using both methods. In the ^{1}H{^{1}H} STD experiments, the STD build-up curves were obtained at various saturation times. The slope of the STD build-up curve at a saturation time of 0 s was obtained by fitting to the monoexponential equation: $STD = STD_{max}(1 - e^{(-ksat \times t)})$, where STD stands for the STD signal intensity at saturation time t, STD_{max} is the maximal STD intensity at long saturation times, and k_{sat} stands for the observed saturation constant. The values of $k_{sat} \times STD_{max}$ correspond to the slope of the curve at zero saturation time with an elimination of T_1 bias. In the DIRECTION experiments, ^{1}H-T_1 were measured with and without the selective irradiation of protein, and its reciprocals, corresponding to the inversion recovery rates, were calculated for each separated ^{1}H signal.

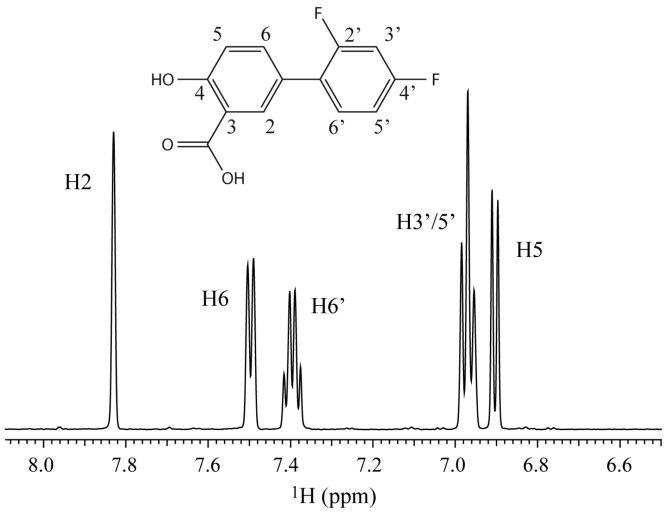

Figure 1. Structure and ^{1}H NMR spectrum of diflunisal.

The values of the STD effect were normalized by referencing the signal of H6 with the largest STD effect. The relative values are shown in Figure 2a. The values of the STD effect were larger in H5 and H6, indicating that these protons contributed as the binding epitopes. The smallest value was obtained in H2, which made less contact with the protein. The binding epitopes were also investigated using the DIRECTION method, evaluating the difference between the ^{1}H-inversion recovery rates with and without the irradiation of protein [10]. The large differences reflect the proximity to the protein surface. H6 and H6′ showed relatively large values (Figure 2b). Since the H3′ and H5′ signals overlapped, H6′ was the only signal available for analysis in a 2′,4′-difluoro ring (Figure 1), indicating that the incomplete information was obtained in the ^{1}H-detection NMR methods. To obtain more detailed information of the binding epitopes for the 2′,4′-difluoro ring, the ^{19}F{^{1}H} STD spectra were acquired with the arrayed saturation times (Figure 3). The ^{19}F{^{1}H} STD experiment was more insensitive than the ^{1}H{^{1}H} STD experiment. It can be considered that the saturation transfer from ^{1}H to ^{19}F is much less effective than that from ^{1}H to ^{1}H. However, the ^{19}F{^{1}H} STD experiment provided the useful information regarding the ^{19}F binding epitopes. The normalized values of the STD effect of F2′ and F4′ were 100% and 41.9%, respectively, and the values of ^{19}F-T_1 were 0.82 s and 1.6 s in the aforesaid order. This result indicated that F2′ made more close contact to HSA than F4′. It can be considered that a portion comprising H6, H6′, and F2′ could play a key role as the binding portion of diflunisal. The H2 made less close contact, which could be caused by an interruption of the carboxyl group at position 3. In the X-ray crystal structure of HSA complexed with diflunisal (pdb: 2BXE), three molecules of

diflunisal were bound with one molecule of HSA, where various close contacts were made in each binding site between two fluorine atoms of diflunisal, and protons of HSA. Since information from an epitope mapping that was obtained by the NMR experiments revealed average contacts in three HSA binding sites, some differences in the close contacts need to be considered between crystal and solution states.

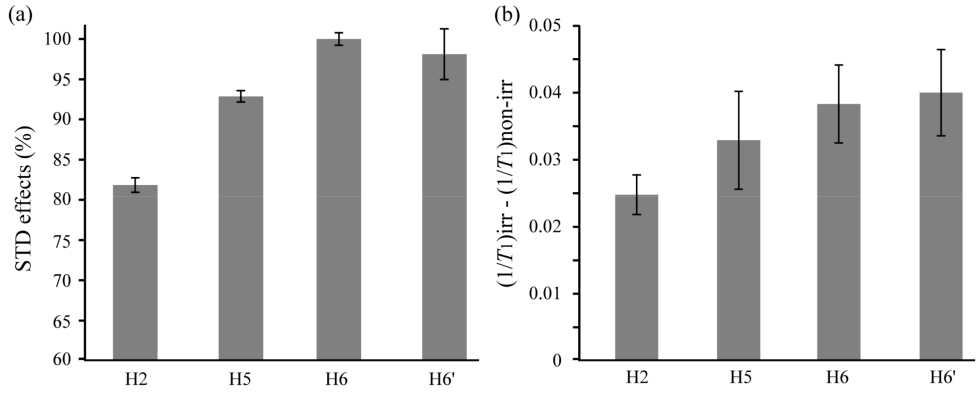

Figure 2. (**a**) The values of the saturation transfer difference (STD) effect of diflunisal. The values were normalized by referencing the signal of H6 with the largest STD effect; (**b**) The difference of inversion recovery rate with and without target irradiation.

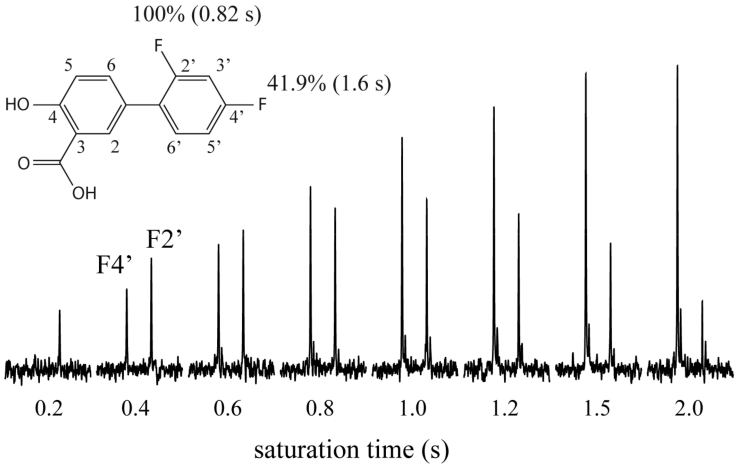

Figure 3. The $^{19}F\{^1H\}$ STD spectra acquired with the arrayed saturation times. The normalized values of the STD effect (%) and the values of ^{19}F-T_1 (s) are shown.

3. Materials and Methods

3.1. Instrumentation and Chemicals

All of the NMR spectra were recorded at 20 °C on a Varian 600 MHz NMR system (Vaian, Palo Alto, CA, USA) or JEOL ECA-500 MHz spectrometer (JEOL Ltd., Tokyo, Japan). Diflunisal and HSA were purchased from Sigma-Aldrich (Tokyo, Japan). A 600-μL of solution containing 0.05 mM HSA and 5.0 mM diflunisal was prepared in 100% ^2H_2O.

3.2. NMR Spectroscopy

The experimental parameters of the $^1H\{^1H\}$ STD experiment were as follows: data points = 16,384, spectral width of ^1H = 8012 Hz, number of scans = 1024, recycle time = 1.0 s. The saturation times for

the selective excitation of proteins were arrayed in the range of 0.2–3.5 s, and the arrayed spectra were acquired five times. The on and off resonance frequencies of ^{1}H were 0.6 and −20 ppm, respectively. Those of the ^{19}F{^{1}H} STD experiment were as follows: data points = 8192, spectral width of ^{19}F = 6012 Hz, number of scans = 10,240, recycle time = 1.0 s. The saturation times for the selective excitation of protein were arrayed in the range of 0.2–2.0 s. The on and off resonance frequencies of ^{1}H were 0.6 and −20 ppm, respectively. The values of the initial slope in the STD build-up curves were obtained by the least-square fitting in both of the STD experiments [9]. The experimental parameters for measuring ^{19}F-T_1 were as follows: data points = 8192, spectral width of ^{19}F = 6012 Hz, number of scans = 128, recycle time = 5.0 s. The inversion recovery pulse sequence was used. In measurements of ^{1}H-T_1 with and without the selective excitation of protein resonance (DIRECTION method) [10], the measurements were repeated five times, and the program in the JEOL Delta software (JEOL Ltd., Tokyo, Japan) was used for calculation of ^{1}H-T_1. The on and off resonance frequencies of ^{1}H were 0.6 and −20 ppm, respectively. The exponential window function was used with zero-filling by a factor of 2. The ^{1}H and ^{19}F chemical shifts were relative to 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) and trichlorofluoromethane, respectively, as external standards.

4. Conclusions

Although the sensitivity of the ^{19}F{^{1}H} STD experiment was lower than that of the ^{1}H{^{1}H} STD experiment, the obtained information was useful for the fluorinated compounds with the degenerated ^{1}H signals. Comprehensive interpretations for the binding epitope mapping are essential, while considering some discrepancies in the results of various NMR experiments. The ^{19}F{^{1}H} STD experiment can be a complimentary method for the ^{1}H detection methods.

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Conflicts of Interest: The authors declare no conflict of interest.

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