Journal of Biomolecular NMR (2006) 34: 221–227
DOI 10.1007/s10858-006-0005-y

Article

Structural studies of Bcl-xL/ligand complexes using ^{19}F NMR

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Received 21 November 2005; Accepted 11 January 2006

Key words: ^{19}F, apoptosis, Bcl-xL, NMR, structure

Abstract

Fluorine atoms are often incorporated into drug molecules as part of the lead optimization process in order to improve affinity or modify undesirable metabolic and pharmacokinetic profiles. From an NMR perspective, the abundance of fluorinated drug leads provides an exploitable niche for structural studies using ^{19}F NMR in the drug discovery process. As ^{19}F has no interfering background signal from biological sources, ^{19}F NMR studies of fluorinated drugs bound to their protein receptors can yield easily interpretable and unambiguous structural constraints. ^{19}F can also be selectively incorporated into proteins to obtain additional constraints for structural studies. Despite these advantages, ^{19}F NMR has rarely been exploited for structural studies due to its broad lines in macromolecules and their ligand complexes, leading to weak signals in ^{1}H/^{19}F heteronuclear NOE experiments. Here we demonstrate several different experimental strategies that use ^{19}F NMR to obtain ligand–protein structural constraints for ligands bound to the anti-apoptotic protein Bcl-xL, a drug target for anti-cancer therapy. These examples indicate the applicability of these methods to typical structural problems encountered in the drug development process.

Abbreviations: 1D – one-dimensional; 2D – two-dimensional; 3D – three-dimensional; HMQC – heteronuclear multi-quantum coherence; HOESY – heteronuclear Overhauser effect spectroscopy; NMR – nuclear magnetic resonance; NOE – nuclear Overhauser effect; NOESY – nuclear Overhauser effect spectroscopy; SAR – structure–activity relationship.

Introduction

Fluorine atoms are present in 17% of the drugs or drug-like molecules in the MDDR database (Muegge et al., 2001; Carosati et al., 2004). Incorporating fluorine atoms into drug molecules often produces positive effect on both potency and pharmacokinetic properties (Rowley et al., 2001; Kalgutkar et al., 2003a, b; Zamora et al., 2003). It is also possible to selectively incorporate ^{19}F-Phe, ^{19}F-Tyr, ^{19}F-Trp, ^{19}F-His, or ^{19}F-Met into proteins and ^{19}F-adenine, ^{19}F-cytidine, ^{19}F-uridine, or 2′-^{19}F-ribose into nucleic acids, providing an additional spectroscopic handle for NMR studies of their structures and functions (Arseniev et al., 1987; Drake et al., 1993; Rastinejad et al., 1995; Danielson and Falke, 1996; Lee et al., 2000; Duewel et al., 2001; Bann et al., 2002; Salopek-Sondi et al., 2003; Bann and Frieden, 2004; Frieden et al., 2004; Hamstra et al., 2004; Scott et al., 2004; Wi et al., 2004; Anderluh et al., 2005; Kreutz et al., 2005; Neerathilingam et al., 2005; Shu and Frieden, 2005; Spees et al., 2005). Given the lack of background signals and high natural abundance of ^{19}F, fluorine NMR has the potential for widespread application in the drug discovery process. While the use of ^{19}F NMR has been reported for detecting ligand binding to proteins (Kitamura et al., 2004; Shikii et al., 2004; Tengel

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et al., 2004), ^{19}F NMR has not yet been exploited for obtaining structural data on protein/ligand complexes to aid in structure-based drug design.

Here we report on the application of ^{19}F NMR for measuring intermolecular NOEs of a fluorinated drug lead complexed to either wild type or ^{19}F-Phe-labeled receptor protein. Using the anti-apoptotic protein Bcl-xL, intermolecular heteronuclear ^{1}H–^{19}F and homonuclear ^{19}F–^{19}F NOEs have been obtained between Bcl-xL and bound ligands. The ^{19}F NMR derived restraints are completely consistent with the known high-resolution structures of these complexes, demonstrating that ^{19}F NMR can be a reliable tool for obtaining structural information on protein–ligand complexes.

Materials and methods

Sample preparation

Bcl-xL was expressed in *Escherichia coli* and purified as previously described (Sattler et al., 1997). A loop-deleted form of the protein was employed in which the long unstructured loop connecting α1 to α2 was truncated (Sattler et al., 1997). Wild type [U-^{13}C]-labeled Bcl-xL sample was prepared by growing cells in media that contained [U-^{13}C]-glucose (3 g/l). The *para*-^{19}F-Phe-labeled sample was prepared by growing cells in media that contained $^{15}NH_4Cl$ (1 g/l), [U-^{13}C]-glucose (3 g/l), *para*-^{19}F-Phe (200 mg/l, Sigma), unlabeled Tyr and Trp (200 mg/l for each) and a specific inhibitor glyphosate (1 g/l) that inhibits the biosynthesis pathway of Phe, Tyr, and Trp aromatic amino acids (Kim et al., 1990). The purified protein was dialyzed against a buffer containing 40 mM sodium phosphate (pH 7.0) and 5 mM ^{2}H-dithiothreitol and concentrated to 1 mM for NMR studies.

NMR spectroscopy and structural models

The NMR spectra were collected at 30 °C on a Bruker DRX600 NMR spectrometer. Heteronuclear ^{19}F/^{1}H NOESY (HOESY) spectra (Rinaldi, 1983; Metzler et al., 1988) were collected in phase-sensitive mode by using States-TPPI (States et al., 1982; Marion and Wuethrich, 1983). Heteronuclear ^{13}C/^{1}H HMQC and homonuclear ^{19}F/^{19}F NOESY (Battiste et al., 2004) were collected as previously described. 3D ^{13}C-resolved ^{1}H/^{19}F HOESY (HMQC-HOESY) spectra were collected by inserting a ^{13}C/^{1}H HMQC in front of a phase-sensitive HOSEY pulse sequence. ^{19}F chemical shifts were referenced to 5-fluorotryptophan (5F-Trp) at −49.6 ppm (Drake et al., 1993).

Results and discussion

Bcl-xL is an anti-apoptotic protein of the Bcl-2 family and inhibits programmed cell death (Danial and Korsmeyer, 2004). As such, it represents an attractive drug discovery target for treating human cancers (Oltersdorf et al., 2005). Several groups have reported inhibitors of Bcl-xL (Degterev et al., 2001; Lugovskoy et al., 2002; Kazi et al., 2002; Oltersdorf et al., 2005; Shiau et al., 2005; Yin et al., 2005). Figure 1 shows the chemical structures of two fluorinated ligands used in this study. Compound **1** binds to Bcl-xL with a binding affinity (K_d) of 200 μM and represents a hit identified from fragment-based screening of compound

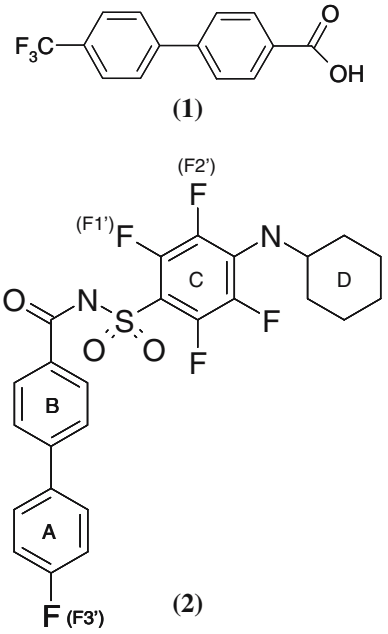

Figure 1. Chemical structures of the compounds used in the current study of the Bcl-xL/ligand complexes. For compound **2**, the fluorine atoms are labeled as F1′, F2′ and F3′, and the aromatic and aliphatic rings are labeled as A, B, C, and D.

libraries against Bcl-xL. Compound **2** binds to Bcl-xL with a binding affinity (K_d) of 20 μM and represents a typical initial lead in the drug discovery process. High-resolution structures of Bcl-xL complexed to similar molecules (e.g. where the fluorine atoms are replaced by protons) have been reported (Oltersdorf et al., 2005; Petros et al., 2006). Thus, these compounds serve as ideal tools to investigate the reliability of using ^{19}F NMR to derive protein–ligand distance restraints.

Fluorinated ligand/Bcl-xL complex

When compound **1** was titrated into ^{13}C-labeled Bcl-xL, selective chemical shift perturbations in the ^{13}C/^{1}H HMQC spectra were observed for the residues of A93, Y101, A142, and V135, indicating binding of the compound to the Bak peptide binding site on Bcl-xL. To obtain intermolecular distance restraints in this system, an approach analogous to that utilized for detecting NOEs between aliphatic protons and water molecules (Grzesiek and Bax, 1993) was explored. In this case, two ^{13}C/^{1}H HMQC spectra were collected in an interleaved fashion with and without saturation (1.0 s) of the ^{19}F peak (13.57 ppm) of the CF_3 group of compound **1** during the relaxation delay period. The difference spectrum of these two HMQC spectra contains only those cross peaks that correspond to protein residues near the CF_3 group of the ligand (Figure 2). Significantly, the spectrum has no interference or artifacts that are often observed when trying to selectively irradiate ^{1}H peaks. Thus, the difference spectrum shown in Figure 2b provides unambiguous ligand-to-protein intermolecular NOE-derived distance restraints.

The NMR resonances of Bcl-xL have been assigned and its structure has been determined previously by NMR (Sattler et al., 1997; Oltersdorf et al., 2005). When compared to the full reference ^{13}C/^{1}H HMQC spectrum shown in Figure 2a, the NOE peaks in Figure 2b can be identified as A104 $C_\beta H_3$, L108 $C_\delta H_3$ and L130 $C_\delta H_3$, indicating that these residues are in close proximity to the CF_3 group of ligand **1**. Indeed, when the acid and biphenyl of compound **1** is superimposed onto the high-resolution NMR structure of 4′-fluoro-biphenyl-4-carboxylic acid (IUPAC) when bound to Bcl-xL (Oltersdorf et al., 2005), the trifluoro-methyl group of compound **1** is less than 5 Å away from A104, L108, and L130 (Figure 3), which is consistent with the observed ^{1}H–^{19}F NOE contacts.

Fluorinated Bcl-xL protein

^{19}F-labeled aromatic residues can be incorporated efficiently into proteins by selective inhibition of the biosynthesis pathway for the aromatic residues in *E. coli* (Kim et al., 1990). This type of labeling can be quite useful since aromatic residues are

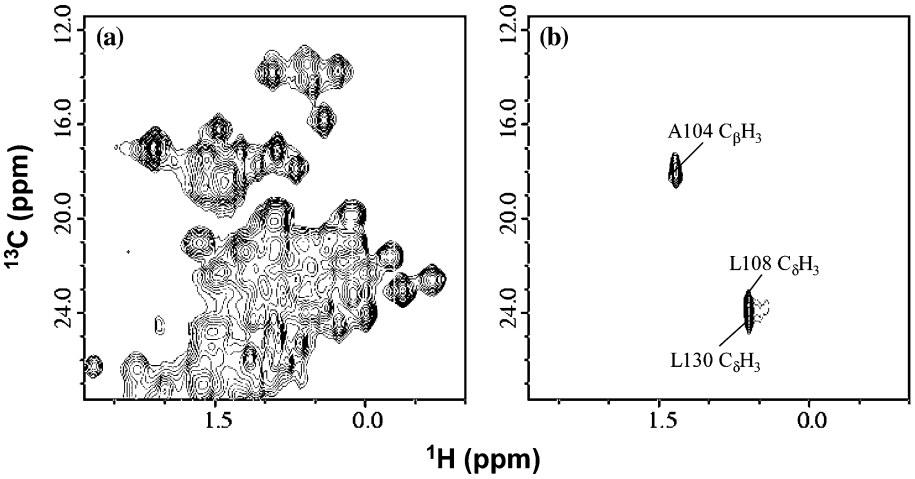

Figure 2. Intermolecular NOEs detected via ^{13}C/^{1}H HMQC for the fluorinated ligand/Bcl-xL complex. (a) Reference ^{13}C/^{1}H HMQC spectrum of the Bcl-xL/ligand **1** complex. (b) Difference of the ^{13}C/^{1}H HMQC spectra of the Bcl-xL/ligand **1** complex collected with and without saturation (1.0 s) of the CF_3 ^{19}F signals on ligand **1** during the relaxation delay period. Spectra were collected with 16 and 1024 scans per increment for a total of 1.8 h and 3.5 days for (a) and (b), respectively.

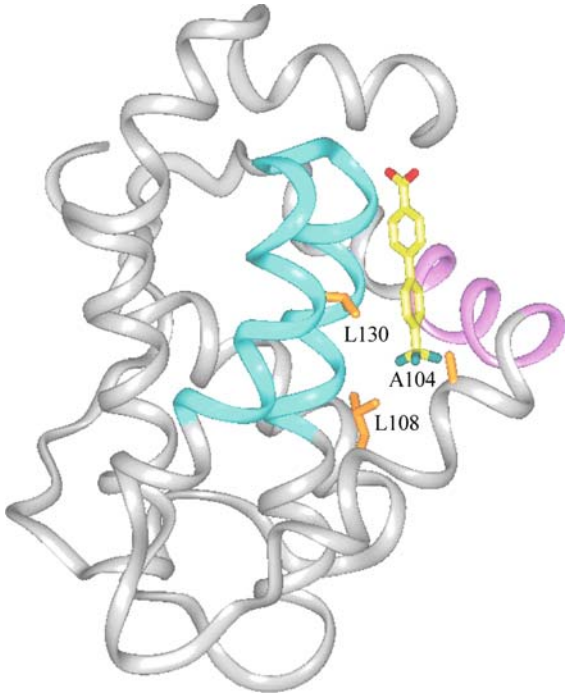

Figure 3. Ribbon depiction of the model of Bcl-xL (residues 1–44 and 85–197) in complex with compound **1**. This model was obtained by superimposing compound **1** onto the high-resolution NMR structure of 4′-fluoro-biphenyl-4-carboxylic acid (IUPAC) when bound to Bcl-xL (Oltersdorf et al., 2005). The compound **1** is colored by atom type except for the carbons which are colored in yellow. Bcl-xL residues that exhibit NOEs to the CF_3 group of the compound are rendered as sticks and colored in gold for carbon atoms. The BH1 and BH3 domains of the Bcl-xL are colored in cyan and pink, respectively.

often found in the hydrophobic core of proteins and at the interface of intermolecular interactions (Yu et al., 1995; Hajduk et al., 2004). Our Bcl-xL construct contains 10 phe residues. When Bcl-xL is labeled with *para*-^{19}F-Phe residues, only minor chemical shift changes are seen between the $^{13}C/^{1}H$ HMQC spectra of the wild type and the labeled proteins. This indicates that the *para*-^{19}F-Phe labeling does not significantly alter the structure of the protein. However, selective chemical shift perturbations were observed for the residues that are in close contact with the labeled *para*-^{19}F-Phe residues likely due to the different electronic properties of ^{19}F and ^{1}H atoms. For example, M170 $C_\varepsilon H_3$ is in van der Waals contact with the aromatic rings of F123 and F143, and has one of the biggest chemical shift perturbations in the spectrum. Similarly, the methyl groups of V135 and L178 are close to the aromatic ring of F131 and also exhibit chemical shift perturbations.

To assign the ^{19}F signals of the [U-^{13}C] and *para*-^{19}F-Phe-labeled Bcl-xL, a ^{13}C-resolved $^{1}H/^{19}F$ HOESY (HMQC-HOESY) spectrum was collected with a mixing time of 100 ms. Consistent with the high-resolution structure of Bcl-xL, NOEs were observed between the fluorine atom of *para*-^{19}F-Phe 12 and G94 H_α's (Figure 4a). Similarly, NOEs were observed between the fluorine atom of *para*-^{19}F-Phe 123 and I166 $C_{\gamma 2}H_3$ (Figure 4b) and between the fluorine atom of *para*-^{19}F-Phe 131 and L178 $C_\delta H_3$ (Figure 4c). The *para* $C_\zeta H$ groups of these Phe residues were not fully assigned before due to spectral overlap, and the ^{19}F–^{1}H NOEs obtained here thus represent new structural restraints for the protein.

Fluorinated ligand/fluorinated Bcl-xL complex

A fluorinated Bcl-xL inhibitor, compound **2** (Figure 1), was titrated into the *para*-^{19}F-Phe-labeled

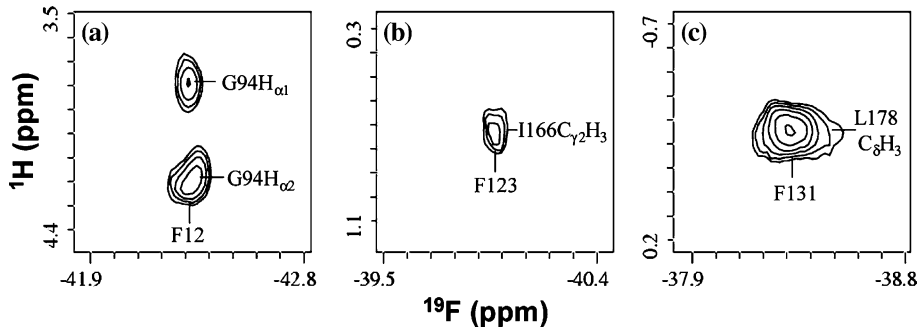

Figure 4. Selected slices of the ^{13}C-resolved $^{1}H/^{19}F$ HOESY spectra. Inserts from ^{13}C slices of G94 C_α (a), I166 $C_{\gamma 2}$ (b), and L178 C_δ (c). The mixing time used in this experiment was 100 ms. The 3D spectrum was collected with 144 scans per increment for a total acquisition of 6 days.

Bcl-xL. The addition of the fluorinated compound caused selective chemical shift perturbations for residues L130, V135, A142, T190, and L194. This pattern of chemical shift perturbations induced by binding of the compound is also consistent with the binding site located at the junction of BH1 and BH3 regions (Oltersdorf et al., 2005). Since both the ligand and Bcl-xL protein are fluorine-labeled, contacts between ^{19}F atoms of the ligand and protein can be observed in $^{19}F/^{19}F$ NOESY spectra of the complex. The fluorine atoms F1′ and F2′ of the ring C of compound **2** (Figure 1) resonate uniquely at −66.4 and −85.5 ppm. Strong intramolecular NOEs between the adjacent F1′ and F2′ fluorine atoms are observed as expected (Figure 5). Interestingly, intermolecular NOEs between the fluorine atoms F1′ and F2′ of the compound and the fluorine at −40.6 ppm (Phe 97) of *para*-^{19}F-Phe-labeled Bcl-xL are also clearly present, although their peak intensities are much weaker than the intramolecular NOEs of the adjacent F1′ and F2′ fluorine atoms. Nevertheless, the observation of these intermolecular NOEs orients ring C of compound **2** towards the *para*-fluorine of Phe 97 of Bcl-xL.

Figure 6 shows the model of Bcl-xL/compound **2** complex obtained by superimposing this ligand onto the high-resolution structure of a homologous compound (differing only on ring C with a *meta*-nitro group and protons instead of the all fluorine atoms) (Petros et al., 2006; as well as unpublished results). The fluorine atoms F1′ and F2′ of ring C of compound **2** are located in close proximity to the fluorine atom of the *para*-^{19}F-Phe 97, again consistent with the observed intermolecular $^{19}F/^{19}F$ NOE data.

Conclusions

Using ^{19}F NMR, we have studied fluorinated ligands in complex with either wild type Bcl-xL or *para*-^{19}F-Phe-labeled-Bcl-xL. Intermolecular heteronuclear NOEs between the ^{19}F atoms of the ligand and ^{1}H of the protein were detected via a $^{13}C/^{1}H$ HMQC experiment that was run with and without irradiation of the ^{19}F atoms of the ligand. Additional structural constraints were obtained from homonuclear ^{19}F–^{19}F NOEs between the fluorinated ligand and the *para*-^{19}F-Phe-labeled Bcl-xL. All of these ^{19}F-derived intermolecular contacts are completely consistent with the known high-resolution structures of Bcl-xL complexed to highly similar compounds. This information can therefore be utilized to augment traditional structure determination of protein–ligand complexes.

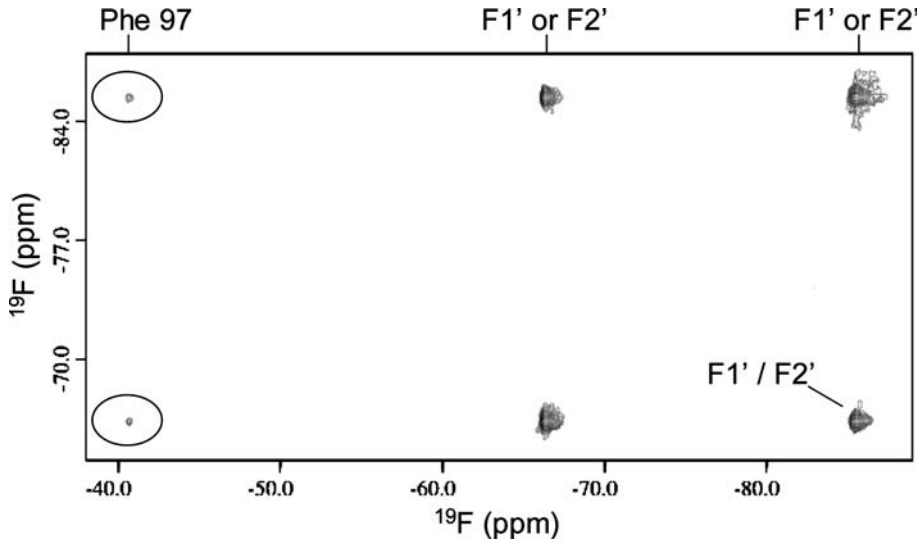

Figure 5. Two-dimensional $^{19}F/^{19}F$ NOESY spectrum of the *para*-^{19}F-Phe-labeled Bcl-xL in complex with compound **2**. The spectrum was collected with a mixing time of 250 ms. ^{19}F assignments are indicated along the top of the figure. F1′ and F2′ correspond to the two fluorine atoms in compound **2** as indicated in Figure 1. Intraligand NOEs between F1′ and F2′ atoms are labeled. Intermolecular NOEs between the fluorine atoms (F1′ and F2′) of the compound and the fluorine atom of the *para*-^{19}F-Phe 97 of Bcl-xL are circled. This spectrum was collected with 256 scans per increment for a total time of 1.2 days.

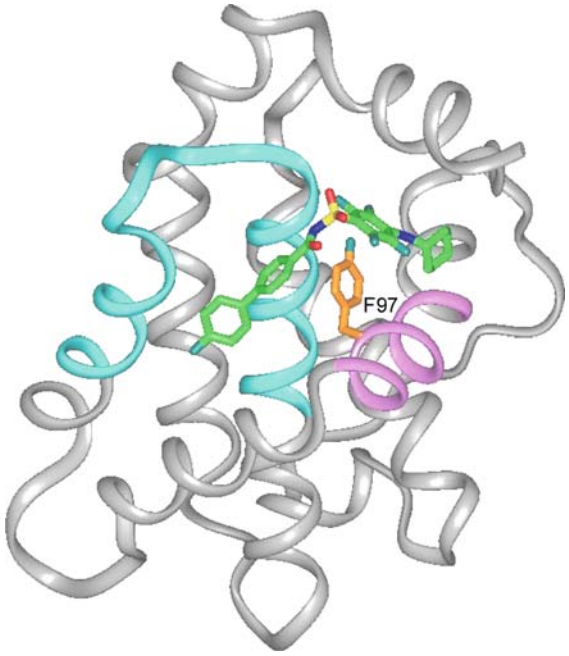

Figure 6. Ribbon depiction of the model of Bcl-xL (residues 1–44 and 85–197) in complex with compound **2**. This model was obtained by superimposing compound **2** onto the high-resolution NMR structures of homologous compounds (Petros et al., 2006). The compound **2** is colored by atom type. *Para*-^{19}F-Phe 97 of Bcl-xL is rendered as sticks and colored by atom type where the carbon atoms are colored in gold. The BH1 and BH3 domains of the Bcl-xL are colored in cyan and pink, respectively.

In many cases, a series of such experiments performed on compounds with fluorine atoms incorporated at different positions of the compound will be sufficient to uniquely determine the binding mode of the ligand complexed to the protein.

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