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Use of ^{19}F NMR spectroscopy to screen chemical libraries for ligands that bind to proteins

Tobias Tengel,[a] Tomas Fex,[b,c] Hans Emtenäs,[a] Fredrik Almqvist,[a] Ingmar Sethson*[a] and Jan Kihlberg*[a,c]

[a] *Organic Chemistry, Department of Chemistry, Umeå University, SE-901 87 Umeå, Sweden. E-mail: jan.kihlberg@chem.umu.se*

[b] *Orbichem AB, Ole Römers Väg 12, SE-223 70 Lund, Sweden*

[c] *AstraZeneca R&D Mölndal, SE-431 83 Mölndal, Sweden*

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Identification of compounds from chemical libraries that bind to macromolecules by use of NMR spectroscopy has gained increasing importance during recent years. A simple methodology based on ^{19}F NMR spectroscopy for the screening of ligands that bind to proteins, which also provides qualitative information about relative binding strengths and the presence of multiple binding sites, is presented here. A library of fluorinated compounds was assembled and investigated for binding to the two bacterial chaperones PapD and FimC, and also to human serum albumin (HSA). It was found that library members which are bound to a target protein could be identified directly from line broadening and/or induced chemical shifts in a single, one-dimensional ^{19}F NMR spectrum. The results obtained for binding to PapD using ^{19}F NMR spectroscopy agreed well with independent studies based on surface plasmon resonance, providing support for the versatility and accuracy of the technique. When the library was titrated to a solution of PapD chemical shift and linewidth changes were observed with increasing ligand concentration, which indicated the presence of several binding sites on PapD and enabled the assessment of relative binding strengths for the different ligands. Screening by ^{19}F NMR spectroscopy should thus be a valuable addition to existing NMR techniques for evaluation of chemical libraries in bioorganic and medicinal chemistry.

Introduction

NMR spectroscopy has long been used to detect binding of small molecules to biomolecular targets, and has recently been employed for the screening of libraries of compounds in drug discovery. A wide range of parameters determined by NMR spectroscopy have been used in screening, including the nuclear Overhauser effect (NOE),[1–4] chemical shift perturbations,[5] diffusion rates,[6–8] relaxation rates[9] and saturation transfer.[10–12] However, screening of chemical libraries using traditional methods produces complex ^{1}H NMR spectra and/or requires labelled protein samples. In this paper we present a simple and direct screening approach based on ^{19}F NMR spectroscopy which does not require labelled protein.

A wide range of ^{19}F labelled compounds and chemical building blocks are commercially available, hence fluorine is easily incorporated into a ligand. The fluorine atom is relatively non-sterically demanding and can, in many cases, replace a hydrogen atom in a ligand with minimal, or beneficial, influence on an event of molecular recognition and the subsequent biological response. Since incorporation of fluorine often improves the pharmacokinetic properties of biologically active compounds, fluorinated compounds are widely prepared in medicinal chemistry projects. ^{19}F has a natural abundance of 100%, a sensitivity of 83% compared to that of ^{1}H, and the ^{19}F chemical shifts are sensitive to the surrounding environment. Because ^{19}F has a large chemical shift dispersion (~200 ppm in organofluorine compounds) it should be possible to screen large libraries without chemical shift overlap becoming a significant problem. Moreover, since ^{19}F does not occur naturally in proteins, there is no interfering background which simplifies spectral interpretation and allows high sensitivity. This nucleus therefore appears to be suitable as a reporter in screening of libraries of compounds which may have affinity for target proteins. In biological systems, ^{19}F NMR spectroscopy has already found use in studies of protein structure and dynamics upon ligand binding,[13–21] conformational preferences and mobility of proteins,[22–24] as well as protein folding and unfolding events.[25] These studies have all relied on fluorine labeling of one or a few of the amino acids in the proteins investigated. Recently, a method has been reported by Dalvit *et al*. where screening of chemical libraries is performed by using a ^{19}F-labelled binder as a probe to detect more efficient binders by the disappearance of the ^{19}F NMR resonance in the spectrum.[26,27] A slight drawback with this method is that the deconvolution process of large mixtures can become lengthy. However, once deconvolution is complete and the hit has been identified the binding constant can be obtained rapidly. Independently, we have developed a different approach which relies on observation of changes in the ^{19}F NMR spectra of ^{19}F-labelled ligands upon binding to a receptor. The method requires labelling of more than one ligand but it provides direct information on the identity of the hit(s) if a library of appropriate size is screened. It also directly gives additional information, such as relative binding strengths of the different ligands and the presence of multiple binding sites on the receptor.

When a fluorinated ligand interacts with a target, the fluorine chemical shift should change due to the difference in chemical environment, as long as the ^{19}F nucleus experiences a difference between the free and bound state. Fluorine NMR spectroscopy has previously been used in a few studies of the binding of single, fluorinated ligands to human serum albumin (HSA), and large induced chemical shifts were then observed.[28,29] It should also be possible to use the linewidth of fluorine nuclei to identify bound ligands. Upon binding to a large target, such as a protein, the ligand will move and tumble at a slower rate. This will result in reduction of the T_2 relaxation rate for the ligand, which consequently will increase the linewidth of the NMR resonances as compared to the free form.

In the present study we have used ^{19}F NMR spectroscopy to screen a small library of ligands for affinity for the chaperones PapD and FimC. These chaperones are involved in the

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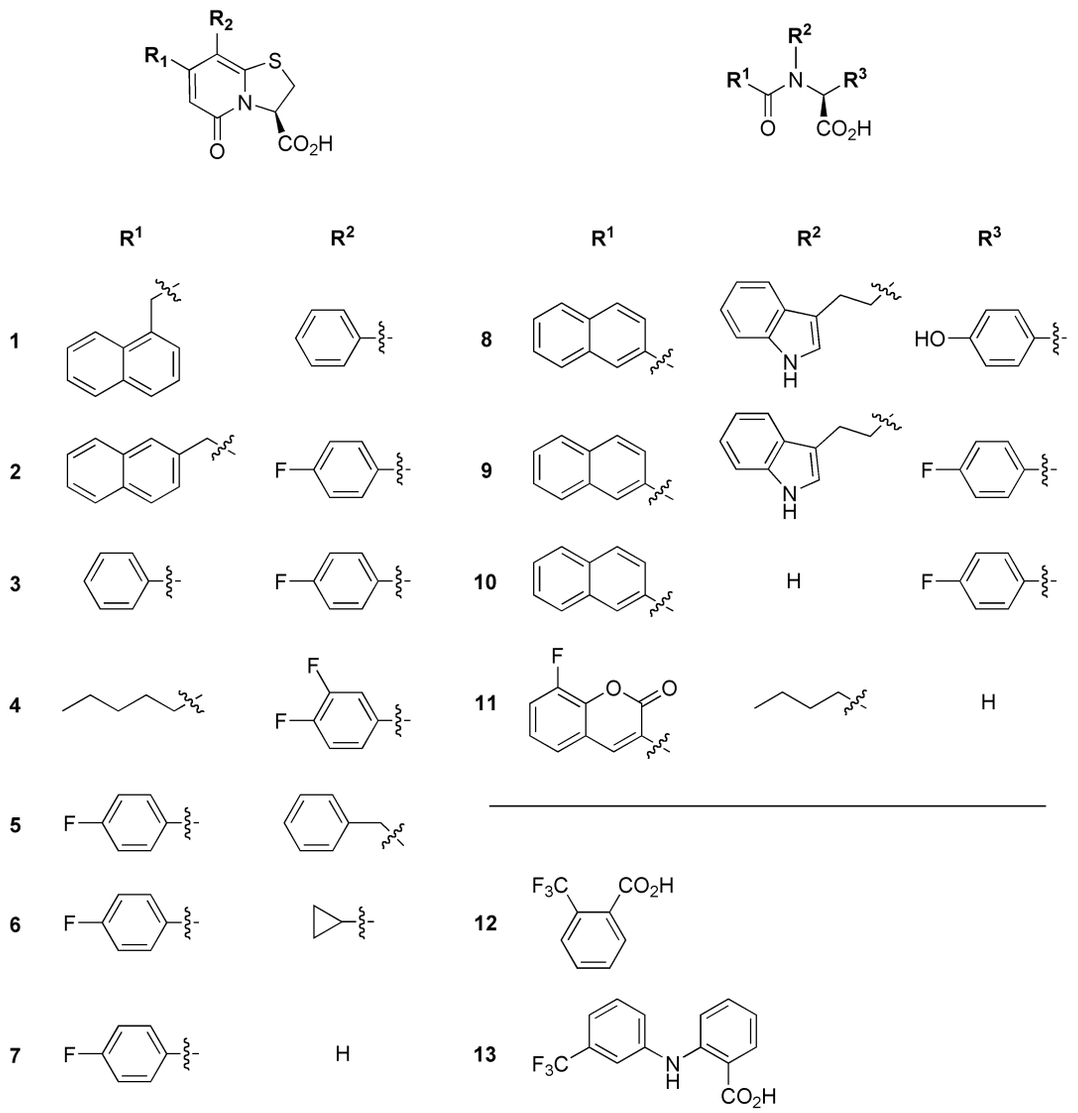

Fig. 1 2-Pyridinones **2**–**7** and amino acid derivatives **9**–**11** were investigated for binding to the chaperones PapD and FimC, and to HSA. 2-(Trifluoromethyl)benzoic acid (**12**) and flufenamic acid (**13**) were included as controls.

assembly of virulence-associated, hair-like protein structures termed pili on the surface of uropathogenic *E. coli*.[30,31] The ability of *E. coli* to adhere to host cell tissue and to cause disease is dependent on functional pili, and PapD and FimC thereby constitute potential targets for development of new antibacterial agents. The library evaluated for binding to PapD and FimC was also screened for affinity to HSA.

Results and discussion

Screening for ligands with affinity for the PapD chaperone

Previous studies using surface plasmon resonance and ^{1}H NMR spectroscopy have identified 2-pyridinone **1** and amino acid derivative **8** (Fig. 1) as ligands with strong affinity for the chaperones PapD and FimC.[32] A collection of ^{19}F-labelled analogues of **1** and **8**, *i.e.* substances **2**–**7** [33] and **9**–**11**, were therefore selected to investigate the versatility of ^{19}F NMR spectroscopy in screening of ligands for binding to these two proteins. Compound **11** was included in the study since it was recently found to have a low affinity for PapD.[34] In addition, 2-(trifluoromethyl)benzoic acid (**12**) and flufenamic acid (**13**), which have unrelated structures were added as controls to the library. An equimolar mixture of these eleven compounds was dissolved in an aqueous solution, which contained 5% DMSO to allow the less soluble 2-pyridinones **2**–**7** to go into solution. The ^{1}H NMR spectrum of this small library was, as expected, complex with several overlapping resonances (Fig. 2). Consequently, it would be difficult to identify ligands that bind to an added protein directly from the change in appearance of the one-dimensional ^{1}H NMR spectrum. In addition, proton signals from an added protein would have to be filtered out of the spectrum, which would require use of a costly ^{15}N and ^{13}C-labelled protein. In contrast the proton-decoupled ^{19}F NMR spectrum of the solution of the mixture of **2**–**7** and **9**–**13** was substantially better resolved (Fig. 3), illustrating the potential advantage of ^{19}F as a probe in studies of ligand–protein interactions. For the mixture of **2**–**7** and **9**–**13**, the ^{19}F resonances were assigned from the spectra of each individual compound, but use of chemical shift information and deconvolution procedures could be envisaged to simplify identification of ligands from larger libraries. Due to the presence of different conformers two resonances were observed for each of compounds **4**, **9** and **11**. In addition to multiple resonances, compound **4** displays splitting of both resonances due to a fluorine–fluorine *J*-coupling.

After having assigned the resonances the ligand mixture was added to a sample of the PapD chaperone so that a 1 : 1 molar ratio between PapD and each ligand was obtained. As a result of binding to PapD, significant line broadening was observed for several of the compounds, as revealed in the one-dimensional ^{19}F NMR spectrum (Fig. 4 and Table 1). All

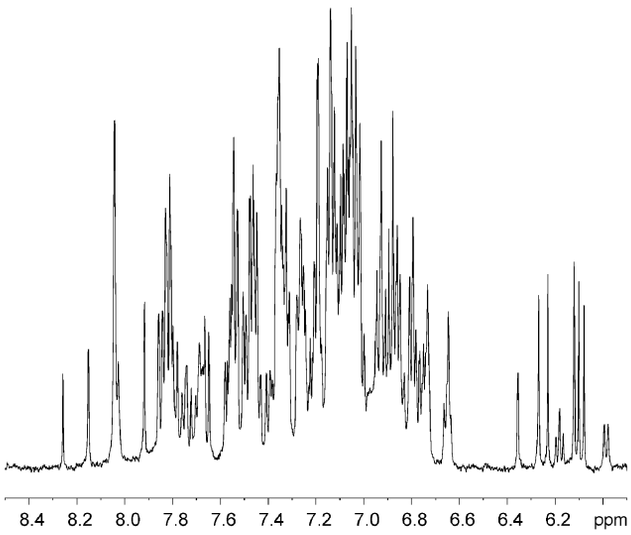

Fig. 2 ^{1}H NMR spectrum of an equimolar mixture of **2**–**7** and **9**–**13** (each at 100 μM) in aqueous solution containing 5% DMSO, at pH 6.3 and at 25 °C. The spectrum was recorded at 500 MHz with 256 scans.

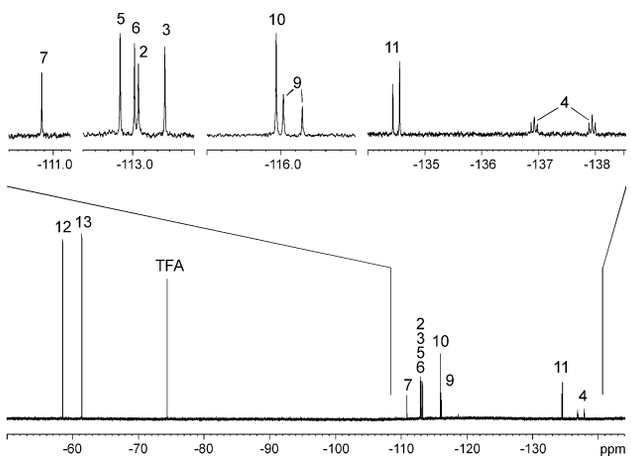

Fig. 3 ^{19}F NMR spectrum of an equimolar mixture of **2**–**7** and **9**–**13** (experimental conditions are given in the legend for Fig. 2). The spectrum was recorded at 375.9 MHz with 1024 scans.

ligands are in fast exchange since only one resonance is observed for each compound, representing the average between the free and bound forms. The observed linewidths can be compared with the values expected for a ligand bound tightly to PapD. The contribution to T_2 relaxation from dipolar and chemical shift anisotropy mechanisms for a ligand that experiences the same rotational correlation time as PapD (estimated from the size and shape of PapD to be between 10–20 ns) should provide linewidths in the range 30–50 Hz.[27] Compounds **2** and **9** experience very large induced line broadenings, which were estimated to be well above 50 Hz. This extensive line broadening therefore suggests that there are other mechanisms than the decreased molecular mobility that contribute to the observed linewidths. It can however be concluded that the extensive line broadening is not caused by aggregation effects since the linewidths in the ^{1}H NMR spectrum of PapD are hardly affected, indicating that the aggregation state of PapD is unchanged. The remaining compounds, the resonances of which display line broadening below 30 Hz, do not experience the same rotational correlation time as PapD. Most likely this is due to less restricted mobility when they are bound to PapD or to that they are only partially bound. Of these compounds **3**–**5** and **10** are those having linewidths that are affected the most. Finally, compounds **11** and **12** each display only a small induced line broadening (1.6 and 0.4 Hz, respectively) and do not seem to have any affinity for PapD, since this small increase in linewidth can mainly be attributed to changes in the viscosity

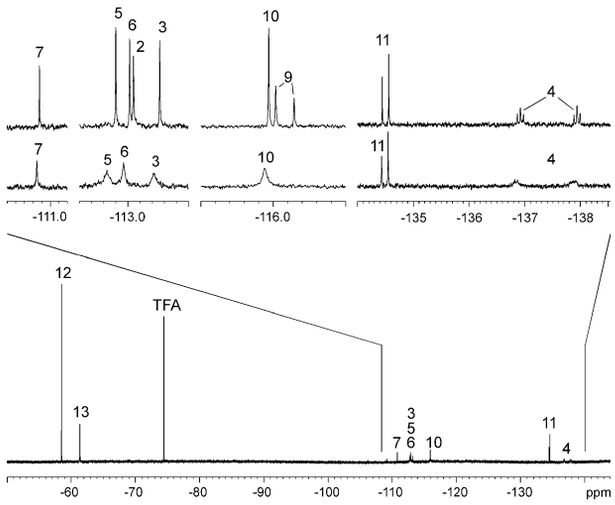

Fig. 4 ^{19}F NMR spectrum of an equimolar mixture of **2**–**7** and **9**–**13** (each at 100 μM) in aqueous solution in the presence of 100 μM PapD. The solution contained 5% DMSO and the spectrum was recorded at pH 6.3 at 25 °C. The expansions are: ligands in presence of PapD (lower expansion) and ligands in absence of PapD (upper expansion).

of the solution when protein is added. Although different causes for the various linewidths can be envisaged, our titration experiments (see below) do corroborate that the increase of the linewidths in this case are correlated to the strength of the binding.

The induced chemical shift on addition to PapD is small for the resonances from the observable, weakly binding ligands in the library. The largest induced chemical shift is as small as 0.07 ppm and was observed for 2-pyridinones **4** and **5** (Table 1). For this library of compounds it is therefore difficult to make a completely clear distinction between ligands that bind with high, intermediate or low affinity to PapD based on the induced chemical shifts. It should, however, be noted that the strongest binders as suggested from line broadening, *i.e.* **2** and **9**, are not observable in the ^{19}F spectrum in the presence of PapD. Even though the induced chemical shifts observed in presence of PapD are small, there is a correlation between linewidth and induced chemical shift. The resonances with the largest induced chemical shifts belong to ligands having significant line broadening (*i.e.* **4** and **5**), whereas ligands identified as having no affinity for PapD from line broadening (*i.e.* **11** and **12**) have smaller induced chemical shifts. Thus, for this particular protein–ligand system, line broadening appears to be a more accurate parameter for identification of compounds with affinity for PapD than the induced chemical shift. In contrast, the chemical shifts changed up to ~1 ppm when HSA was used as target protein (*cf*. section on screening for ligands with affinity for HSA, below).

It should be noted that the use of line broadening to separate weak and strong binders could in some cases be misleading due to chemical exchange. This is because an increased linewidth could be caused by slower molecular tumbling as well as by chemical exchange. Consequently, an increased linewidth can safely be interpreted as a proof of binding, but there may be occasions when the induced linewidth is not properly correlated to the strength of the binding. For instance, if a ligand which is involved in fast exchange has a large difference in chemical shift between the free and bound form, this would contribute to a larger line broadening than a similar exchange rate for a ligand with a small chemical shift difference. In addition, bound ligands may experience different restrictions in mobility.

As mentioned above linewidths in large excess of what is expected for a tightly bound ligand are observed for **2** and **9**. This is an indication that exchange mechanisms are simultaneously contributing to the observed linewidths. One such exchange mechanism is when a ligand switches between the free

Table 1 ^{19}F chemical shift, linewidth, relaxation rates and surface plasmon resonance data for substance **2**–**7** and **9**–**13**. Chemical shift and linewidth obtained for free ligand and in presence of PapD (1 : 1 ratio)

Ligand	Linewidth free/Hz	Linewidth bound/Hz	δ free/ppm	Induced δ /ppm	T_2 relaxation[a] /ms	SPR response[b]
2	1.6	19[c]	−113.05	0.13		77
3	1.0	17	−113.26	0.05	82	23
4	2.5	[d]	−136.92	0.07	[d]	35
	2.4		−137.94	0.06		
5	1.4	22	−112.90	0.07	57	23
6	1.4	10	−113.02	0.06	120	11
7	1.2	4.0	−110.87	0.03	163	9
9	1.7	25[c]	−116.02	0.05		77
	1.5	28[c]	−116.15	0.06		
10	1.2	15	−115.97	0.03	78	17
11	1.4	3.0	−134.44	0.01	373	4
	1.4	2.3	−134.56	0.01		
12	0.8	1.2	−58.50	0.00	987	4
13	0.9	10	−61.41	0.03	127	33

[a] Relaxation times measured at 1 : 1 ligand : PapD ratio. [b] Determined with surface plasmon resonance using a BIACORE 3000 instrument. The normalized responses were calculated using substance **1** as a reference at 100% and have been corrected for differences in molecular weight. [c] Chemical shifts and linewidths were measured in a sample containing this ligand and PapD only in order to avoid spectral overlap. In addition, the number of scans was increased from 1000 to 20000 to improve the signal to noise ratio. [d] Linewidth and T_2 relaxation rate not measured due to F–F coupling.

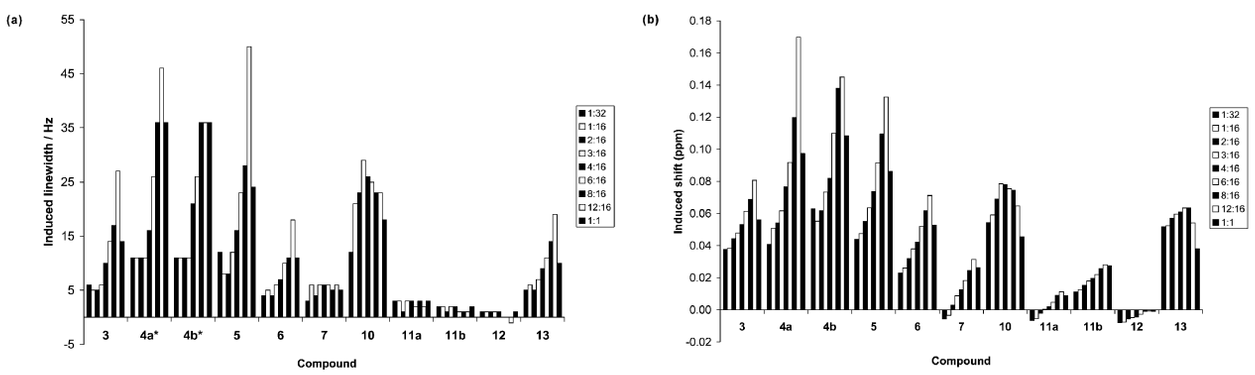

Fig. 5 Induced linewidth and chemical shift for each ligand when titrated to a sample of PapD in aqueous solution (200 μM, pH 6.3, 5% DMSO) at 25 °C. The titration was performed so as to give ratios of 1 : 32, 1 : 16, 2 : 16, 3 : 16, 4 : 16, 6 : 16, 8 : 16, 12 : 16 and 1 : 1 for each ligand relative to PapD. (a) Induced linewidth for the members of the library of compounds. The linewidth for **2** and **9** could not be measured due to extensive line broadening. * Denotes a linewidth determined by simulation. (b) Induced chemical shifts for the library of compounds. The chemical shift for **2** and **9** could not be measured due to extensive line broadening.

and bound form. An even larger line broadening would result for ligands that have access to more than one binding site on the protein. In the case of PapD the existence of multiple binding sites is suggested by the fact that even though the ratio between PapD and each of the ligands in the library is 1 : 1, all but **11** and **12** show affinity for the chaperone.

Titration of ligands to PapD

In an effort to determine if ligands **2**–**7** and **9**–**13** bind to the same or different sites on PapD, and to evaluate the relative binding strengths, ^{19}F NMR spectra were recorded after addition of aliquots of the equimolar mixture of **2**–**7** and **9**–**13** to PapD (Fig. 5). This titration was performed so as to give relative concentrations ranging from 1 : 32 to 1 : 1 for each ligand relative to PapD, with the PapD concentration being kept constant at 200 μM. Assuming that there is only one binding site on PapD one would expect to observe an increased line width for the ligands early in the titration series, followed by a reduction in the latter titration points when the available binding site is occupied. Such a behavior can be observed only in the last titration point for the weakly binding ligands **3**–**6** and **13** (Fig. 5A). As the linewidths of these compounds are broadened up to a 3 : 4 ratio of each ligand relative to PapD, this indicates the presence of several binding sites on the chaperone. However, compound **10** behaves differently with a reduction in linewidth taking place at a 1 : 4 ratio relative to PapD. The latter observation could be explained by ligand **10** having affinity for one distinct site on PapD, and being involved in competitive binding with ligands having higher affinity, such as **2** and **9**. As shown above (Fig. 4), compounds **2** and **9** experience extensive line broadening in the presence of PapD and due to this their linewidths could not be determined in the mixture. However, on further addition of **2** or **9** to the 1 : 1 ligand/protein sample the resonances of **2** and **9** reappeared in the spectrum, demonstrating that chemical exchange processes are the most likely explanation for their disappearance earlier in the titration series.

Using the line widths as criteria of binding, the titration experiment indicates that all available binding sites are in use only when each ligand has a concentration of 3 : 4 relative to PapD. This behavior is compatible with **2** and **9** having high affinities for PapD and that several binding sites are available on the protein. Moreover, it supports the conclusion from the one-dimensional spectrum recorded at a 1 : 1 ratio between each ligand and PapD (Fig. 4), *i.e.* that all the other compounds in the library can be classified as ligands with lower affinity although there is some variation in the strength of their interaction with PapD. This conclusion is also supported by the induced chemical shifts which show the same general pattern with increased ligand concentration as the induced linewidths (Fig. 5b). The chemical shifts for ligands **3**–**6** first experience a downfield shift and then, in the final titration point, shift upfield towards the value of the free ligand. Compound **10** again behaves differently, with chemical shift changes decreasing earlier in the titration series.

The correlation between induced linewidths and chemical shifts for the titration series thus supports the conclusion that multiple binding sites exist for the ligands on binding to the PapD chaperone. In fact, recently initiated studies in our laboratory using ^{15}N labelled chaperone indicate that compounds such as pyridinones **1**–**7** and amino acid derivatives **8**–**11** bind to at least two different sites on the chaperone.[35] From the ^{19}F NMR studies presented herein it is not possible to determine which compounds compete with each other at a specific site. However, it is evident that compound **10**, and also **3**–**6**, are involved in competitive binding with other ligands having higher affinity such as **2** and **9**.

Comparison with surface plasmon resonance

The affinity of compounds **1**–**7** and **9**–**13** for PapD was also investigated in an independent manner using surface plasmon resonance (SPR) after immobilization of PapD on a dextrane-coated sensor chip. From previous SPR studies **1** and **8** are known to bind tightly to PapD.[32] Compound **1** was therefore used as reference and the other responses were calibrated with the response of **1** set at 100%. In this assay, **2** and **9** were found to interact strongly with PapD whereas **3**–**5** along with **10** and **13** showed intermediate binding to PapD (Table 1). The remaining compounds (**6**, **7**, **11** and **12**) displayed low affinity for PapD. These data are in good agreement with the conclusions reached by ^{19}F NMR spectroscopy, which identified **2** and **9** as ligands with high affinity for PapD, whereas **11** and **12** were found to be non-binders, and the remaining compounds were classified as weak ligands. In the group of weak binders, as revealed by NMR spectroscopy, compounds **3**–**5** and **10** were found to have the highest affinity for PapD as compared to the other ligands in the group. This ranking corroborates well with the SPR responses. However, some discrepancies were found between the two methods. Substance **13** was found to be a relatively strong binder in the SPR measurement, while NMR indicated it to have an affinity comparable to that of **6**.

Screening for ligands with affinity for the FimC chaperone

For the FimC chaperone screening of the library was conducted at a concentration of 100 μM using a 1 : 1 molar ratio between FimC and each of compounds **2**–**7** and **9**–**13**. As FimC is a homologous chaperone to PapD with equivalent size, similar binding properties would be expected for the compounds in the library. Indeed, ^{19}F NMR spectroscopy did reveal similar effects on linewidth and chemical shift for the members of the library in the presence of FimC as found for PapD (Fig. 6). That is, ligands identified as strong or weak binders for PapD showed the same characteristics in their interactions with FimC. However, the data suggest all ligands to have a slightly weaker affinity for this chaperone as compared to PapD, since both line broadenings and the induced chemical shifts were less pronounced upon binding to FimC. These findings are in agreement with previous studies based on surface plasmon resonance for compounds **1** and **8**.[32]

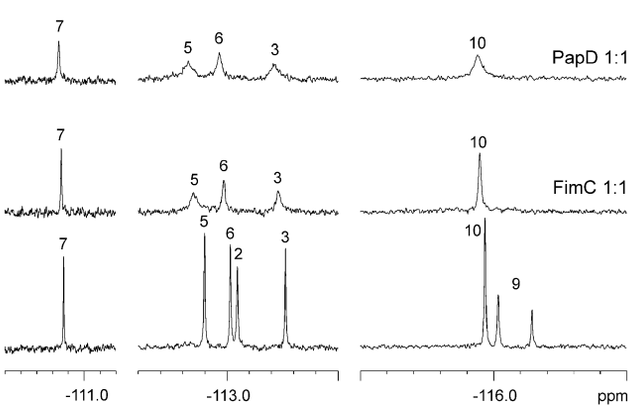

Fig. 6 Selected regions from the ^{19}F NMR spectra of an equimolar mixture of **2**–**7** and **9**–**13**, alone or in presence of 100 μM FimC or 100 μM PapD. The spectra were recorded for aqueous solutions containing 5% DMSO at pH 6.3 and at 25 °C. Only the regions with resonances for ligands **2**–**3**, **5**–**7** and **9**–**10** are shown. The expansions are; ligand mixture alone (lower spectrum), ligands with FimC (middle spectrum), and ligands with PapD (upper spectrum).

Screening for ligands with affinity for HSA

Human serum albumin (HSA) is one of the most abundant proteins in the circulatory system and possesses a unique capability to bind a large number of various endogenous and exogenous compounds. The primary function of HSA is to transport fatty acids, but HSA also has the ability to interact with a large number of metabolites, drugs and organic compounds. There are thought to be five principal sites for medium or long-chain fatty acids on HSA.[36–38]

Screening of the library of compounds **2**–**7** and **9**–**13** for binding to HSA was performed under the same conditions as for PapD and FimC, *i.e.* at a concentration of 100 μM for all compounds with a molar ratio of 1 : 1 between HSA and each ligand. As revealed by the induced line broadening, all compounds in the library had at least some affinity for HSA, and most of them appeared to bind tightly (Fig. 7). Since HSA is a larger protein than PapD and FimC, a larger induced line broadening would be expected in these experiments due to the slower molecular tumbling of HSA. Although it is hard to assign some of the resonances in the spectrum due to extensive line broadening, large induced chemical shifts were observed for several resonances (Fig. 7). Flufenamic acid, **13**, which is known to have affinity for HSA,[28] displays an induced downfield chemical shift of ~0.4 ppm, along with an extensive line broadening. The two ligands identified as non-binders for PapD and FimC, **11** and **12**, also appear to interact with HSA. However, line broadening as well as induced chemical shifts suggest weak interactions for these two substances. Compound **4** appears to be one of the ligands with the strongest affinity for HSA as its resonances are shifted the most (1.2 and 1.3 ppm). Compound **7** displays an induced shift of less than 0.1 ppm although one observes a large line broadening, identifying it as a ligand with affinity for HSA. The resonances of compounds **2**, **3**, **5** and **6** can not be assigned in presence of HSA due to the broad lines which result in extensive overlap, but it is clear that most or all of them bind well to albumin. The same is true for **9** and **10** since the resonances are significantly broadened, with one of them having an induced shift of 0.7 ppm.

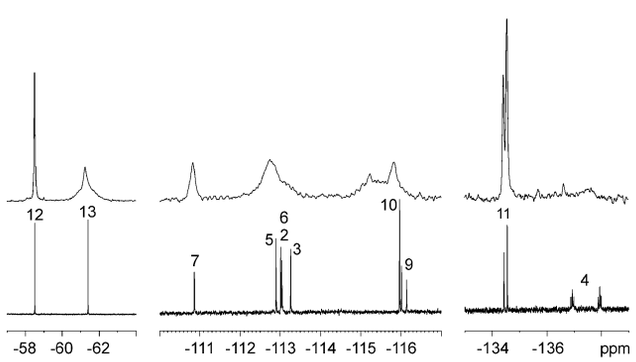

Fig. 7 ^{19}F NMR spectrum of an equimolar mixture of **2**–**7** and **9**–**13** (each at 100 μM) in aqueous solution in the presence of 100 μM HSA (upper spectrum, processed with 10 Hz line broadening). The solution contained 5% DMSO and the spectrum was recorded at pH 6.3 at 25 °C. The lower spectrum was obtained for the ligand mixture in the absence of HSA (*cf*. Fig. 3).

Implications for screening of libraries in medicinal chemistry

In the present study a complete assignment of all resonances in the library was conducted before screening for protein binding. This should not be necessary when the method is applied to screening of larger libraries, because ^{19}F has characteristic

chemical shifts which depend on the chemical environment of the fluorine atom, as has been extensively studied.[39] For instance, compounds with fluorinated phenyl groups typically have chemical shifts between −110 and −130 ppm, depending on other substituents as well as their position. CF_3 groups on aromatic compounds will instead be found at about −60 ppm. Thus, the chemical shift of resonances that are broadened and/or shifted will identify which type of ligand binds to the target. Deconvolution of larger libraries would thus be expected to require screening of smaller libraries from which hits could be identified directly.

The results presented here suggest that ^{19}F NMR spectroscopy should be a valuable addition to existing NMR techniques for screening of chemical libraries in medicinal chemistry projects. This depends on the fact that ^{19}F NMR spectra are, in general, well resolved with little chemical shift overlap since the resonances are distributed over a range of ~200 ppm. Ligands that bind to the target protein can therefore be detected simply by inspection of line broadening and/or induced chemical shifts in a one-dimensional proton-decoupled ^{19}F NMR spectrum. Qualitative information about relative binding strengths and the presence of multiple binding sites is also obtained. Quantitative determination of binding constants can then be determined by other methods based on NMR spectroscopy.[40,41] Since fluorine does not occur naturally in proteins, there are no interfering background resonances, which simplifies spectral interpretation and allows high sensitivity. Screening by ^{19}F NMR spectroscopy, as described here, does depend on access to ^{19}F labelled compounds, but a wide range of fluorinated compounds and building blocks are commercially available making this nucleus suitable as a reporter in screening. For instance, ~12% of the compounds found in the available chemical directory of screening compounds (ACD-SC) contain fluorine. In addition, the fluorine atom is relatively non-sterically demanding and can, in many cases, replace a hydrogen atom in a ligand with minimal influence on the biological response.

Conclusions

The ^{19}F NMR screening technique presented in this paper is simple to carry out since it relies on identification of binding ligands directly from ^{19}F NMR spectra, which are usually well resolved. Members of a library, which bind to a target protein, can be detected simply by inspection of line broadening and/or induced chemical shifts in a single, one-dimensional ^{19}F NMR spectrum. When binding of the members of a small library of 11 fluorinated compounds to the two bacterial chaperones PapD and FimC was investigated, changes in linewidths appeared to be a more sensitive way to detect binding than changes in chemical shifts. Screening of the same library for binding to HSA revealed larger induced chemical shifts for some ligands, which may reflect a different environment for the fluorine nucleus when binding to HSA. The results obtained for binding to PapD using ^{19}F NMR screening, in general, agreed well with independent binding studies performed using surface plasmon resonance. In addition it was found that the titration experiments provided information about the presence of multiple binding sites on PapD. The titration experiments also enabled the evaluation of relative binding strengths for the various compounds. Thus, the methods presented in this work have the potential to screen chemical libraries for compounds that bind to a receptor, as well as for evaluation of relative binding strengths and detecting the presence of multiple binding sites.

Experimental

General

Pyridinones **1**–**7**,[32,33] and amino acid derivatives **8** and **11**,[32,34] were synthesized as described in the cited references. Amino acid derivatives **9** and **10** were prepared using the same route as for **8** and **11**. The chaperones PapD and FimC were a gift from Washington University School of Medicine in St. Louis and human serum albumin (HSA, 97–99%) was purchased from ICN. 2-(Trifluoromethyl)benzoic acid (**12**) and flufenamic acid (**13**) were purchased from Acros Chimica.

Surface plasmon resonance

The binding of the ligands **1**–**7** and **9**–**13** to the chaperone PapD was studied by surface plasmon resonance using a BIACORE 3000 instrument. The chaperone was immobilized on sensor chip CM5 using a standard thiol coupling procedure. This involved activation of the carboxylic acid moieties of the dextrane surface of the sensor chip with *N*-ethyl-*N*-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide, followed by coupling of cystamine in the presence of dithioerythritol. Native carboxyl groups of PapD were modified with 2-(2-pyridinyldithio)ethylamine hydrochloride in the presence of EDC prior to immobilization. Modified PapD (50 μg mL^{-1} in 10 mM NaOAc buffer, pH 5.5) was then reacted with the cystamine derivatized dextrane surface to give immobilization levels of 10 000 RU. Unmodified dextrane in one channel was used as reference surface.

Each of the pilicides **1**–**7**, **9**–**13** were diluted from 2 mM stock solutions to a final concentration of 30 μM in phosphate running buffer (6.7 mM, pH 7.4) supplemented with 5% DMSO and TWEEN-20 (0.01%). The compounds were injected over the sensor chip at a flow rate of 30 μL min^{-1} at 25 °C. The injection was made in triplicate and in random order for all compounds and the binding to immobilized PapD was observed in real time. After injection of each compound the surface of the sensor chip was regenerated by injection of aqueous glycine hydrochloride (10 mM, pH 2.0) followed by washing with a 1 : 1 mixture of DMSO and water.

In order to allow comparison of the binding of the compounds to PapD the response from the BIACORE instrument (determined as response units, RU) was divided with the molecular weight of each ligand to correct for signal enhancement due to differences in molecular size. Normalized responses were then calculated using pilicide **1** as a reference at 100%.

NMR spectroscopy

Samples of PapD, FimC and HSA for NMR spectroscopy were prepared in 40 mM sodium phosphate buffer at pH 6.3 which contained 10% D_2O and 3 mM NaN_3. All samples were prepared with the addition of 5% DMSO-d_6 in order to prevent precipitation of pyridinones **2**–**7**. For the screening experiments equimolar amounts of **2**–**7** and **9**–**13** were dissolved in DMSO-d_6 and this solution was then added to the samples of PapD, FimC or HSA to give a final ligand : protein ratio of 1 : 1 (0.1 mM for all substances). In the titration experiment equimolar amounts of **2**–**7** and **9**–**13** were dissolved in DMSO-d_6 and this solution was then added to a 200 μM sample of PapD. The titration was performed so as to give relative concentrations of 1 : 32, 1 : 16, 2 : 16, 3 : 16, 4 : 16, 6 : 16, 8 : 16, 12 : 16 and 16 : 16 for each ligand relative to PapD, with the PapD concentration being kept constant at 200 μM.

^{19}F NMR experiments were performed on a Bruker DRX spectrometer operating at a fluorine frequency of 375.9 MHz, whereas a Bruker AMX2 spectrometer operating at a proton frequency of 500.13 MHz was used for the ^{1}H NMR experiments. Trifluoroacetic acid was used as an internal ^{19}F reference in all experiments and calibrated to −74.225 ppm using $CFCl_3$ as an external reference at 0 ppm. The chemical shift of DMSO-d_5 at 2.5 ppm was used as a reference in the proton experiments. ^{19}F NMR experiments were conducted with proton decoupling using 1024 scans. An interpulse delay of 1.5 s was used in the ^{19}F NMR experiments. The ^{19}F screening experiment on HSA was acquired using 25000 scans and conducted without proton

decoupling, since decoupling decreased the signal to noise ratio due to NOE effects. All NMR experiments were conducted at 25 °C.

Data were processed on a Silicon Graphics workstation using the XWINNMR software (Bruker). Fluorine NMR experiments were typically processed with 3 Hz line broadening, a Lorentzian lineshape was fitted to each resonance, and the added linewidth was taken into account when measuring the linewidth.

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