# Solvent selection for insoluble ligands, a challenge for biological assay development: a TNF-α/SPD304 study

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**Contents:**



***TNF-α expression host and media***

The TNF-α was expressed in *E. coli* as a GST-fusion protein encoding the extracellular domain of human TNF-α from valine 77 to leucine 233, as previously described (1). *E.coli* BL21(DE3) pLysS was used as expression host. All media were supplemented with 100 μg/mL ampicillin (sodium salt) and 34 μg/mL chloramphenicol.

***Expression and purification of recombinant human TNF-α***

*E. coli* BL21(DE3) pLysS transformed with pGEX/TNF-α was grown to mid log phase ($A_{600nm}$=0.6) in Luria Bertani medium. Expression of TNF-α was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) at 25 $^{o}$C for 3h. Cells were

harvested by centrifugation at 3500x g for 20 min and washed with phosphate buffer saline (PBS) and stored at -80$^{o}$C.

GST-TNF-α was purified using a glutathione agarose column (Macherey-Nagel, Germany) after cells' disruption as previously described (2). Separation of TNF-α from its GST fusion partner was accomplished by proteolytic cleavage with the type-14 human rhinovirus 3C protease (America Pharmacia Biotech) performed at 4 $^{o}$C for 16h (2). Uncleaved fusion protein as well as the GST-tagged protease was removed by passage over a glutathione affinity matrix.

***Solubility screening of SPD304***

A simple method to determine the solubility of SPD304 in aqueous solutions containing low (<10% v/v) concentrations of organic solvents was developed. This method was based on the observation that SPD304 has a strong absorption band at 252nm. Before beginning, it was verified that this compound can form greater than 30 mM solutions in each of the pure solvents tested. Different compounds are likely to have different absorption maxima, but these are readily obtained by UV/visible spectroscopy. As the hydrochloride salt of SPD304 is soluble in dd-H2O, a stock solution (10 mM) was prepared from this form. The solubility of SPD304 is significantly reduced at mild acid to neutral pHs. We diluted SPD304 to a final concentration of 100 μM (estimated according to weight) either in pure solvent (solvent solutions) or in 10 mM citrate-phosphate buffer (pH 6.5) containing 2.5%, 5% or 10 % of each solvent (aqueous solutions). Samples remained under continuous agitation for 12-16 h at 25 $^{o}$C in the dark; thereafter the soluble fraction was recovered by centrifugation at 15,000 x g for 30 min at room temperature. In each case precipitated ligand was re-suspended in a fresh sample of the corresponding pure

solvent in order to quantify the insoluble material. It should be noted that maximum concentration of SPD304 in pure solvent was 100μM. As linearity between $A_{252nm}$ and SPD304 concentration (in both pure solvent and aqueous solutions) was observed (data not shown), solubility of SPD304 (in aqueous solutions) can be easily determined as follows:

$$\frac{A_{252nm} \text{ of SPD304 in pure solvent}}{100\,\mu M} = \frac{A_{252nm} \text{ of SPD304 in aqueous solution}}{\text{aqueous (unknown) solubility } (\mu M)}$$

Rearrangement of the terms of the above equations results in the following equation:

$$\text{aqueous solubility } (\mu M) = \frac{A_{252nm} \text{of SPD304 in aqueous solution}}{A_{252nm} \text{of SPD304 in solvent solution}} \times 100(\mu M) \quad \text{(S.1)}$$

All measurements were corrected using the appropriate blank sample (buffer without SPD304).

In addition, aqueous solubility of a small molecule can be determined by measuring the amount precipitates (after ligand's suspension in aqueous solution and centrifugation in order to separate soluble from insoluble material). Consequently, by measuring the amount of precipitated ligand we can (i) check whether our determinations are correct (the sum of the soluble and insoluble material should be equal to the initial amount of ligand as estimated according to weight) (ii) verify the amount of ligand remained into aqueous solution. This approach becomes very useful, in cases of extreme insoluble compounds or compounds with very low extinction coefficient. In those cases UV absorbance of aqueous solutions would be near to spectrophotometer limits. For example 1 mg of a compound is dissolved in a total volume of 1 mL of aqueous solution. Assuming that 0.02 mg are soluble (but not

detectable) when precipitate is re-suspended in equal volume of pure organic solvent, 0.98 mg of this compound must be detected.

In the case of SPD304, aqueous solubility can be determined taking as follows:

The sum of the soluble and insoluble material should be equal to the initial amount of ligand (estimated according to weight). Thus:

$$SPD304_{total}=SPD304_{aqueous}+SPD304_{prec} \text{ or}$$

$$SPD304_{aqueous}=SPD304_{total}-SPD304_{prec} \qquad (S.2)$$

where:

$SPD304_{total}$: is the total amount of SPD304 (μM)

$SPD304_{prec}$: is the precipitated amount of SPD304 (μM)

$SPD304_{aqueous}$: is the soluble amount of SPD304 (μM)

As 100 μM of SPD304 were initially dissolved, Eq. (S.2) becomes:

$$SPD304_{aqueous}=100\,(\mu M) - SPD304_{prec} \qquad (S.3)$$

Assuming that:

$$\frac{A_{252nm} \text{ of } SPD304_{total} \text{ in pure solvent}}{100\,\mu M}=\frac{A_{252nm} \text{ of } SPD304_{prec} \text{ in pure solvent}}{SPD304_{prec}\,(\mu M)}$$

then,

$$SPD304_{prec}\,(\mu M)=\frac{A_{252nm} \text{ of } SPD304_{prec} \text{ in pure solvent}}{A_{252nm} \text{ of } SPD304_{total} \text{ in pure solvent}}\times 100\,(\mu M)$$

and Eq. (S.3) becomes:

$$SPD304_{aqueous}\,(\mu M)=100\left(1-\frac{A_{252nm} \text{ of } SPD304_{prec} \text{ in pure solvent}}{A_{252nm} \text{ of } SPD304_{total} \text{ in pure solvent}}\right)(\mu M) \quad (S.4)$$

Therefore, aqueous solubility of SPD304 can also be determined by measuring the $A_{252nm}$ of the insoluble amount of ligand using Eq. (S.4)

It should be noted that all measurements were corrected using the appropriate blank sample (buffer without SPD304).

***Stability of TNF-α in the presence of various solvent***

The effect of various solvents' concentration on TNF-α stability was examined by monitoring both the changes of fluorescence emission intensity values and the changes of absorbance at 280nm ($A_{280nm}$) of the protein. For this, TNF-α was incubated (at 4 $^{o}$C) in 10 mM citrate-phosphate buffer containing 1.25, 2.5, 5, 7.5 or 10%, v/v of one of the following solvents: DMSO; DMF; glycerol, methanol, PEG3350 and PEG5000. The pH in all cases was adjusted to 6.5. All sample solutions were prepared 12-16 h prior to taking measurements. Samples were centrifuged at 15000 x g for 30 min in order to remove precipitated protein, and the fluorescence emission intensity (at 304 nm) of the resulting supernatants was measured.

Data obtained in each case were compared with a protein solution containing only buffer while both the fluorescence intensity and $A_{280nm}$ values were corrected using the corresponding blank solution. The effect of solvent concentration on the Tyr fluorescence emission intensity was also studied.

***Fluorescence binding assay***

Fluorescence intensity was measured with a Hitachi F-2500 fluorescence spectrophotometer in 1.0 x 4.5 cm quartz cuvettes at 25 $^{o}$C. The excitation and emission wavelengths were 274 nm and 304 nm respectively. The slits were set at 5 and 20 nm in the excitation and emission respectively. The following procedure was used for titration of TNF-α with SPD304: 1.5 mL of protein solution (0.5 μM) was placed in a cuvette and equilibrated at 25 $^{o}$C for 1h. After equilibration, small

increments (2 to 7.5 μL) of the ligand solution were injected in the cuvette. The experiments were performed in 10 mM citrate-phosphate pH 6.5 containing 5% DMSO (or a different concentration of various solvents as indicated in the text). In order to determine dilution effect of TNF-α (due to ligand addition) and any fluorescence effect by unbound ligand, a blank sample containing Tyr with the same fluorescence signal, was titrated with ligand additions as described above. The sample absorbance was kept below 0.1 to minimize the inner filter effect (3).

Alternatively, binding of SPD304 to TNF-α using fluorescence spectroscopy was studied by preparing a series of solutions (0.5 mL) in separate test tubes (eppendorfs), containing 0.5 μM of TNF-α and increasing amounts of the ligand. During preparation of the solutions the buffer containing the appropriate concentration of each solvent, was first added to all tubes followed by the SPD304 solution and finally the protein was added. All solutions were mixed by pipetting and equilibrated for 1h before measurement. All measurements were corrected using a Tyr blank as above.

***Determination of dissociation constant ($K_d$) from fluorescence measurements***

The dissociation constant ($K_d$) is an indicator of binding strength between two molecules. For the reaction:

$$P + L \leftrightarrow PL$$

$K_d$ is expressed by the equation:

$$K_d = \frac{[P][L]}{[PL]} \quad \text{(S.5)}$$

where [P] is the concentration of free Protein, [L] is the concentration of free ligand and [PL] is the Ligand-bound-Protein.

Assuming that $P = P_F$ and $L = L_F$ then:

$$K_d = \frac{[P_F][L_F]}{[PL]}$$

Rewriting the above equation in terms of total ligand ($L_T$) and Protein concentrations ($P_T$) and applying the conservation of mass assumption $[L_F] = [L_T] - [PL]$ and $[P_F] = [P_T] - [PL]$, gives:

$$K_d = \frac{([P_T] - [PL])([L_T] - [PL])}{[PL]} \qquad \text{(S.6)}$$

Rearrangement of the terms of eq. (S.4) as follows:

$$K_d[PL] = ([P_T] - [PL])([L_T] - [PL])$$

$$K_d[PL] = [P_T][L_T] - [L_T][PL] - [P_T][PL] + ([PL])^2$$

$$0 = ([PL])^2 - [L_T][PL] - [P_T][PL] - K_d[PL] + [P_T][L_T]$$

$$0 = ([PL])^2 - ([L_T] + [P_T] + K_d])[PL] + [P_T][L_T]$$

Leads to the following equation:

$$[PL] = \frac{([L_T] + [P_T] + K_d]) \pm \sqrt{([L_T] + [P_T] + K_d])^2 - 4[P_L][L_T]}}{2} \qquad \text{(S.7)}$$

It should be noted that:

$$[L_F] = [L_T] - [PL] \qquad \text{(S.8)}$$

According to Bujalowski and Lohman (4) the observed fluorescence intensity can be expressed by the equation:

$$F_{obs} = MF_{P_F} P_F + \sum MF_i P_i \qquad \text{(S.9)}$$

where:

$MF_{P_F}$ and $P_F$ are the molar fluorescence and concentration of free protein, respectively, and

$MF_i$ and $Pi$ are the molar fluorescence and concentration of the protein bound in state "i", respectively.

The molar fluorescence of free protein is estimated as the slope of a plot of fluorescence intensity versus protein concentration (dilution of protein) measured in the absence of ligand. Note that the molar fluorescence is dependent on the experimental conditions (e.g buffer composition, pH, temperature, etc). Therefore, it is essential that the value of molar fluorescence should be determined under exactly the same conditions (and in the same experiment) as the binding reaction. Thus in each case for each solution used, a different $MF_{PF}$ was determined.

Note, too, that any background signal $F_{BG}$ (fluorescence or scattered light and/or background fluorescence in the absence of protein) from the buffer should be subtracted (5). Assuming that $MF_i = FR \times MF_{P_F}$, where FR is the fluorescence ratio of bound protein, Eq. (S.9) becomes:

$$F_{obs} = F_{BG} + MF_{P_F}[P_F] + FR \cdot MF_{P_F} \cdot [PL] \qquad \text{(S.10)}$$

The dissociation constant can then be calculated by fitting the data in Eqs. (S.7), (S.8) and (S.10) as illustrated in Table S.1. Data were analysed using Prism V.5 (GraphPadSoftware, San Diego, CA).

**Table S.1.** Equation model for the determination of dissociation constant ($K_d$)

| Parameter | Equation |
|---|---|
| Bound ligand | $[PL] = \frac{([L_T] + [P_T] + K_d]) \pm \sqrt{([L_T] + [P_T] + K_d])^2 - 4[P_L][L_T]}}{2}$ |
| Free ligand | $[L_F] = [L_T] - [PL]$ |
| Observed Fluorescence | $F_{obs} = F_{BG} + MF_{P_F}[P_F] + FR \cdot MF_{P_F} \cdot [PL]$ |
| **Constants:** | **Parameters to fit:** |
| $P_T$: Total protein concentration | $K_d$: Dissociation constant |
| $F_{BG}$: Background fluorescence of free protein | *FR:* fluorescence ratio |
| $MF_{P_F}$ : Molar fluorescence of free protein | *FR >0 binding increases fluorescence* |
| | *FR<0 binding quenches fluorescence* |

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