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Macrocyclic mimetics of the Suppressor of Cytokine Signaling 1 and 3 proteins (SOCS1-SOCS3)

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BACKGROUND and AIM

In numerous inflammatory, immune, and oncogenic processes, the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is selectively upregulated in response to cytokine stimulation. Of particular interest are the endogenous proteins that modulate this pathway: the suppressors of cytokine signaling (SOCS) proteins, which operate via a negative feedback mechanism [1] . Notably, SOCS1 and SOCS3 are downregulated in chronic inflammatory diseases and cancer [2] . In this study, aiming to develop SOCS1 and SOCS3 mimetics, we designed and synthesized cyclic analogues using the CLIPS strategy. This method involves the formation of non-native bonds between the thiol side chains of cysteines and xylene-based scaffolds [3].

METHODS

Peptides were prepared by the Solid Phase Peptide Synthesis (SPPS) on a 25μmol scale following the fluorenylmethoxycarbonyl (Fmoc) strategy. Cyclization was performed by mixing crude linear peptidomimetics (1mM) with 1.1 equivalents of xylene scaffolds in 50% CH_3CN and 50% K_2HPO_4 buffer 0.1M pH 7.6, under stirring overnight at room temperature (RT). The purity and identity of the peptides were confirmed by LC-MS. The cyclic peptides were then characterized by Circular Dichroism (CD) and Nuclear Magnetic Resonance (NMR) spectroscopies. In addition, Micro Scale Thermophoresis (MST) experiments, serum stability studies and phosphorylation inhibition assays were performed.

DESING OF CYCLIC ANALOGUES OF SOCS1 AND SOCS3 PROTEINS

For the design of these new xylene-based cyclic analogues of SOCS1, two different scaffolds were used: α,α'-dibromo-m-xylene and 1,3,5-tribromomethylbenzene to provide a monocyclic and a bicyclic version of previously identified lead compounds, in this case icPS5(Nal1) (**Figure 1A**). In the case of SOCS3, KIRCONG chim was used as a template and reacted with α,α'-dibromo-m-xylene to form partially flexible cycles by cyclizing only the N-terminal (KIR region) or C-terminal (CONG region), allowing the development of monoright and monoleft cycles, respectively. As these new cycles are highly insoluble, one or two PEG were inserted sequentially to give monoright PEG_1 and PEG_2 and monoleft PEG_1 and PEG_2 (**Figure 1B**).

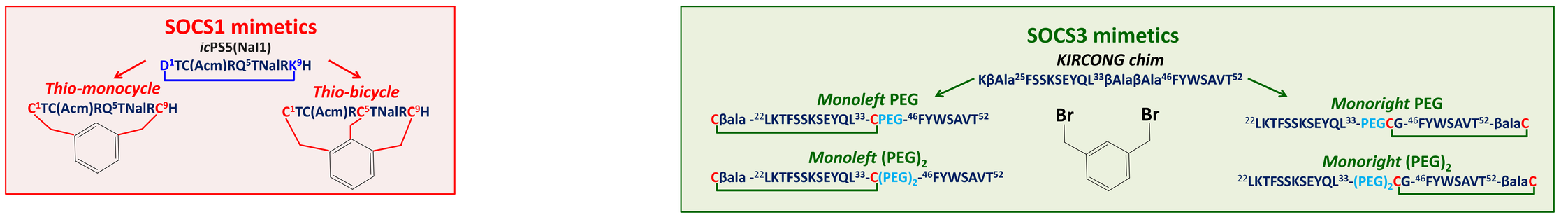

Figure 1. Template sequence (**A**) (*ic*PS5(Nal1)) for the design of thio-analogues of SOCS1 protein (**B**) (KIRCONG chim) for the design of monocyclic mimetics of SOCS3 protein.

RESULTS OF THIO-ANALOGUES OF SOCS1 PROTEIN

Binding Assay-MicroScale Thermophoresis (MST)

Fitting the experimental data for the thio-monocycle PS5(Nal1) (**Figure 2A**) resulted in a very good K_D in the low Micromolar range (~7μM), even 5-fold lower than the *ic*PS5(Nal1) template (K_D ~ 36μM), whereas the thio bicycle PS5(Nal1) has a high K_D value of 130 μM and does not reach full saturation (**Figure 2B**). It is hypothesized that the introduction of a bicyclic scaffold reduces the ability to recognize JAK2 due to partial masking of the interaction hotspots [4] .

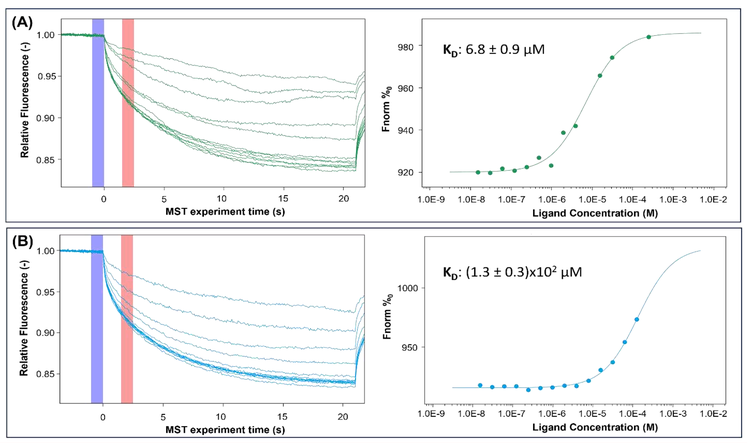

Figure 2. In vitro binding assays. Binding isotherms for MST signals versus peptides concentrations (left) and thermophoretic traces of MST assays for the binding to JAK2 (right) of peptide (**A**) thio-monocycle PS5(Nal1) and (**B**) thio-bicycle PS5(Nal1).

Inhibition of Phosphorylation Assay

The SOCS1 mimetic activity of two thio-analogues was studied using phosphorylation inhibition assays on a substrate called Srctide. LC-MS experiments measured the decrease in p-Srctide peak area with increasing amounts of thio-analogues. The IC_{50} for the thio-monocycle was found to be 2.2 ± 0.3 μM, and for the thio-bicycle, it was 1.0 ± 0.5 x 102 μM (**Figure 3**) [4] .

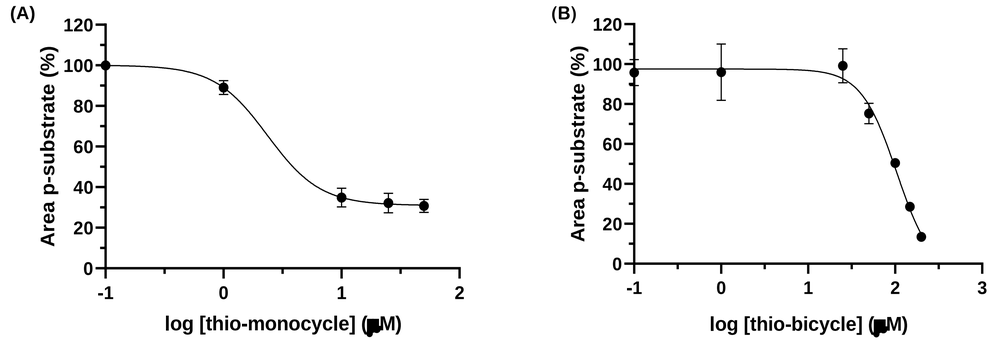

Figure 3. The SOCS1 mimetic activity of thio-analogues PS5(Nal1) was measured by analyzing the percentage area of phosphorylated Srctide (p-Srctide) at different concentrations for both (**A**)thio-monocycle and (**B**)thio-bicycle forms. The data, averaged from two experiments and expressed in logarithmic form, use 100% as the p-Srctide area without the inhibitor.

Circular Dichroism

The thio-monocycle PS5(Nal1) lacks an aromatic band and shows two minima at 224 nm and 203 nm, which shift to 218 nm and 197 nm with 50% TFE, indicating no helical structure (**Figure 4 A**). The thio-bicycle has a minimum below 200 nm and a band at 230 nm, indicating an aromatic contribution enhanced by the naphthyl group of Nal1 (**Figure 4 B**). The addition of 50% TFE does not significantly alter the CD profiles or increase the secondary structure content [4] .

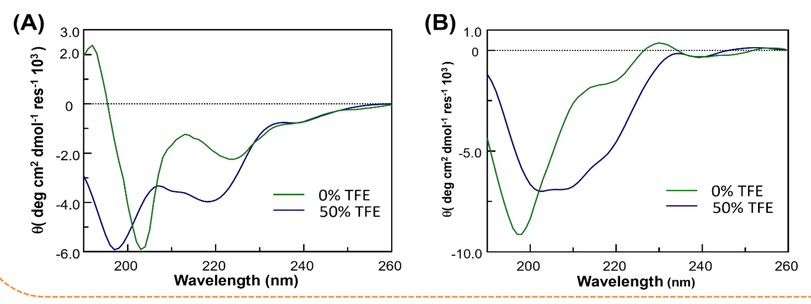

Figure 4. Overlay of CD spectra of (**A**) thio-monocycle PS5(Nal1); (**B**) thio-bicycle PS5(Nal1) compounds.

Serum stability

Serum stability assays showed that the new thio-cyclic analogues of icPS5(Nal1) degraded faster over time compared to icPS5(Nal1) (**Figure 5**). Nevertheless, both analogues retained 25% of their presence after 30 Hours [4] .

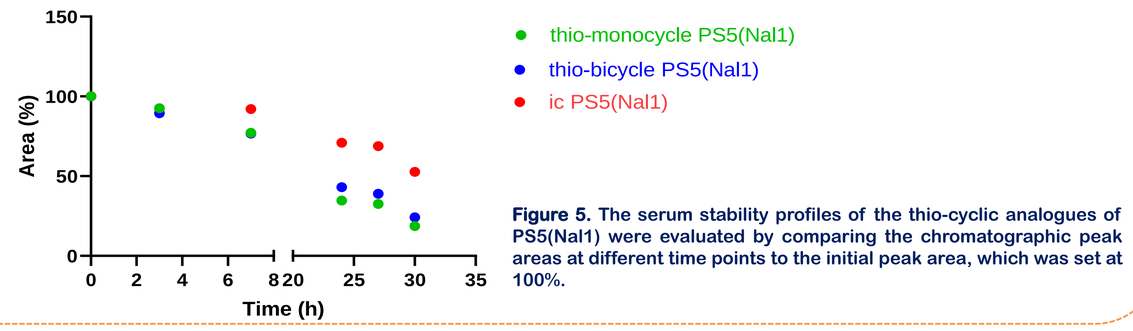

Figure 5. The serum stability profiles of the thio-cyclic analogues of PS5(Nal1) were evaluated by comparing the chromatographic peak areas at different time points to the initial peak area, which was set at 100%.

RESULTS OF MACROCYCLIC MIMETICS OF SOCS3 PROTEIN

ONGOING studies

The main problem with these mimetics is their poor solubility, which has made purification difficult and led to low synthetic yields. Of the MST assays (**Table 1**), only *Monoright* PEG_1 gave a good K_D (**Figure 6**), while the other compounds showed no binding, and even *Monoright* PEG_2 showed aggregation (**Figure 7**) . In view of the above issues, macro-cycle characterization studies are still ongoing.

Table 1. Results of MST binding assays of macrocyclic mimetics of SOCS3 protein

PEPTIDE	BINDING
Monoleft PEG	No binding
Monoleft $(PEG)_2$	No saturation
Monoright PEG	K_D= (5±2)*10 μM
Monoright $(PEG)_2$	Insoluble

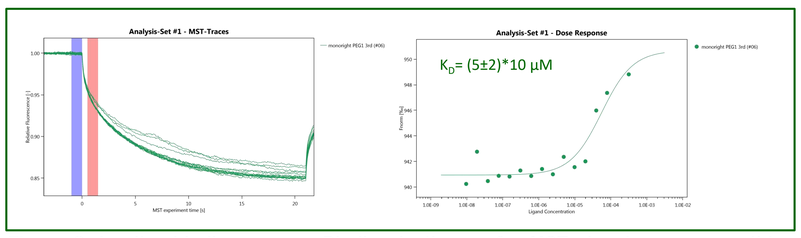

Figure 6. In vitro binding assays. Binding isotherms for MST signals versus peptides concentrations (left) and thermophoretic traces of MST assays for the binding to JAK2 (right) of peptide *Monoright* PEG_1 .

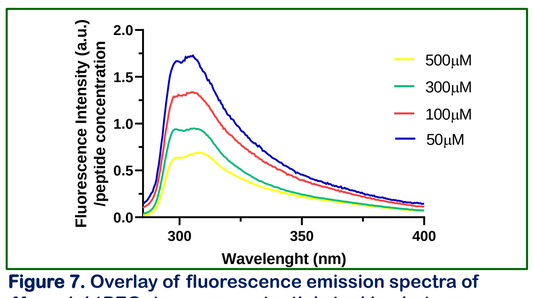

Figure 7. Overlay of fluorescence emission spectra of *Monoright* PEG_2 to assess potential stacking between monomers.

CONCLUSION

In summary, with respect to SOCS1 protein mimetics, the results demonstrated that the *ic*PS5 peptide is a bioactive sequence, making it highly adaptable and ideal for the chemical modifications necessary to convert KIR-SOCS1 peptidomimetics into specific JAK2 inhibitors. For SOCS3 mimetics, synthetic difficulties and poor solubility have slowed down studies, but MST suggest that cyclization at the CONG end is more favorable for interaction with JAK2.

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