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Dynamic Combinatorial Self-Assembly of Cyclophilin hCyp-18 Ligands through Oxorhenium Coordination

Cécile Clavaud,[a, b] Julien Le Gal,[a, c] Robert Thai,[a] Mireille Moutiez,[a] and Christophe Dugave*[a]

*The dynamic combinatorial assembly of independent modules **A** and **B** through oxorhenium(V) coordination by a NS_2+S motif in the presence of cyclophilin hCyp-18—an important peptidyl-prolyl isomerase—was investigated. Increasing glutathione (GSH) concentrations were used to dissociate [**A**·$Re^{V}O$·**B**] complexes that displayed low affinity for hCyp-18. Conversely, coordinates that displayed submicromolar affinities for hCyp-18 were protected against thiol exchange and could be detected by LC-MS. Determination of the GSH concentration that decreased the extracted ionic current of the complex by 50% (CC_{50}) enabled the selection of three oxorhenium coordinates that were shown to bind to the active site of hCyp-18 and to inhibit its peptidyl–prolyl isomerase activity in the micromolar to submicromolar range.*

Introduction

We recently reported the synthesis of libraries of rhenium complexes based on coordination of two sets of independent modules.[1] For this purpose, *n* modules **A** that were linked to a recurrent $N(CH_2–CH_2S)_2$ motif (called NS_2), and *m* modules **B** that had a thiol moiety, assembled unambiguously after disulfide reduction through chelation with a ReO^{3+} core to give all combinations that corresponded to $n\times m$ complexes [Equation (1)]. Screening of the libraries of complexes for specific ligands for cyclophilin hCyp-18, an important human peptidyl–prolyl isomerase,[2] selected two oxorhenium coordinates that bound cyclophilin with affinities more than one order of magnitude better than those of model peptide substrates.[1] In our model, a specific interaction of the oxorhenium coordinates with the protein was anticipated to involve the two subsites of hCyp-18: the S1–S1′ subsite was expected to bind motif **A**, which contains a proline analogue, whereas subsite S2′–S3′ was assumed to recognize the **B** moiety, which contains an amino acyl-(*p*-nitroanilide) surrogate[3,4] (Scheme 1).

$$\mathbf{A}-N(SH)_2 + \mathbf{B}-SH + ReO^{3+}\cdot 3\,L^- \rightarrow [\mathbf{A}-NS_2\cdot ReO\cdot S-\mathbf{B}] + 3\,L - H \quad (1)$$

$$[\mathbf{A}-NS_2\cdot ReO\cdot S-\mathbf{B}] + GSH \rightleftharpoons [\mathbf{A}-NS_2\cdot ReO\cdot S-G] + \mathbf{B}-H \quad (2)$$

Assembly of the modules was carried out either in organic solvents in the presence of commercially available oxorhenium salts[1,4] or in buffers by transchelation of oxorhenium gluconate,[3,4] and takes place in the presence of hCyp-18 in aqueous solutions. As anticipated, bimolecular complexes are sensitive to glutathione (GSH) and other thiols, which reversibly substitute the **B** moiety.[4,5]

It would therefore be anticipated that all complexes should dissociate readily through GSH-mediated substitution [Eq. (2)],[4,6] whereas complexes that interact with the protein (i.e., hCyp-18) should be protected against thiol exchange. A thermodynamic step is thus combined with the kinetically controlled coordination process[5] in order to introduce partial reversibility and, therefore, to select high-affinity cyclophilin ligands. In addition, the reversible substitution of the **B** moiety by GSH might lead to amplification of the best cyclophilin ligands to the detriment of rhenium complexes that do not bind to hCyp-18.

Here we report the dynamic selection of new rhenium coordinates that bind specifically to hCyp-18 under GSH-mediated discrimination conditions (reaction under thermodynamic control), their identification from dynamic combinatorial libraries (DCLs)[7] by LC-MS,[8] and their biochemical characterization.

Results and Discussion

Validation of the strategy

We first tested our strategy on a limited library of sixteen known compounds. Modules **A1**–**4** were combined with modules **Ba** and **Bi** to give the corresponding complexes (Figure 1). These bound to cyclophilin in the submillimolar range, except for complex **4i**, which displayed an apparent K_d of 11 ± 2 μM.

[a] *C. Clavaud, Dr. J. Le Gal, Dr. R. Thai, Dr. M. Moutiez, Dr. C. Dugave*
CEA, Institut de Biologie et de Technologie de Saclay (iBiTecS)
Service d'Ingénierie Moléculaire des Protéines (SIMOPRO)
Bâtiment 152, 91191 Gif-sur-Yvette (France)
Fax: (+33) 1-6908-9071
E-mail: christophe.dugave@cea.fr

[b] *C. Clavaud*
Present address: Unité des Aspergillus, Institut Pasteur
25 rue du Dr. Roux, 75015 Paris (France)

[c] *Dr. J. Le Gal*
Present address: CHELATEC, Institut de Biologie
9 quai Moncousu, 44093 Nantes Cedex (France)

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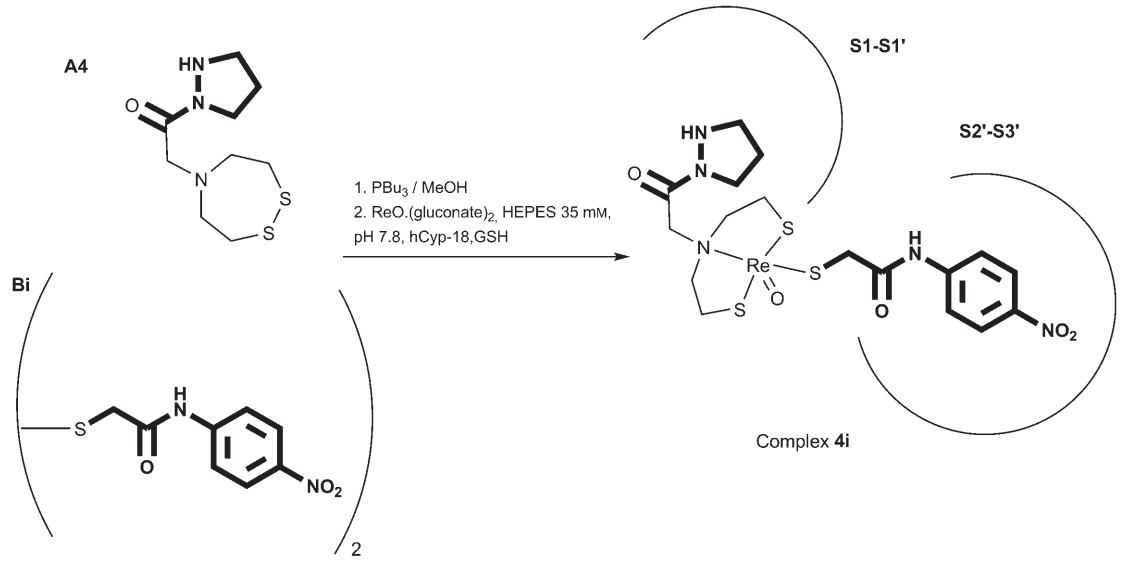

Scheme 1. Synthesis of complex **4i** in a buffer. The putative interaction with the two subsites of hCyp-18 is shown.

Conversely, most of the complexes in series **Bl** and **Bo** were inactive.[1]

Self-assembly of the complexes in the presence of oxorhenium gluconate, after reduction of the corresponding mixture of modules with tributylphosphine in methanol, gave all sixteen complexes. The UV chromatograms could not be efficiently exploited because of the multiplicity of compounds (i.e., starting components and their oxidized forms, as well as GSH adducts). We preferred to monitor the evolution of the mixture by LC-MS (ES/MS in the positive mode). The expected rhenium coordinates were identified by extracting the characteristic isotopic $^{32-34}S/^{185-187}Re$ motif (Figure 2).[1,4] Selective integration of the corresponding extracted ionic current peaks reflected the yields of complexation and the ability of rhenium complexes to be ionized by the electrospray ionization technique in the positive mode. We checked that the spectrometer response was linear in the 10^{-8} to 10^{-4} M range (concentration in oxidized modules) without significant "memory effect".[10] As expected, all sixteen complexes appeared as mixtures of two diastereomers due to *syn*/*anti* isomerism (Figure 2).[1,4,11]

To overcome the problems resulting from variability in complexation yields, intrinsic stability of complexes to GSH and buffer, and unpredictable ionization capacity, the intensity of the ionic current for each complex was monitored with increasing GSH concentrations (from 10^{-6} to 10^{-2} M) and the results were standardized by applying a correction as follows:

$$CC_{50} = C_{50} \times (IIC_{reference}/IIC_{complex})$$

where CC_{50} and C_{50} are the corrected GSH concentration and the experimentally observed GSH concentration necessary to decrease the observed ionic current by 50%, respectively. IIC is the experimentally measured integrated ionic current of the pair of diastereomers. Complex **4i** was chosen as a reference.[1]

We ensured that addition of rhenium gluconate did not affect cyclophilin: incubation of hCyp-18 (64 μM) with [ReO·gluconate$_2$] (5 mM) did not significantly inhibit the PPIase activity of the protein.

The synthesis of the 16-component library was carried out by reduction and incubation of modules **A1**–**A4** (1.0 equiv) on the one hand, and **Ba**, **Bi**, **Bl**, and **Bo** (0.5 equiv in dimers) on the other hand with oxorhenium gluconate at 20 °C. Complexation yields varied only slightly upon addition of recombinant hCyp-18 (final concentration: 64 μM),[9] except in the case of complex **4i**, which was formed more efficiently when cyclophilin was added to the mixture (Figure 2A, graphs a and b), an effect that we called the "cyclophilin-enhancing effect". As expected, all complexes readily dissociated upon addition of GSH in the absence of hCyp-18 (Figures 2B and D, graph d). Conversely, complexes that displayed an affinity for cyclophilin also showed resistance to higher GSH concentrations ("cyclophilin-protecting effect"), and their apparent resistance was related to their affinity for the protein. In particular, compound **4i** (K_d = 11 μM) displayed a CC_{50} value of 200 μM, whereas complex **2o** (no affinity) was not protected by cyclophilin, with a CC_{50} value below 1 μM (Figures 3B and D, graph c).

The positive effects of hCyp-18 both on the formation of a complex and on its resistance to GSH strongly suggest that the oxorhenium coordinate **4i** specifically interacted with the protein. In contrast, heat-denatured hCyp-18 and bovin serum albumin had no protective effect towards GSH and were not able to facilitate the formation of complex **4i**. Active hCyp-18 inhibited with cyclosporine (10 μM), a potent inhibitor of PPIase activity,[2] was also unable to protect complex **4i** against GSH substitution. This result is likely to reflect competition between cyclosporine and **4i**, and strongly points to a specific interaction of the coordinate inside the active site of the protein. In turn, there is no experimental evidence that the PPIase activity is implicated in the selection process.

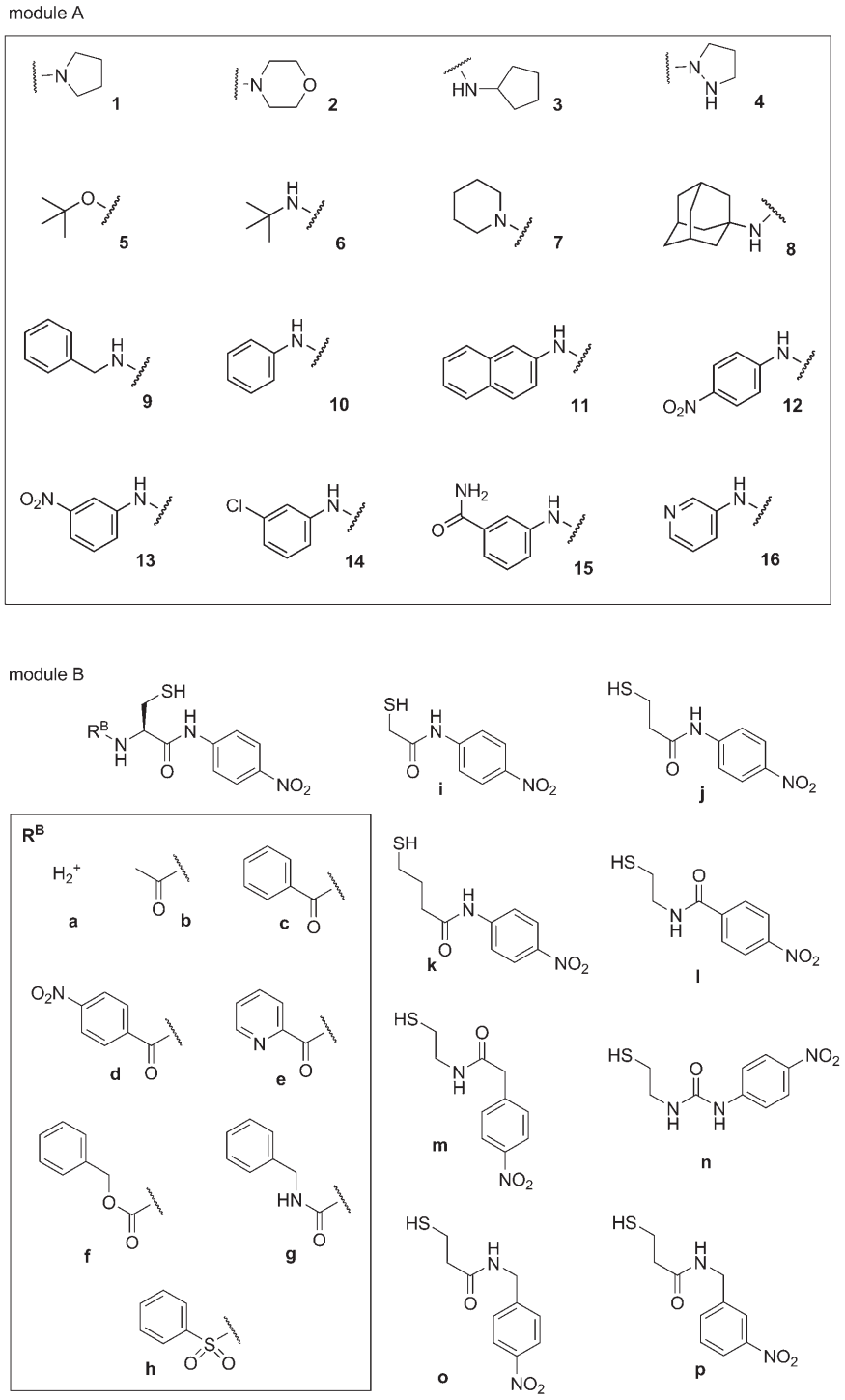

Figure 1. Proline analogues and Cys-*p*NA surrogates selected for the synthesis of DCLs.

Positive results were confirmed with binding experiments and enzyme kinetic assays.[3] The specific binding of compound **4i** to hCyp-18 shown by fluorescence titration ($K_d = 11$ μM) was confirmed by monitoring the PPIase activity of hCyp-18 with increasing concentrations of oxorhenium complex. Thus, an IC_{50} of 12 μM was calculated from the trypsin-coupled PPIase assay (Table 1).[12]

Application to the selection of new inhibitors of hCyp-18

In a second step, we investigated cyclophilin-directed selection within a series of 192 possible oxorhenium coordinates. Analysis of the complex mixtures was difficult, due to large differences in complexation yields in the buffer, nonequivalent abilities of coordinates to ionize by ESI-MS in the positive mode, and formation of numerous isomassic species. Therefore, the library was divided into sixteen parallel DCLs. Each DCL was obtained by combination of twelve **A** modules (compounds **5**–**16**) with one of the sixteen **B** modules with increasing GSH concentrations (10^{-5} to 10^{-1} M). LC-MS analysis of the resulting mixtures showed that compounds **5b**, **9b**, and **16n** displayed higher resistance to GSH ($CC_{50} \geq 1$ mM) in the presence of hCyp-18, and so these were selected as potential cyclophilin ligands (Figure 3).

In these experiments, four complexes were not observed by LC-MS and probably did not assemble properly. This might be the consequence of the fast reoxidation of modules, a reaction that competes with putative slow coordination in the buffer, though all reactions were carried out in thoroughly degassed solvents.

The marked "cyclophilin-enhancing effect" reported with compound **4i** in the absence of GSH was also observed with complex **16n** and suggests that assembly of these compounds was assisted by the protein and is likely to take place at the active site of cyclophilin (in situ coordination chemistry). Although cyclophilin assistance in the assembly of complexes **4i** and **16n** could explain these results, a hCyp-18-related protecting effect towards spontaneous dissociation of the complex cannot be ruled out. Additional experiments are needed to decide this point. Curiously, the expected "cyclophilin-enhancing effect" was not observed with all selected complexes (in particular complex **5b**), suggesting

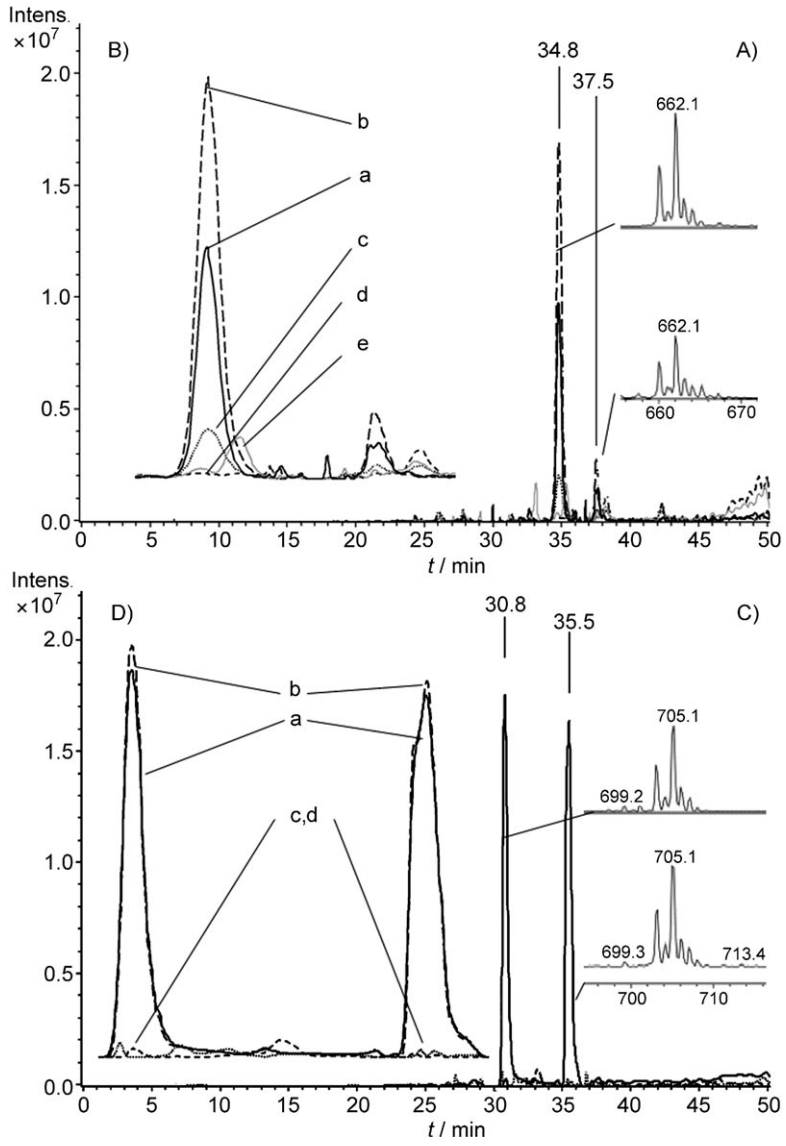

Figure 2. Extracted ionic current (LC-MS) for *m/z* 662 (complex **4i**, A and offset B) and *m/z* 705 (complex **2o**, C and offset D); a: no effector (——); b: with hCyp-18 (-----); c: with GSH and hCyp-18 (····); d: with GSH only (- - - -); e: with GSH, hCyp18, and cyclosporine (····).

that the "enhancing effect" and the "protecting effect" are independent processes.

Complexes **5b**, **9b**, **10i**, and **16n** were synthesized in methanol in milligram amounts, with use of commercial tetrabutylammonium tetrachlorooxorhenate.[1,4] The obtained diastereomers were not separated and were tested simultaneously, due to slow *syn–anti* interconversion in the buffer.[11] As expected, fluorescence titration experiments[3] showed that complex **5b** binds to cyclophilin in the micromolar range. Complexes **9b** and **16n** displayed submicromolar affinities and interesting IC_{50} values (Table 1) similar to those of previously reported inhibitors of hCyp-18.[2,13]

Conclusions

We describe a simple and straightforward method for the identification of new protein ligands assembled through oxorhenium coordination. The development of the 192-component DCL in the presence of hCyp-18 and GSH clearly favored the selection of some compounds with micro- to submicromolar affinities for the protein. Studies to determine the exact mode of interaction between the oxorhenium complexes and hCyp-18—in particular a possible preference for a given diastereomer—are underway. The possible coexistence of two selection pathways—that is, "protecting effect" and "enhancing effect"—will also be investigated (Figure 4).

Although sensitivity to GSH and other endogenous thiols might be a major drawback to the biological use of such compounds to target intracellular proteins, this strategy allows rapid identification of nonpeptide motifs that can interact with protein subsites and might be used as starting building blocks for the design of nonrhenium inhibitors of hCyp-18. The utilization of presynthesized modules that can be stored and reused in other combinations is simple and attractive. Although they are not commercially available, modules **A** can easily be obtained by standard peptide coupling between a set of amines and the NS_2 motif, which is readily available at preparative scales.[4] The strategy might be applied to other proteins as well, in particular surface proteins, provided that they tolerate addition of GSH and are not sensitive to oxorhenium chelates. Moreover, the ReO^{3+} core might be replaced with $^{99m}TcO^{3+}$, a radioisotope commonly used for molecular imaging.[14]

Experimental Section

Preparation of aqueous [ReO·gluconate$_2$] (50 mM): A solution of $SnCl_2$ (63 mg, 1.05 equiv) in HCl (0.05 M, 1.5 mL) was added under argon in portions of 50 μL to a suspension of sodium of $NaReO_4$ (92 mg, 318 μmol) and sodium gluconate (503 mg, 8.8 equiv) in water (4.8 mL), and the solution was stirred for 1–2 h at room tem-

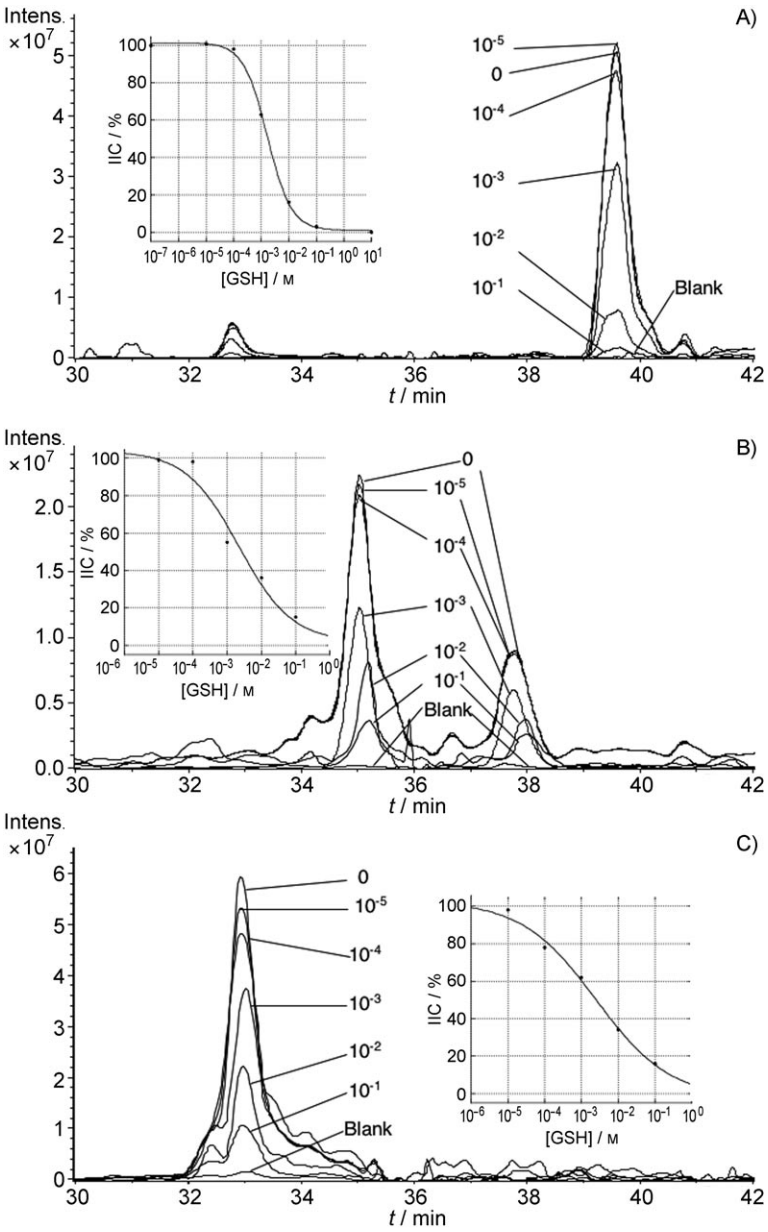

Figure 3. Effect of increasing concentrations in GSH on the extracted ionic current (LC-MS) for A) *m/z* 735 (complex **5b**), B) *m/z* 768 (complex **9b**), and C) *m/z* 713 (complex **16n**).

perature until the color had turned dark blue. The solution can be used during the following 2 h, except if a black precipitate appears.

Complexation in DCLs (96 compounds): For each set of twelve complexes, the modules (**A**, 12× 1 μmol and **B**, 6 μmol) were mixed together, and were then dissolved in methanol (800 μL) and reduced with a methanolic solution of PBu_3 (1 M, 20 μL, 1.1 equiv). The solution was then split into eight portions (100 μL), which were added to pure **B** and stirred until complete dissolution of the solid and then for 1 h at room temperature under argon. Series of eight tubes containing increasing concentrations in GSH (from 10^{-4} to 10^{-2} M for the 16-component DCL and from 10^{-5} to 10^{-1} M for the 12-component DCLs) were incubated at 20 °C for 24 h. A typical experimental mixture contained: reduced **A**+**B** (15 μL), [ReO·gluconate$_2$] (40 μL), hCyp-18 (512 μM, 50 μL), GSH (20 μL), and HEPES (pH 7.8, 35 mM, to a final volume of 400 μL). The results were compared with references (ref. [1]: no GSH, no hCyp-18; ref. [2]: no GSH, 64 μM hCyp-18). The reaction was quenched by sudden addition of methanol (400 μL) and freezing.

LC-MS analysis: The LC-MS system used for the assays comprised an Agilent 1100 Series LC system (Santa Clara, CA) coupled on-line to an Esquire HCT ion trap mass spectrometer equipped with an orthogonal Atmospheric Pressure Interface-ElectroSpray Ionization (AP-ESI) source (Bruker Daltonik, GmbH, Germany). LC separation was carried out on an analytical octadecyl column (Atlantis dC18, 4.6×150 mm, 3 μm, 100 Å; Waters, Milford, MA, USA) at a flow rate of 600 μL min^{-1} with a 40 min linear gradient from 0 to 100% acetonitrile/MilliQ water with 0.1% formic acid after a 5 min step in the initial conditions for column equilibration and sample desalting. Elution from the LC column was split into two flows: one at 550 μL min^{-1} was directed to UV monitoring at 214 nm, and the remaining flow (50 μL min^{-1}) was directed to the electrospray mass spectrometer for MS analyses.

An aliquot of sample (100 μL) was injected for each run. The LC flow was directed to the waste through a switching valve for the first 7 min after the injection before entering the source to minimize contamination of the AP-ESI source from potential interference from the sample buffer.

Table 1. Apparent K_d values of rhenium complexes and IC_{50} values of selected rhenium complexes and related molecules that bind to hCyp-18.

Compound	CC_{50} [μM]	$K_d \pm$ SD [μM]	$IC_{50} \pm$ SD [μM]
Suc-Ala-Ala-Pro-Phe-*p*NA	–	118±6	540±70
2o	<0.5	>10000	n.i.[a]
4i	200	11±1	12±2
5b	1000	2±0.1	5.3±2
9b	2200	0.3±0.03[b]	0.3±0.05
10i	<0.5	>10000	n.i.[a]
16n	1250	0.3±0.04[c]	0.2±0.02
cyclosporine	–	0.3±0.04[c]	0.02

[a] No inhibition; [b] fluorescence quenching at 345 nm; [c] fluorescence enhancement at 345 nm; [b,c] limit of detection with [hCyp-18] = 320 nM.

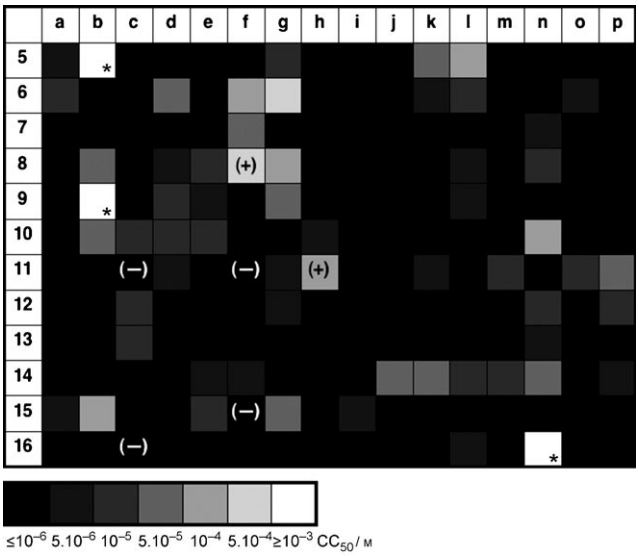

Figure 4. Titration of the GSH sensitivity of the 192-component DLC after calculation of the CC_{50}. Lead compounds are indicated by an asterisk; (+): false positive; (−): complexes that were not observed.

Nitrogen served as the drying and nebulizing gas, while helium gas was introduced into the ion trap for efficient trapping and cooling of the ions generated by the ESI ionization. Ionization was carried out in positive mode with a nebulizing gas set at 35 psi, a drying gas set at 8 μL min^{-1}, and a drying temperature set at 340 °C for optimal spray and desolvation. Ionization and mass analyses conditions (capillary high voltage, skimmer and capillary exit voltages, and ion-transfer parameters) were tuned for optimal detection of compounds in the 100–1000 *m/z* range. Full scan MS and MS/MS spectra were acquired with EsquireControl software, and all data were processed with Data Analysis software (Bruker Daltonik, GmbH, Germany).

Selection of complexes: The complexes were unambiguously identified on the basis of their time of retention (relative to a reference synthesized with [*n*Bu$_4$N]ReOCl$_4$) and both their *m/z* and their typical isotopic profiles in mass spectrometry. All data were processed as follows: the integrated ionic current (IIC in arbitrary units) was calculated by use of the integration software in order to determine the detection efficiency for each complex. The GSH concentration necessary for a 50% decrease in the IIC (C_{50}) was evaluated and was corrected by use of the formula: $CC_{50} = C_{50} \times (IIC_{reference}/IIC_{complex})$.

Complexes **4i** and **2o** were prepared as described previously.[1]

Synthesis of complexes 5b, 9b, 10i, and 16n: Solutions of modules **A** (0.1 mmol) and **B** (0.05 mmol) in methanol or acetone (5 mL) were treated under argon with tributylphosphine (10%, 42 μL, 0.165 mmol) for 30 min before addition of [*n*Bu$_4$N]ReOCl$_4$ and triethylamine in methanol (10%, 40 μL). A green-brown precipitate immediately formed. After stirring for 2 h, the crude mixture was centrifuged. The precipitate was washed twice with methanol, dissolved in DMSO, and purified by HPLC.

Complex 5b: Semipreparative RP-HPLC $t_R = 28.5$ min; purity 98% (analytical RP-HPLC $t_R = 28.5$ min); ^{1}H NMR (CD_3OD): $\delta = 1.54$ (s, 9H; CH_3 *t*Bu), 2.03 (s, 3H; CH_3 Ac), 2.96–3.01 (m, 4H; CH_2S NS_2), 3.66–3.71 (m, 4H; CH_2N NS_2), 3.97–4.07 (2 × brs, 2H; $CH_2\beta$ Cys), 4.44 (s, 2H; NCH_2CO NS_2), 4.80 (brt, $J = 6.8$ Hz, 1H; CHα Cys), 7.78–7.82 and 8.19–8.21 ppm (2 × d, $J = 9.6$ Hz, 4H; CH ar. *p*NA); ES/MS (positive mode): $m/z = 732.8$ (60%) + 734.8 (100%) $[M+H]^+$; HRMS calcd for $C_{21}H_{31}N_4O_7ReS_3$: 734.0913; found: 734.0943.

Complex 9b: Semipreparative RP-HPLC $t_R = 23.0$ (minor) + 25.1 (major) min; purity 96% (major) (analytical RP-HPLC $t_R = 27.4$ min); ^{1}H NMR (CD_3OD, major isomer): $\delta = 1.99$ (s, 3H; CH_3 Ac), 2.93–3.06 (m, 4H; CH_2S NS_2), 3.73 (m, 4H; CH_2N NS_2), 3.96 and 4.05 (2 × brs, 2H; $CH_2\beta$ Cys), 4.43 (m, 2H; CH_2–Ph), 4.47 (s, 2H; NCH_2CO NS_2), 4.80 (brt, $J = 7.3$ Hz, 1H; CHα Cys), 7.27–7.38 (m, 5H; H Ph), 7.80 and 8.20 ppm (2 × d, $J = 9.0$ Hz, 4H; CH ar. *p*NA); ES/MS (positive mode): $m/z = 766.1$ (60%) + 768.1 (100%) $[M+H]^+$ for ^{187}Re; HRMS calcd for $C_{24}H_{31}N_5O_6ReS_3$: 767.0916; found: 767.0939.

Complex 10i: Semipreparative RP-HPLC $t_R = 25.2$ min; purity 98% (analytical RP-HPLC $t_R = 27.7$ min); ^{1}H NMR (CD_3OD, major isomer): $\delta = 3.02$–3.17 (m, 4H; CH_2S NS_2), 3.59–3.91 (m, 6H; CH_2N NS_2 + SCH_2–CO), 4.63 (s, 2H; NCH_2CO NS_2), 7.10–7.16 (m, 1H) + 7.30–7.36 (m, 2H) + 7.58–7.62 (m, 2H) (H Ph), 7.80 (d, $J = 9.3$ Hz, 2H; H *p*NA), 8.19 ppm (d, $J = 9.3$ Hz, 2H; H pNA); ES/MS (positive mode): $m/z = 681.0$ (60%) + 683.0 (100%) $[M+H]^+$; HRMS calcd for $C_{20}H_{23}N_4O_5ReS_3$: 682.0389; found: 682.0366.

Complex 16n: Semipreparative RP-HPLC $t_R = 21.2$ (minor) + 21.7 (major) min; purity 97% (analytical RP-HPLC $t_R = 23.9$ min); ^{1}H NMR (CD_3OD): $\delta = 2.99$–3.13 (m, 4H; CH_2S NS_2), 3.65–3.90 (m, 8H; CH_2N NS_2 + SCH_2–CH_2NCON), 4.72 (s, 2H; NCH_2CO NS_2), 7.58 (d, $J = 9.3$ Hz, 2H; *p*NA), 7.77 (dd, $J = 5.1$, $J' = 8.5$ Hz, 1H; Pyr), 8.15 (d, $J = 9.3$ Hz, 2H; *p*NA), 8.34 (brdd, $J = 1.5$, $J' = 8.5$ Hz, 1H; Pyr), 8.47 (dd, $J = 1.5$, $J' = 5.1$ Hz, 1H; Pyr), 9.14 ppm (brs, 1H; Pyr); ES/MS (positive mode): $m/z = 711.0$ (60%) + 713.0 (100%) $[M+H]^+$; HRMS calcd for $C_{20}H_{25}N_6O_5ReS_3$: 712.0606; found: 712.0584.

Biochemical assays: The apparent dissociation constant (K_d) was obtained from the titration of the cyclophilin Trp121 at 345 nm.[3] The trypsin-coupled PPIase assay was carried out as previously reported.[11]

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Keywords: combinatorial chemistry · combinatorial methods · cyclophilins · PPIase · rhenium

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