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Chemical Biology with Organometallics

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Abstract

Inorganic and organometallic moieties are explored as structural scaffolds for the design of biologically active compounds. In this strategy, a metal center plays a structural role by organizing the organic ligands in the three-dimensional receptor space. It is hypothesized that this approach allows to access unexplored chemical space, thus giving new opportunities for the design of small molecules with unprecedented biological properties. Along these lines, our group pioneered the design of organometallic scaffolds for the inhibition of protein kinases. These compounds are formally derived from the class of ATP-competitive indolocarbazole alkaloids and allow to access novel structures with very defined and rigid shapes in an economical fashion, having resulted in the discovery of picomolar inhibitors for protein kinases with impressive selectivity profiles. This article summarizes the most important results over the last few years.

Introduction

The identification of compounds with novel and defined biological functions is of high importance for the development of small-molecule pharmaceuticals and as tools (“molecular probes”) for the investigation of biological processes. Nowadays, established technologies such as combinatorial chemistry and high-throughput screening allow to synthesize and identify initial lead structures in a rapid fashion and, in this respect, the chemical diversity of scaffolds is a key factor for success. The stark majority of synthetic efforts is focused on purely organic compounds. Surprisingly, a recent publication revealed that the diversity of

organic molecules is however quite limited: half of the 24 million organic compounds registered in the Chemical Abstract Service (CAS) Registry can be classified with only 143 scaffolds [1]. Similarly, the diversity of topological shapes of known organic drugs is also extremely low: a report on the analysis of the Comprehensive Medicinal Chemistry (CMC) database demonstrated that out of more than 5000 compounds analyzed, half of the known drugs fall into only 32 shape categories [2]. Most of the 32 frameworks contain at least two six-membered rings linked or fused together. Thus, it can be concluded that current efforts to obtain biologically active compounds draw from a limited set of structural scaffolds. Strategies to increase the molecular structural diversity are therefore demanded and promise to provide opportunities for the discovery of compounds with novel and unprecedented properties.

Metal Complexes as Structural Scaffolds

A few years ago, our group hypothesized that structural diversity might be increased by complementing organic elements with single metal atoms and to explore the opportunities of metals to help building small compounds with defined three dimensional structures [3]. Transition metals appear especially appealing for this purpose since they can support a multitude of coordination numbers and geometries which reach beyond the linear (sp-hybridization), trigonal planar (sp^2-hybridization), and tetrahedral (sp^3-hybridization) binding geometries of carbon (Fig. 1). For example, it is intriguing that an octahedral center with six different substituents is capable of forming 30 stereoisomers compared to just two for an asymmetric tetrahedral carbon. Thus, by increasing the number of substituents from four (tetrahedral center) to six (octahedral center), the ability of the center to organize substituents in the three dimensional space increases substantially. In addition, using a hexavalent center may provide new synthetic opportunities for accessing globular shapes by building structures from a single center in six different directions.

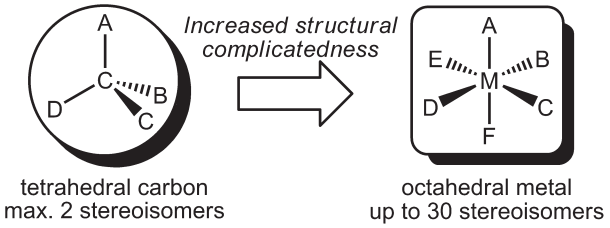

Figure 1. Structural opportunities with an octahedral metal center.

Ruthenium as a Priviledged Metal

Current efforts in our laboratory are concentrating predominately on ruthenium. In our opinion, ruthenium possesses a combination of properties that render it ideal for the design of chemically inert bioactive organometallic species. Main reasons are a high kinetical

inertness of coordinative bonds to ruthenium if certain basic design principles are considered (all here discussed compounds are air-stable, stable in water, and can withstand millimolar concentrations of thiols and thus should stay intact within the biological environment), the availability of inexpensive metal precursors (currently US$ 20/g for $RuCl_3$), and a predictable and established synthetic chemistry. A significant concern in using metal-containing compounds is the potential toxicity of the metal. In this respect, it is our opinion that a metal complex that has a substitutionally inert coordination sphere should not display any "metal-specific" toxicity itself and the biological properties of the metal complex should be instead determined by the entire entity. However, toxic effects due to metabolic conversion of coordinated ligands followed by the exposure of the metal to biological ligands cannot be excluded in individual cases. It is therefore desirable that the metal itself or derived metal salts have a low toxicity. In this respect, data from phase I clinical trials of two (substitutionally labile) ruthenium anticancer drugs, KP1019 and NAMI-A, are encouraging since these compounds have proven to be much less toxic compared to the anticancer drug cisplatin [4, 5].

Staurosporine as a Lead Structure

We selected the natural product staurosporine as a structural inspiration (lead structure) for the design of ATP-competitive organometallic kinase inhibitors **1** (Figs. 2 and 3) [6]. Staurosporine is a member of the class of indolocarbazole alkaloids, many of which are potent ATP-competitive protein kinase inhibitors. This family of inhibitors shares the indolo[2,3-a]carbazole moieties **2** (lactam form) or **3** (imide form, arcyriaflavin A) which bind to the adenine binding site by establishing two hydrogen bonds to the backbone of the hinge between the N-terminal and C-terminal kinase domain (Figs. 2b and 3). Staurosporine adopts a defined globular structure with the carbohydrate moiety being oriented perpendicular to the plane of the indolocarbazole heterocycle (Fig. 3). The indolocarbazole moiety occupies the hydrophobic adenine binding cleft with the lactam group mimicking the hydrogen bonding pattern of the adenine base, and the carbohydrate moiety forming hydrophobic contacts and hydrogen bonds within the globular ribose binding site (Fig. 2). Thus, staurosporine closely matches the shape of the ATP-binding site which makes it a highly potent albeit unselective inhibitor for many protein kinases.

In order to match the overall shape of the ATP-binding site of protein kinases in a fashion similar to staurosporine, but with less synthetic effort and more extended structural options, we replaced the indolocarbazole alkaloid structure with simple metal complexes in which the main features of the indolocarbazole heterocycle **3** are retained in a metal-chelating pyridocarbazole **4** (Fig. 3). This ligand **4** can serve as a bidentate ligand for ruthenium complexes of type **1**. Additional ligands in the coordination sphere of the metal can now substitute for the carbohydrate moiety of staurosporine, with the metal center serving as a "glue" to unite all of the parts. This approach has resulted in the successful design of nanomolar and even picomolar protein kinase inhibitors.

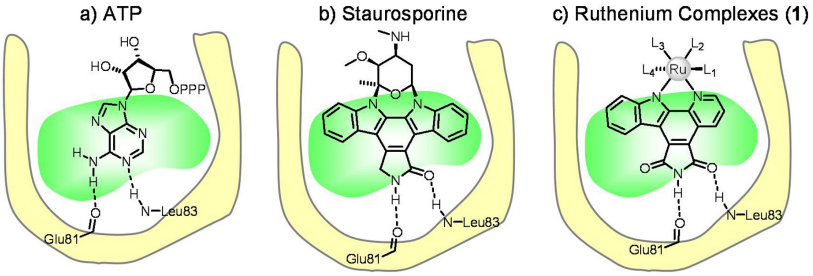

Figure 2. Schematic representation of the ATP-binding pocket of cyclin dependent protein kinase 2 (CDK2) in complex with a) ATP, b) staurosporine, and c) indolocarbazole-derived ruthenium complexes. Both ATP and staurosporine form hydrogen bonds with the backbone amide groups of glutamate 81 and leucine 83. The green area indicates a patch of high hydrophobicity. Adapted from ref. [7].

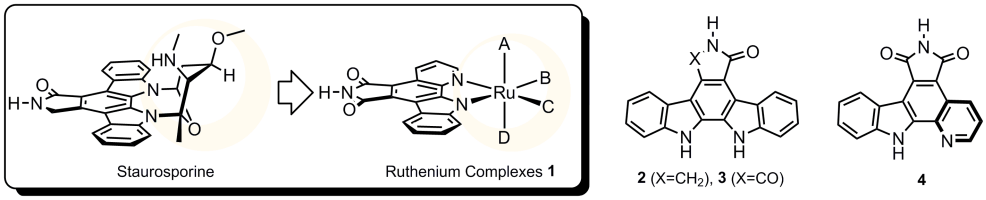

Figure 3. Designing ruthenium complexes of scaffold **1** as protein kinase inhibitors by using the ATP-competitive natural product staurosporine as a lead for the overall three-dimensional shape. The chelating ligand **4** contains the main pharmacophore of the indolocarbazole heterocycles **2** and **3**.

Proof-of-principle: Organoruthenium Inhibitors for the Protein Kinases GSK-3 and Pim-1

We recently identified the cyclopentadienyl-CO sandwich scaffold **5** to be a nanomolar inhibitor for the protein kinases GSK-3 and Pim-1 (Fig. 4). IC_{50} curves of racemic **5** and the corresponding pyridocarbazole ligand **4** against the α-isoform of GSK-3 (GSK-3α) are shown in Figure 4c. Accordingly, the pyridocarbazole ligand **4** itself is 15000-times less potent against GSK-3α compared to the entire complex **5**. Consequently, the activity of **5** requires the complete ruthenium ligand sphere. Methylation of **5** at the imide nitrogen (**Me5**) abolishes this activity completely. Figure 4c furthermore demonstrates that staurosporine is by an order of magnitude less potent for this kinase than **5** [8].

Complex **5** displays metal-centered chirality and potency as well as selectivity for GSK-3 or Pim-1 depend on the absolute configuration and additional substitutions at the periphery of this half-sandwich scaffold. For example, whereas the racemic mixtures of **5** and **6** are dual inhibitors for GSK-3 and Pim-1, (*S*)-**6**, having a additional OH-group at the indole, is very

selective for Pim-1 with an IC_{50} of 0.22 nM at 100 µM ATP compared to 9 nM for GSK-3α (α-isoform) [9]. On the other hand, (*R*)-**7**, having an OH and Br-group at the indole and a methylester at the cyclopentadienyl moiety, displays an IC_{50} of 0.35 nM for GSK-3α compared to 35 nM for Pim-1. In fact, in a panel of 57 kinases, (*R*)-**7** is highly selective for just GSK-3 [10].

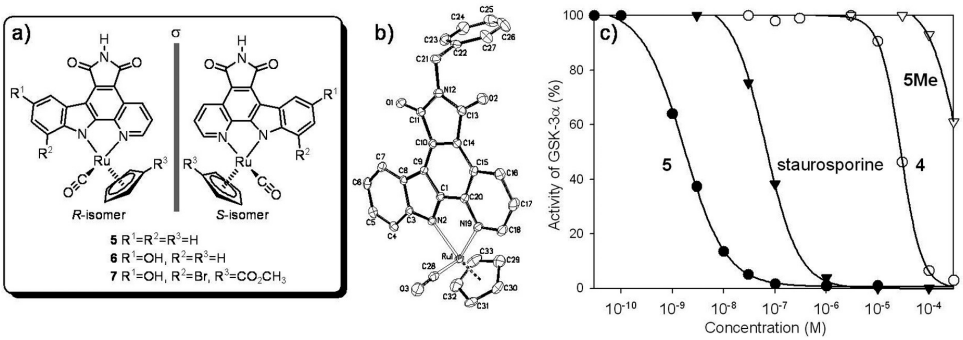

Figure 4. (**a**) The half-sandwich complexes **5**-**7** are potent inhibitors for the protein kinases GSK-3 and/or Pim-1. The half-sandwich compounds **5**-**7** are pseudo-tetrahedral and exist in two mirror-imaged configurations. The absolute configuration at the ruthenium center has been assigned according to the priority order of the ligands being η^5-C_5H_5 > pyridine [N(C, C, C)] > indole [N(C, C, lone pair)] > CO. (**b**) Crystal structure of *N*-benzylated **5** (ORTEP drawing with 35% probability thermal elipsoids). (**c**) IC_{50} curves with GSK-3α measured with 10 µM ATP. **5Me** is the N-methylated derivative of **5**.

Cocrystal Structures of Protein Kinases with Organometallics

In collaboration with Stefan Knapp (Oxford University) and later independently in our own laboratory, we obtained cocrystal structures of ruthenium complexes bound to Pim-1 [9]. For example, Figure 5 shows a cocrystal structure of Pim-1 with (*S*)-**6**. The structure demonstrates that the ruthenium complex (*S*)-**6** binds to the ATP binding site and that the ruthenium centre does not undergo any direct interactions with the active site but has a purely structural role. Furthermore, superimposing cocrystallized (*S*)-**6** and Pim-1 (PDB code 2BZI) with cocrystallized staurosporine and Pim-1 (PDB code 1YHS) reveals how closely the half-sandwich complex mimics the binding mode of staurosporine (Figs. 5b and 5c). The pyridocarbazole moiety of (*S*)-**6** is nicely placed inside of hydrophobic pocket formed between residues from the N-terminal and C-terminal domain, mimicking the binding position of the indolocarbazole moiety of staurosporine, while the rest of the ruthenium complex occupies the binding site of the carbohydrate moiety of staurosporine. Curiously, the oxygen of the Ru-CO ligand is at a very close distance to Gly45 in the glycine-rich loop.

Currently, five structures of ruthenium half-sandwich complexes with protein kinases are available in the protein databank, four with Pim-1 (2BZH, 2BZI, 2OI4, 2BZJ), and one with Pim-2 (2IWI). In addition, a cocrystal structure of an osmium complex with Pim-1 was published recently by us (3BWF) [11]. A structure of (*R*)-**6** with GSK-3β has been solved in our laboratory at 2.4 Å and will be published soon (G. E. Atilla-Gockumen, E. Meggers, unpublished results). The Marmorstein group (The Wistar Institute, Philadelphia, USA) obtained several structures by soaking ruthenium complexes into crystal structures of the lipid kinase PI3Kγ [12].

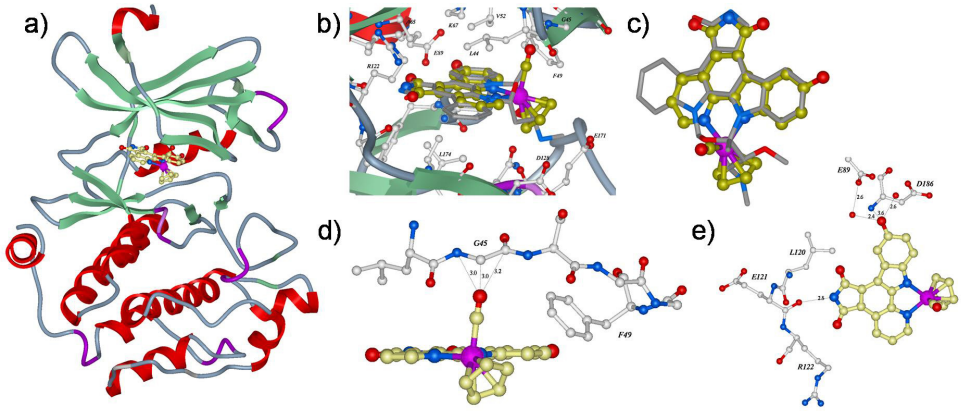

Figure 5. Cocrystal structure of (*S*)-**6** with Pim-1 at 1.9 Å (PDB code 2BZI). (**a**) The ruthenium complex occupies the ATP binding site of Pim-1. (**b**) and (**c**) Superimposed relative binding positions of (*S*)-**6** and staurosporine to Pim-1 (PDB code 1YHS). (**d**) and (**e**) Some important interactions.

Cellular Data: Inhibition of GSK-3β by a Ruthenium Complex within Mammalian Cells

Over the last two year we have repeatedly demonstrated that organometallic GSK-3 inhibitors function inside of living mammalian cells [10, 13, 14]. GSK-3β is a negative regulator of the wnt-signaling pathway by phosphorylating β-catenin. Phosphorylated β-catenin itself is unstable due to rapid degradation by the proteasome. In the presence of a wnt signal, GSK-3β is inactivated, resulting in an accumulation of β-catenin in the cytoplasm, followed by a translocation into the nucleus where β-catenin serves as a transcriptional cofactor. Thus, inhibition of GSK-3β by pharmacological inhibitors or by wnt signaling leads to increased β-catenin levels and activation of wnt dependent transcription.

In order to probe the inhibition of GSK-3β within mammalian cells we routinely use Western blot analysis (qualitative detection of β-catenin upregulation), cellular β-catenin staining experiments (confirmation of β-catenin translocation into the nucleus), and a β-catenin reporter system for a quantitative analysis. For the latter we use human embryonic kidney cells (HEK-293OT) that have stably incorporated a Tcf-luciferase transcription reporter (OT-Luc cells). This transcription reporter generates luciferase in response to increased concentrations of β-catenin. An inhibition of GSK-3β can thus be determined by the luminescence signal upon addition of luciferin to the cell lysate. For example, exposure of OT-Luc cells to varying concentrations of the selective ruthenium GSK-3 inhibitor (*R*)-**7** over a period of 24 hours yielded a strong upregulation of luciferase in the concentration window of 3 μM down to 10 nM (Figure 6a, compare also with (*R*,*S*)-**5** in Figure 6b). Intriguingly, in contrast, the popular organic GSK-3 inhibitors 6-bromo-indirubin-3'-oxime (BIO) (**8**) and kenpaullone (**9**) (Figs. 6c, d) require micromolar concentrations for significant activities.

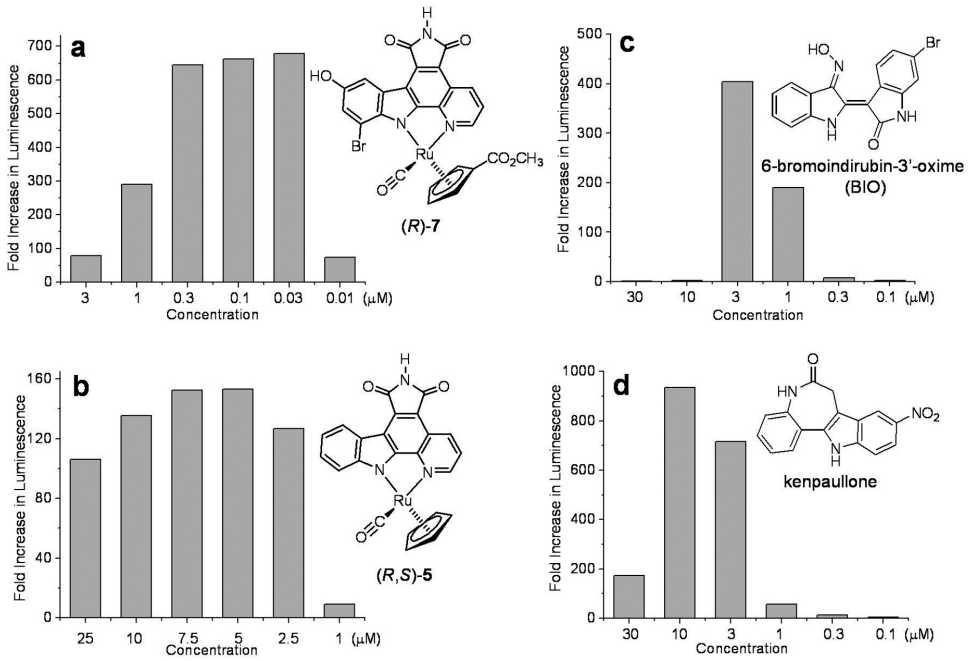

Figure 6. Cellular GSK-3β inhibition of (*R*)-**7** and comparison with organic GSK-3 inhibitors. HEK-293OT cells transfected with a β-catenin-responsive luciferase reporter were treated with different concentrations of inhibitors for 24 hours. After cell lysis, the luminescence signal was measured after addition of luciferin.

It is noteworthy that organometallic compounds of this type can inhibit GSK-3β in frog embryos (demonstrated for (*R*,*S*)-**6**) and zebrafish embryos (demonstrated for (*R*)-**7**) and result in phenotypes that are related to the activation of the wnt pathway. For example, the exposure of zebrafish embryos to LiCl, a known GSK-3 inhibitor, causes a perturbed

development of the head structure with a no-eye-phenotype, among others. Treatment of zebrafish embryos with (*R*)-**7** under analogous conditions, results in a similar phenotyp with a decrease of the head structure without eyes and a stunted and crooked tail. In addition, the yolk is enlarged and misshaped (Fig. 7c). However, zebrafish embryos which were instead treated under identical conditions with the mirror-imaged compound (*S*)-**7**, develop normal (Figs. 7a and 7b). These observations are consistent with an inhibition of GSK-3β by (*R*)-**7**, but not (*S*)-**7** and further demonstrate that the biological activity of (*R*)-**7** is determined by the overall three-dimensional structure of the ruthenium complex and not the ruthenium itself.

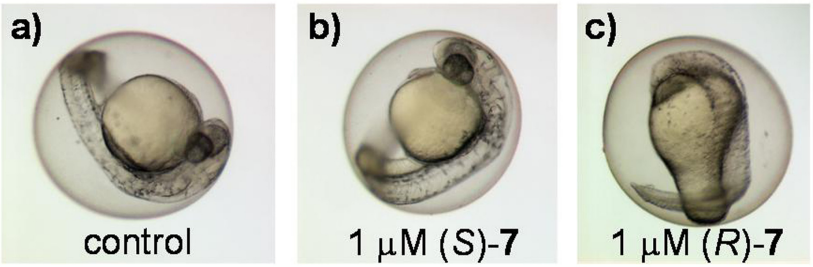

Figure 7. Exposure of zebrafish embryos to (**a**) DMSO (2%), (**b**) 1 μM (*S*)-**7**, and (**c**) 1 μM (*R*)-**7** (see Figure 4 for structures). The embryos were collected and maintained in E3 media at 28.5 °C, compounds added at 4 hours post fertilization (hpf), and the phenotypes were compared at 25 hpf.

In conclusion, organometallic (*R*)-**7** is superior as a molecular probe for the function of GSK-3β compared to established organic GSK-3β inhibitors with respect to binding affinity, cellular potency, and kinase selectivity.

Combinatorial Chemistry with Ruthenium Complexes

We were interested in developing a method to rapidly assemble coordination spheres around the ruthenium center in a combinatorial fashion and for this we deviced a synthesis of complex **8** [15]. Although this compound bears four leaving groups, three acetonitriles and one chloride, it is remarkably stable at room temperature. For example, **18** can be purified on a regular silica gel column (CH_2Cl_2/MeOH) and is stable as a solid on the bench for a few days without decomposition. This behaviour is a demonstration of the kinetic inertness of the ruthenium pyridocarbazole scaffold. However, the acetonitrile ligands and the chloride can be replaced upon heating of **8** in the presence of other ligands at elevated temperatures of 70 – 110 °C (**8**→**9**) (Fig. 8a). Thus, **8** allows a rapid access to a diversity of novel structures **9** by ligand replacement chemistry in the final synthetic step.

Figure 8b shows a selection of octahedral ruthenium complexes **10-13** that we synthesized through this route. Compounds **10**, **11**, and **12** are interesting protein kinase inhibitors. For example, the racemic compound **11**, bearing a CO and 1,4,7-trithiacyclononane ligand, is a subnanomolar inhibitor for the protein kinase Pim-1 with an IC_{50} of 0.45 nM at 100 µM ATP. Intriguingly, the nature of this monodentate ligand has a dramatic effect on kinase inhibition. For example, substituting the CO for $P(OMe)_3$ (**13**) renders the compound completely inactive (Fig. 8b,c). Other ligands such as DMSO, cyanide, and ammonia also result in a significant decrease in potency for Pim-1 (data not shown). Interestingly, substituting the CO for an azide (**12**) yields a fairly potent MSK-1 inhibitor ($IC_{50} = 70$ nM).

The bar diagram in Figure 8c further demonstrates that the CO complex **11** is most potent against Pim-1 but also inhibits GSK-3α to a significant extent at 100 nM. Apparently, the CO ligand, which is in the plane perpendicular to the pyridocarbazole chelate, is an important pharmacophore both for Pim-1 and GSK-3, but not for most other kinases. The rest of the ligand sphere can then be used to render the complex either selective for Pim-1 or GSK-3. This is well illustrated by the compounds **10** and **11**. Whereas complex **11** significantly prefers Pim-1, the complex **10** is instead selective for GSK-3α with an IC_{50} of 8 nM at 100 µM ATP.

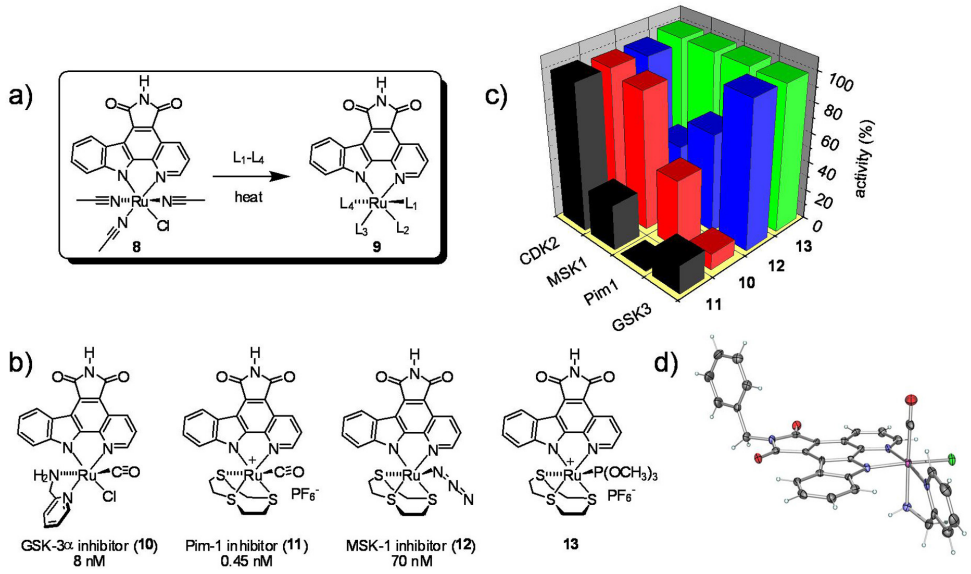

Figure 8. (**a**) Synthesis of ruthenium pyridocarbazole complexes **9** by ligand substitution chemistry from the common precursor **8**. (**b**) Selected complexes derived from **8**. IC_{50} values were measured at 100 µM ATP. (**c**) Inhibitory activities of the organoruthenium compounds **10-13** at a concentration of 100 nM against the protein kinases GSK-3α, Pim-1, MSK1, and CDK2/CyclinA. ATP concentration was 100 µM. (**d**) Crystal structure of the *N*-benzylated derivative of **10**. ORTEP drawing with 40% probability thermal elipsoids.

Conclusion

With GSK-3 and Pim-1 as model kinases we have established that organometallic scaffolds are highly promising scaffolds for the design of kinase inhibitors. Accordingly, we have identified low nanomolar and even picomolar inhibitors for these kinases, demonstrated their exceptional kinase selectivities, have verified their usefulness in mammalian cell culture and model organisms such as zebrafish and frog embryos, and we confirmed their binding modes with cocrystal structures of ruthenium inhibitors bound to the ATP-binding site of Pim-1. Furthermore, we have developed tools that allow to rapidly synthesize a diverse selection of organometallic compounds and to screen them for their kinase activities. The discovered organometallic GSK-3 and Pim-1 inhibitors are some of the most potent and selective inhibitors for kinases know to date and therefore these results validate the strategy of exploring organometallic scaffolds for the design of enzyme inhibitors. Future work has to focus on octahedral metal complexes and expand the investigation to other biological targets.

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