Enzyme Inhibitors

Switching on a Signaling Pathway with an Organoruthenium Complex**

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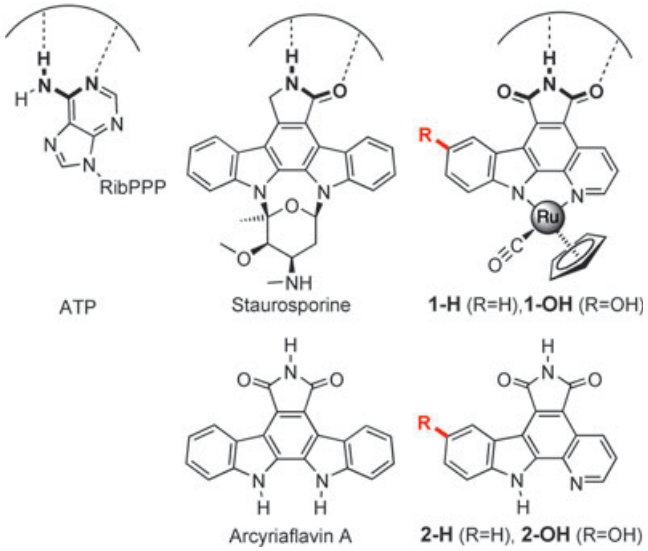
Altering cellular function with small synthetic molecules is a general approach for the design of drugs (medicinal chemistry) and molecular probes (chemical genetics). Medicinal chemistry and chemical genetics are focused predominately on the design of organic molecules, whereas inorganic compounds find applications mainly for their reactivity (e.g., cisplatin as DNA-reactive therapeutic) or imaging properties (e.g., gadolinium complexes as MRI diagnostics).[1,2] We are exploring a new direction that aims at utilizing the unique structural opportunities of metal complexes for the design of "organiclike" small-molecule probes and drugs.[3,4] We recently described an organoruthenium compound to be a low-nanomolar inhibitor for the protein kinase glycogen synthase kinase 3 (GSK-3).[4] Here we disclose our success in developing this compound into a molecular probe for cellular signal transduction pathways involving GSK-3.[5]

Our strategy for the design of metal complexes as protein kinase inhibitors uses the class of indolocarbazole alkaloids (e.g., staurosporine) as a lead structure. Accordingly, these metal complexes bear a bidentate ligand which retains the structural features of the indolocarbazole heterocycle. This targets the metal complexes to the ATP-binding site by enabling two hydrogen bonds to the backbone of the hinge between the N-terminal and C-terminal kinase domains, analogous to ATP and conventional organic indolocarbazole inhibitors (Scheme 1).[6] The remaining ligand sphere of the ruthenium atom gives the opportunity to create interactions with other parts of the ATP-binding site. Following this strategy, we recently described ruthenium compound **1-H** as a selective low-nanomolar and ATP-competitive inhibitor of GSK-3.[4] The key component of the design was the novel pyridocarbazole ligand **2-H**, derived from arcyriaflavin A by just replacing one indole moiety with a pyridine residue.



Scheme 1. Designing ruthenium complexes as protein kinase inhibitors by mimicking ATP-competitive indolocarbazoles.

Here we introduce the ruthenium complex **1-OH** (Scheme 1), derived from **1-H** by adding a single hydroxy group to the indole moiety. Molecular modeling suggests that this OH group can occupy a small hydrophilic cavity within the ATP-binding site of GSK-3. Indeed, this modification not only increases the affinity for GSK-3 by an order of magnitude, but at the same time increases water solubility considerably, and thus makes it a promising molecular probe for GSK-3. GSK-3 is a component and negative regulator of the wnt signaling pathway, and we demonstrate in the following that **1-OH** can switch on the wnt signaling pathway by inhibiting GSK-3 inside living cells and in *Xenopus* embryos.

We devised a short and economic synthetic route to this class of metalated pyridocarbazoles (Scheme 2). Starting with aryl hydrazine hydrochloride **3**, Fischer indole synthesis and protecting-group exchange lead to pyridoindole **4**, which is deprotonated and treated with the TBS-protected dibromomaleimide **5** to yield monosubstitution product **6**. The key step of the synthetic scheme is the following smooth anaerobic photocyclization to the TBS-protected pyridocarbazole **7** in 78% yield.[7] Compound **7** can be deprotected with TBAF to give **2-OH** or treated with $[Ru(Cp)(CO)(CH_3CN)_2]PF_6$ in the presence of K_2CO_3 to yield cyclometalated ruthenium complex **8**.[8] Subsequent TBS deprotection with TBAF gives **1-OH**. Complex **1-OH** is stable in aqueous solution, in cell culture medium, under air, and can even withstand a 5 mM solution of 2-mercaptoethanol for 12 h without any sign of decomposition, as determined by 1H NMR spectroscopy. Furthermore, **1-OH** also has favorable solubility in water. For example, it can be dissolved in 3% DMSO/water at a concentration of 1 mM.

We next examined the potency of **1-OH** by determining the concentration required for 50% inhibition (IC_{50}) of GSK-3α (the α isoform) and GSK-3β (the β isoform). The IC_{50} of **1-OH** is 300 pM and 500 pM for GSK-3α and GSK-3β, respec-

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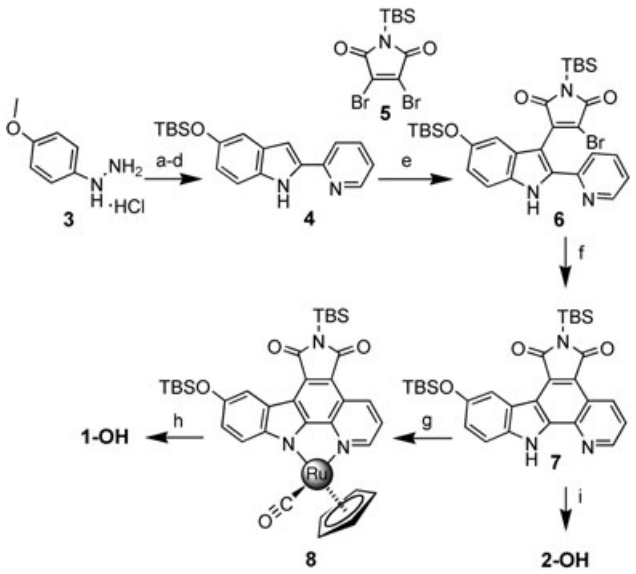

Scheme 2. Synthesis of ruthenium complex **1-OH** and pyridocarbazole **2-OH**. a) 2-Acetylpyridine, *t*BuOH, 4 h reflux (100%). b) Trimethylsilyl polyphosphate, 115°C, overnight (63%). c) BBr_3, CH_2Cl_2, −60°C, then RT overnight (87%). d) DIEA, DMF, 0°C, 40 min, then TBS triflate, 0°C, 1 h (71%). e) Li hexamethyldisilazide, THF, −15°C, 45 min, then **5** in THF, −15°C, 15 min, then RT, 45 min (58%). f) *hν*, Pyrex filter, MeCN, 3 h, (78%). g) $[Ru(Cp)(CH_3CN)_2(CO)]PF_6$, K_2CO_3, MeCN, 55°C, overnight (86%). h) TBAF, CH_2Cl_2, RT, 30 min (87%). i) TBAF, CH_2Cl_2, RT, 30 min (80%). DIEA = *N*,*N*-diisopropylethylamine, TBAF = tetrabutylammonium fluoride, TBS = *tert*-butyldimethylsilyl.

tively, approximately an order of magnitude lower than that of the initial complex **1-H**. For comparison, ruthenium compound **1-OH** is at least 100 times more potent against GSK-3 than staurosporine under our experimental conditions (IC_{50} of 40 nM against GSK-3α).[9–12] Methylation of the imide nitrogen atom (**Me1-OH**) reduces the activity dramatically by almost four orders of magnitude (IC_{50} of 2 μM against GSK-3α), as demonstrated by the IC_{50} curves in Figure 1, which

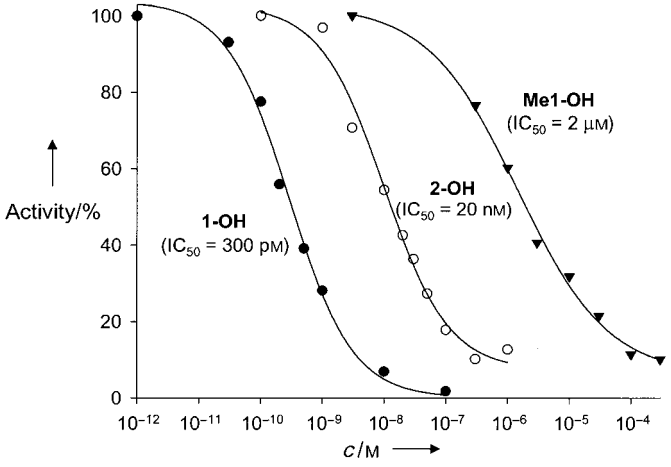

Figure 1. IC_{50} curves with GSK-3α obtained by phosphorylation of phosphoglycogen synthase peptide-2 with [γ-32P]ATP: ruthenium complex **1-OH** (●), ligand **2-OH** (○), and methylated ruthenium complex **Me1-OH** (▼).

reconfirms that the potency of **1-OH** is entirely based on specific recognition. It is also noteworthy that ligand **2-OH** is almost two orders of magnitude less active than the corresponding ruthenium complex **1-OH**, clearly verifying that the entire assembly is necessary for full activity.

Ruthenium complex **1-OH** also shows high selectivity. The protein kinases Abl (IC_{50} = 1 μM), CHK-1 (IC_{50} = 2 μM), Lck (IC_{50} = 500 nM), MAPK-1 (IC_{50} > 3 μM), RSK-1 (25 nM), Src (IC_{50} = 4 μM), and Zap-70 (IC_{50} > 10 μM) are inhibited at concentrations that are 80 to more than 30000 times higher relative to GSK-3α. Even the evolutionarily closely related protein kinase CDK-2 is only inhibited with an IC_{50} of 200 nM.[9–12]

Next, we investigated whether **1-OH** can inhibit GSK-3 inside mammalian cells. GSK-3 is a negative regulator of the wnt signal transduction pathway that phosphorylates β-catenin.[13] Phosphorylated β-catenin is unstable and is degraded rapidly by the proteasome. Wnt signaling inhibits GSK-3, leading to stabilization of β-catenin protein. β-Catenin then accumulates and serves as a transcriptional coactivator through its interaction with the T-cell factor (Tcf) family of transcription factors. Thus inhibition of GSK-3 by pharmacological inhibitors or by wnt signaling leads to increased β-catenin levels and activation of wnt-dependent transcription.

To test for cellular accumulation of β-catenin as a response to inhibition of GSK-3 by ruthenium complex **1-OH**, we used human embryonic kidney cells (HEK293T) 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.[14] Exposure of OT-Luc cells to varying concentrations of **1-OH** over a period of 24 h yields a strong upregulation of luciferase in the concentration window of 3 μM down to 100 nM (Figure 2A). For example, at a **1-OH** concentration of 300 nM, luciferase activity is enhanced by a factor of 1180. Intriguingly, at the same time, methylated control **Me1-OH** and free ligand **2-OH** do not yield any significant increase in luciferase activity at a concentration of 1 μM (1.6- and 2.4-fold, respectively). As a positive control, the established selective GSK-3 inhibitor LiCl leads to an increase of the luminescence signal by a factor of 1190 at a concentration of 30 mM.[14,15]

To verify the accumulation of β-catenin directly, we analyzed the cellular β-catenin concentration by Western blotting after incubation with **1-OH**. Figure 2B demonstrates an increase in β-catenin in the presence of **1-OH**, but not **Me1-OH** or **2-OH**, at 1 μM. These experiments demonstrate that ruthenium complex **1-OH** crosses the cell membrane and activates the wnt pathway at low micromolar and even nanomolar concentrations.

It is also noteworthy that **1-OH** does not show any signs of cytotoxicity at concentrations of 3 μM or less, supporting our assumption that the kinetically inert ruthenium center is only an innocent nonreactive bystander. The integrity of complex **1-OH** inside the cell is also reinforced by the fact that the entire ligand assembly is necessary for the activity inside the cell and that the free ligand **2-OH** does not activate the wnt pathway itself at similar concentrations. This is a remarkable

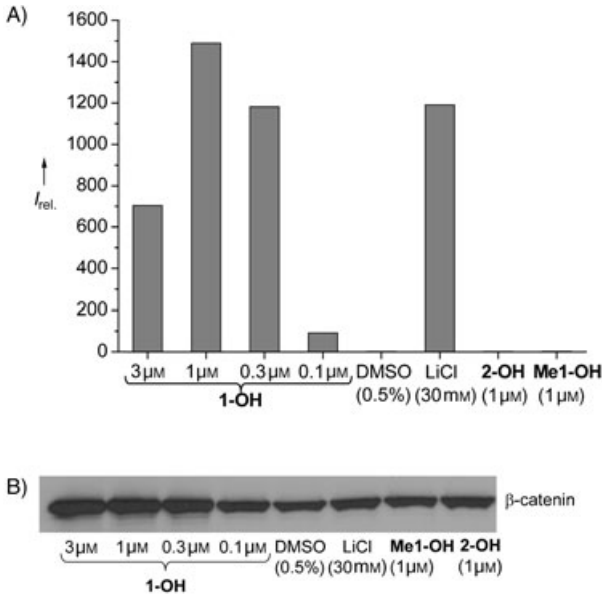

Figure 2. Cells transfected with a β-catenin-responsive luciferase reporter were treated with different concentrations of **1-OH**, ligand **2-OH**, and methylated ruthenium complex **Me1-OH** for 24 h. LiCl served as a positive control, and DMSO as a negative control. A) Relative luciferase activity determined after cell lysis, addition of the substrate luciferin, and luminescence measurement. B) Western blot of the cell lysate qualitatively detecting the amount of β-catenin.

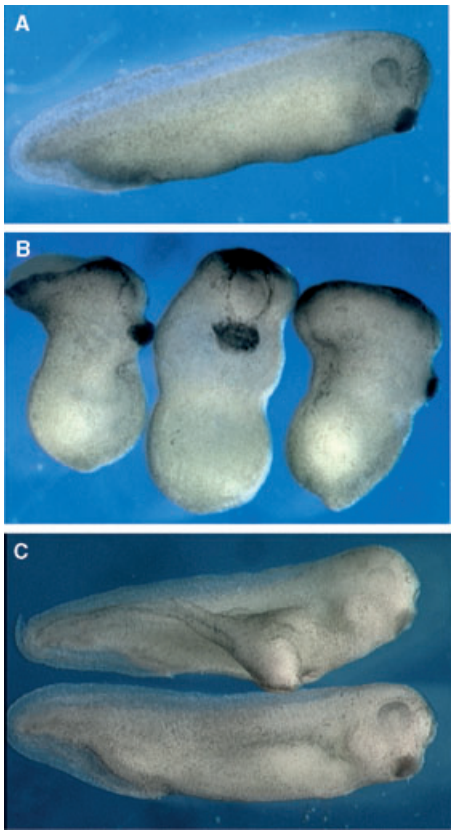

Figure 3. Effect of **1-OH** on dorsal development in *Xenopus laevis*. A) Control tadpole. B) *Xenopus* embryos were treated with **1-OH** (10 μM) added to the culture medium from the 32-cell stage until midblastula stage and then allowed to develop until tadpole stage. 100% of embryos showed this hyperdorsalized phenotype ($n = 29$). A similar effect was observed with **1-OH** at 5 μM ($n = 27$; data not shown). C) **1-OH** (ca. 0.5 mM in 10% DMSO) was injected into a ventral blastomere of 32–64-cell stage *Xenopus* embryos (upper embryo, with twinned dorsal axis). Control injections with water or DMSO did not affect dorsal axis development (lower embryo in C).

observation considering the millimolar concentrations of highly metallophilic thiols such as glutathione, cysteine, and cysteine-containing proteins within the cell.

Finally, we investigated the in vivo effects of **1-OH**. β-Catenin is required for dorsal axis formation in *Xenopus laevis* embryos, while ectopic activation of the wnt pathway, for example, with lithium, induces an ectopic dorsal axis.[16] Thus, we examined the phenotypes of embryos treated with **1-OH**. *Xenopus* embryos were exposed to **1-OH** added to the culture medium from the 32-cell stage until the midblastula stage (stage 8.5), and then were transferred to control medium until the tadpole stage. 100% of embryos ($n = 56$) treated with 5 or 10 μM **1-OH** demonstrated a hyperdorsalized phenotype, with expansion of dorsal and anterior structures and loss of posterior tissues (Figure 3B), highly similar to embryos exposed to lithium or the recently described GSK-3 inhibitor 6-bromoindirubin-3′-oxime.[10] Furthermore, injection of **1-OH** into a ventral blastomere at the 32–64-cell stage caused formation of a complete secondary dorsal axis (Figure 3C), similar to effects observed with injection of LiCl.[15–17] Thus, it can be concluded that ruthenium complex **1-OH** inhibits GSK-3 and activates wnt signaling in vivo in *Xenopus* embryos.

In summary, we have reported a ruthenium complex, **1-OH**, which is a highly potent inhibitor for the protein kinase GSK-3 and demonstrated that **1-OH** can switch on the wnt signal transduction pathway inside living cells and in *Xenopus* embryos. Such properties of an organometallic compound are unprecedented, and this success indicates that our strategy of using kinetically inert metal centers as rigid structural scaffolds for the design of enzyme inhibitors leads to new opportunities for creating highly potent molecular probes and ultimately drugs.

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