Antitumor Agents

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Antagonizing STAT3 Dimerization with a Rhodium(III) Complex**

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Abstract: *Kinetically inert metal complexes have arisen as promising alternatives to existing platinum and ruthenium chemotherapeutics. Reported herein, to our knowledge, is the first example of a substitutionally inert, Group 9 organometallic compound as a direct inhibitor of signal transducer and activator of transcription 3 (STAT3) dimerization. From a series of cyclometalated rhodium(III) and iridium(III) complexes, a rhodium(III) complex emerged as a potent inhibitor of STAT3 that targeted the SH2 domain and inhibited STAT3 phosphorylation and dimerization. Significantly, the complex exhibited potent anti-tumor activities in an in vivo mouse xenograft model of melanoma. This study demonstrates that rhodium complexes may be developed as effective STAT3 inhibitors with potent anti-tumor activity.*

The development of cisplatin and other "shotgun" cytotoxic metal complexes have contributed tremendous advances to the field of inorganic medicinal chemistry over the past few decades.[1] Recently, organometallic compounds have emerged as viable alternatives to organic small molecules as molecularly-targeted agents targeting protein kinases or the binding interfaces of protein–protein interactions.[1b,2] Meggers and co-workers have pioneered the development of kinetically inert transition-metal complexes as potent and selective inhibitors of enzyme activity.[2b,c,3] Sadler and co-workers developed organometallic ruthenium(II) anticancer complexes which exhibit in vitro and in vivo anticancer activities by inhibition of human glutathione-S-transferase π.[4] Recently, our group reported the first examples of rhodium(III) and iridium(III) complexes as inhibitors of the NEDD8-activating enzyme (NAE), tumor necrosis factor-α (TNF-α), and the mammalian target of rapamycin (mTOR).[5]

The signal transducer and activator of transcription (STAT) family proteins mediate a range of cellular responses to cytokines and growth factors.[6] The activation of STAT proteins is initiated by upstream growth factor receptors and cytoplasmic kinases such as Janus kinases (JAKs) and Src family kinases,[7] thus culminating in the formation of activated STAT dimers by reciprocal phosphotyrosine–Src Homology 2 (SH2) domain interactions. The aberrant expression and constitutive activation of one of the STATs, STAT3, has been associated with tumorgenesis through up-regulation of cell survival proteins and cell-cycle regulators,[8] and enhanced angiogenesis of cells.[9] In particular, STAT3 plays an important role in the development of skin cancer.[10] The inhibition of STAT3 dimerization through occupation of the SH2 domain of STAT3 has been demonstrated by a number of small molecules.[11] Notably, S3I-201 (NSC 74859)[12] induced the regression of human breast cancer xenografts in a nude mice model.

In the context of metal-based inhibitors of STAT3, Turkson and co-workers have utilized kinetically liable platinum complexes to inhibit STAT3 activity in living cells putatively, through binding to the STAT3 DNA-binding domain to form irreversible platinum–STAT3 adducts, and demonstrated their ability to induce tumor regression in a mouse colon cancer model.[13] Gunning and co-workers have developed bis(dipicolylamine) copper(II) complexes as functional proteomimetics of the SH2 domain.[14]

In the present study, a library of 11 substitutionally inert rhodium(III) and iridium(III) complexes (**1**–**11**; Figure 1) was screened for their ability to inhibit STAT3 DNA-binding activity by using ELISA. Of these 11 complexes, the cyclometalated iridium(III) complexes **10** and **11** emerged as top candidates (see Figure S1 in the Supporting Information). Based on the structures of **10** and **11**, a focused library of 26

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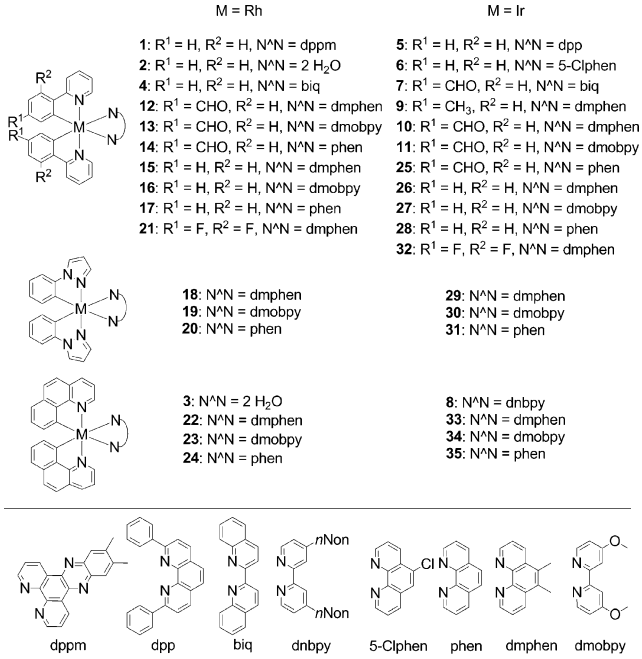

Figure 1. Chemical structures of the cyclometalated rhodium(III) and iridium(III) complexes **1**–**35** which were synthesized and evaluated in this study.

cyclometalated rhodium(III) and iridium(III) complexes (**10**–**35**; Figure 1) was designed and synthesized. This library was enriched in the favorable substructures identified in the first round of screening, such as 4-(pyridin-2-yl)benzaldehyde ligands, while excluding ligands that were observed to result in inferior activity. In the second round of screening, the rhodium(III) complexes **12** and **14** exhibited the greatest ability to inhibit STAT3 DNA-binding activity, and showed superior potency compared to both parent complexes (**10** and **11**) and S3I-201 (see Figure S2).

The synthesis and characterization of the complexes **1**–**35** (as PF_6^- salts) are given in the Supporting Information. The spectroscopic data of the complexes are presented in Table S1. The complexes were stable in a $[D_6]$DMSO/D_2O (9:1) solution at 298 K for at least seven days as revealed by ^{1}H NMR spectroscopy (Figure S3 shows representative spectra for **12**), and in acetonitrile/H_2O (9:1) solution at 298 K for at least seven days as verified by UV/Vis spectroscopy (Figure S4 shows representative spectra for **12**).

Bioactive iridium and rhodium complexes have been reported to interact with a number of biological targets in vitro, including protein and DNA.[2d] The group of Barton has extensively explored the application of rhodium polypyridyl complexes as metallointercalators or metalloinsertors of DNA, including mismatched DNA.[15] Polypyridyl-containing iridium half-sandwich complexes have been demonstrated by the groups of Sadler and Sheldrick to bind to DNA through intercalative and/or covalent binding modes.[16] In contrast, Sheldrick and co-workers have found that a number of other polypyridyl-containing trichloro iridium and rhodium complexes surprisingly did not significantly interact with DNA.[17] To explore this issue further, we investigated the ability of the complexes **10**–**35**, identified from the second round of screening, to interact with DNA by using fluorescence resonance energy transfer (FRET) melting experiments (Figure S5 shows representative spectra for **12**). Our results showed that the complexes exhibited minimal binding to DNA (see Table S2), thus suggesting that the mechanism of action of these complexes is unlikely to involve DNA binding. We hypothesize that the lower cationic charge and substitutional inertness of the complexes lowered their propensity to interact nonspecifically with DNA.

In a preliminary cytotoxicity evaluation, **12** exhibited potent cytotoxicity against A375.S2 ($IC_{50} = 6.6 \pm 3.0$ μM) and A2058 ($IC_{50} < 1$ μM) human melanoma cells, moderate cytotoxicity towards A375 human melanoma cells ($IC_{50} = 17.2 \pm 4.9$ μM), but only low cytotoxicity towards HaCAT human keratinocytes ($IC_{50} > 100$ μM) and normal human dermal fibroblasts ($IC_{50} > 100$ μM; see Figure S6a). In contrast, **14** showed a reduced ability to effectively discriminate between cancerous and normal cells (see Figure S6b).

Given the promising anti-proliferative activity exhibited by **12** in vitro, we investigated the biological efficacy of **12** in a mouse xenograft tumor model. BALB/c nu/nu mice were injected subcutaneously with human malignant melanoma A375 cells, and were treated four times a week with a subcutaneous injection of either **12** (75 mg kg^{-1}) or a control until sacrifice at day 35. Encouragingly, the tumors at sacrifice were visibly smaller in the treatment groups compared to the vehicle control group (Figure 2a). Furthermore, there was a significant difference in the estimated tumor volume in the two groups from day 16 onwards (Figure 2b). The mean tumor weight after sacrifice in the treatment group was reduced by about 60% compared to that of the control group (Figure 2c). We observed that the treated mice exhibited no signs of gross toxicity or weight loss over the course of the experiment (Figure 2d). Additionally, there was no significant difference between the two groups of mice with respect to the mean weights of the heart, liver, and kidney after sacrifice (Figure 2e). Taken together, these results suggest that the rhodium(III) complex **12** was effective at inhibiting the growth of skin cancer tissue in an in vivo model, without causing overt toxicity to the mice.

Because of the critical role of STAT3 in the development of skin cancers, the mechanism of action of **12** on STAT3 signaling was further explored.[10a,18] Complex **12** inhibited the DNA-binding activity of STAT3 ($IC_{50} = 0.83 \pm 0.17$ μM) in a cell-free assay (Figure 3a). Moreover, **12** suppressed STAT3-directed luciferase reporter activity in EGF-stimulated A375 cells ($IC_{50} = 2.4 \pm 0.2$ μM; Figure 3b), thus indicating that **12** could suppress STAT3-driven gene transcription in cells. Furthermore, a fluorescence polarization assay[19] revealed that **12** was able to displace the high-affinity peptide 5′-FAM-GpYLPQTV[20] from the SH2 domain of STAT3 in a dose-dependent manner, with an IC_{50} value of 4.8 μM (Figure 3c), thus suggesting that **12** targets the SH2 domain of STAT3.

While the rhodium(III) complex **12** is expected to be substitutionally inert, the aldehyde groups on its ĈN ligands may be able to react with nucleophilic moieties present in biomolecules. To investigate this issue, we incubated **12** with either cysteine, lysine, or alanine in aqueous buffer solution at

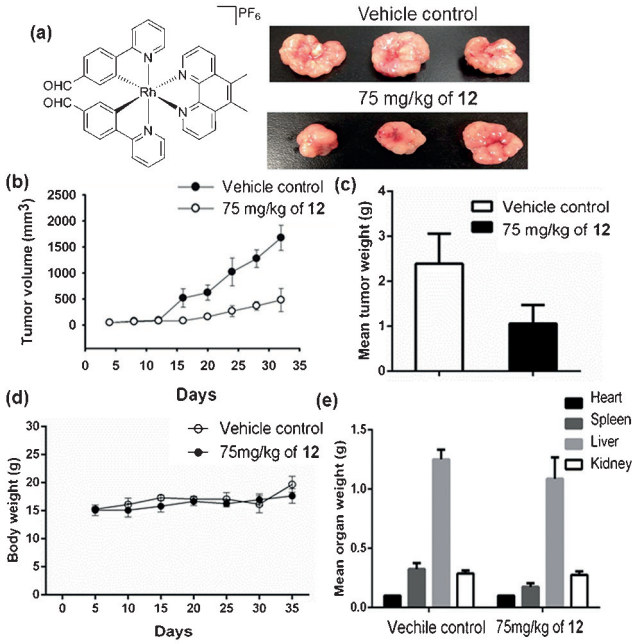

Figure 2. a) Chemical structure of **12** and photographs of dissected tumors from the control (vehicle) and treatment (**12**, 75 mg kg^{-1}). b) Average tumor volume of control group versus treatment group (**12**, 75 mg kg^{-1}). c) Average tumor volume weights measured after sacrifice. Each group contained five mice and results are reported as the values of the mean ± SD. The results were analyzed using the Student's *t*-test. Significantly different at $*p < 0.05$. d) Average body weight of the vehicle control group versus the treatment group (**12**, 75 mg kg^{-1}). e) Average weight of organs (heart, spleen, liver, and kidney) of the two groups. Each group contained five mice, and results were reported as the values of the mean ± SD. The results were analyzed using the Student's *t*-test. Significantly different at $*p < 0.05$.

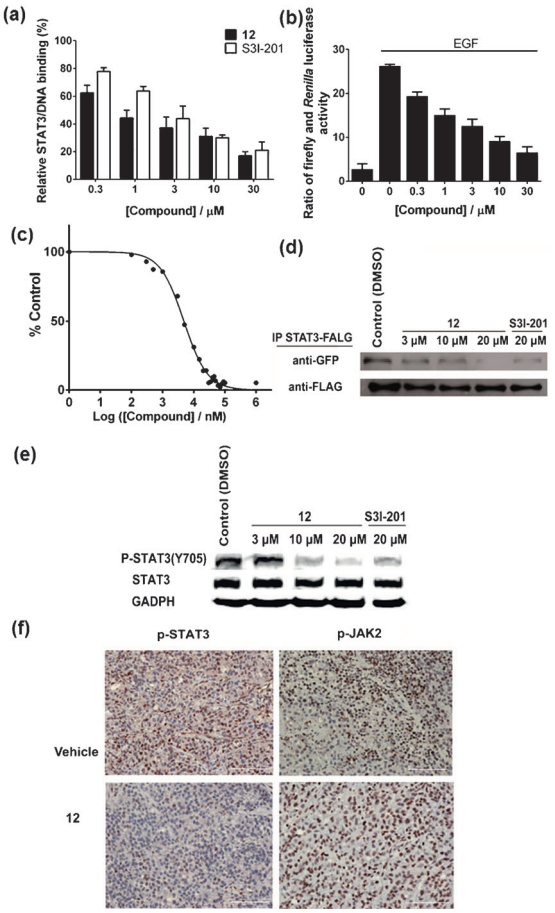

Figure 3. a) **12** inhibits in vitro STAT3 DNA-binding activity in a dose-dependent manner. b) **12** represses the EFG-induced STAT3 transcription in A375 cells as determined by dual luciferase reporter assay. c) **12** prevents STAT3 binding to the fluorescent phosphotyrosine peptide 5′-FAM-GpYLPQTV as revealed by a fluorescence polarization assay. d) **12** reverses the interaction of STAT3-FLAG and STAT3-GFP in transfected A375 cells as analyzed by SDS polyacrylamide gel electrophoresis. e) **12** leads to a dose-dependent decrease of phosphorylation of STAT3 but not total STAT3 in A375 cells. f) Immunohistochemical staining images of xenograft tumors shows the ablility of **12** to inhibit phosphoryation of STAT3 in vivo.

37 °C overnight. However, the resulting MALDI-TOF spectra showed only a single major peak at 675 corresponding to $[\mathbf{12}-PF_6]^+$, and lacked peaks corresponding to covalent **12**–amino acid adducts. This data suggests that the covalent binding of **12** to STAT3 is unlikely to play a role in the mechanism of action of this complex and is consistent with previous work by Lo and co-workers, who showed that the reaction of an iridium(III) complex, which also contained the 4-(pyridin-2-yl)benzaldehyde ĈN ligand, with alanine formed only trace amounts of adducts, thus indicating facile hydrolysis of the imine intermediates.[21]

We next performed a STAT3 pull-down assay using A375 cells co-expressing FLAG-STAT3 and STAT3-GFP to investigate whether **12** could inhibit STAT3 dimerization in cells. In the absence of **12**, STAT3-GFP co-immunoprecipitated with FLAG-STAT3 (Figure 3d). Remarkably, a dose-dependent decrease in the level of STAT3-GFP was observed upon treatment of A375 cells with **12**, thus suggesting that **12** was able to disrupt the interaction between STAT3-GFP and FLAG-STAT3 in cells.

The phosphorylation of STAT3 monomers is essential for dimerization.[22] Treatment of A375 cells with **12** resulted in a dose-dependent reduction in STAT3 tyrosine-705 phosphorylation, but had no effect on total STAT3 content, as observed by Western blotting (Figure 3e). We envision that the inhibition of STAT3 tyrosine phosphorylation could be attributed to the targeting of the SH2 domain of STAT3 by **12**, which prevents its interactions with pTyr residues on cytoplasmic receptor kinases that are essential for the subsequent phosphorylation. The ability of **12** to inhibit STAT3-driven transcription, STAT3 dimerization, and STAT3 phosphorylation in cells was further confirmed in other cell types (see Figures S7–S9). Additionally, unlike previous iridium(III) and rhodium(III) complexes developed by our group, **12** showed no significant effect against JAK2 activity, mTOR activity, or TNF-α binding (see Figures S10, S11, and S12, respectively), thus indicating the importance of chemical structure in determining the selectivity of these substitutionally inert complexes against protein targets. Finally, **12** possessed a log *P* value of −0.596, thus indicating that it is relatively hydrophilic and satisfies Lipinski's lipophilicity criterion ($\log P < 5$) for druglikeness.[23]

To investigate whether **12** inhibited STAT3 signaling in vivo, we performed immunohistochemistry experiments on the xenografted tumor tissues after sacrifice. The treated tumor tissues showed significantly reduced levels of phosphorylated STAT3 compared to the vehicle control group (Figure 3 f), thus suggesting that the anti-tumor activity of **12** against human tumor xenografts could be, at least in part, attributed to the suppression of STAT3 activity in vivo. Moreover, the levels of JAK2 phosphorylation were unaffected, which was consistent with the in vitro data described above. Further experiments showed that inflammatory cytokine COX-2 and inducible nitric oxide synthase (iNOS) expression in tumor tissues were reduced by **12** (Figure 4 a).

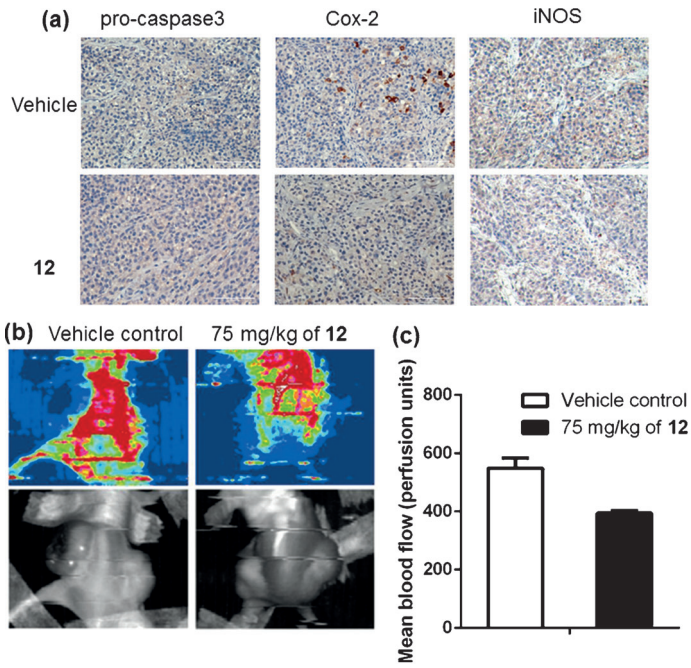

Figure 4. a) Immunohistochemical staining of xenograft tumors using anti-pro-caspase-3, anti-COX-2, and anti-iNOS antibodies. Positive signals are manifested as a brown coloration. b) Laser Doppler flowmetry to assess blood flow around the tumor site in vivo. Photographic image of blood flow from the vehicle control (left) and treatment (right) groups. c) Average blood flow in the control versus treatment groups. The results were subjected to the Student's *t*-test. Significantly different at $*p < 0.05$.

This observation is consistent with previous reports showing that STAT3 promotes the expression of COX-2[24] and iNOS.[25] Given the putative roles of COX-2[26] and iNOS[27] in tumor biology, this result offers another possible avenue by which **12** might exert anti-proliferative activities in the mouse model. In contrast, the levels of pro-caspase-3 were not significantly affected by treatment with **12**.

Angiogenesis is essential for the growth and metastasis of cancers, and blood flow is increased around tumors compared to surrounding normal tissues. Laser Doppler flowmetry[28] results demonstrated that the blood flow around the tumors of mice in the treatment group was reduced compared that of the vehicle control group (Figure 4 b and c). This data suggested that **12** was able to, directly or indirectly, inhibit angiogenesis in the mouse xenograft model, thereby potentially depriving tumor tissues of oxygen and nutrients necessary for proliferation.

Microarray analysis revealed that the focal adhesion, cytokine–cytokine receptor interaction, and leukocyte transendothelial migration pathways contained multiple down-regulated genes in tumor tissues of the treatment group compared to the control group (see Table S3). The down-regulation of vascular endothelial growth factor (VEGF) C is particularly noteworthy as VEGF is a well-known promoter of angiogenesis.[29] Significantly, STAT3 has been reported to up-regulate VEGF expression and tumor angiogenesis in human cancer cell lines[30] and in non-small-cell lung carcinoma patients.[31] We hypothesize that the down-regulation of VEGF expression in tumor tissues could potentially be attributed to the inhibition of STAT3-directed transcription by **12** in vivo, which could, in turn, account for the reduction of blood flow in the treated mice as observed by laser Doppler flowmetry.

In conclusion, we have discovered the novel cyclometalated rhodium(III) complex **12** which represents, to our knowledge, the first example of a substitutionally inert, Group 9 organometallic compound utilized as a direct inhibitor of STAT3. Complex **12** targets the SH2 domain of STAT3, as revealed by a fluorescence polarization assay, and was able to inhibit STAT3 DNA-binding activity in vitro and attenuate STAT3 phosphorylation, dimerization, and signaling activity in cells. Importantly, **12** was able to significantly reduce tumor size and weight in an in vivo mouse xenograft model. Furthermore, tumor tissues treated with **12** showed repressed STAT3 phosphorylation, VEGF expression, and angiogenesis. We hypothesize that the anti-tumor effects of **12** in the mouse model is mediated, at least in part, by the inhibition of STAT3-directed gene expression by **12** in vivo, which could in turn be attributed to its ability to target the SH2 domain of STAT3 and inhibit STAT3 dimerization. We anticipate that this cyclometalated rhodium(III) complex may serve as a useful scaffold for the further development of highly potent inhibitors of STAT3 dimerization as potential anti-neoplastic agents.

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