Organoruthenated Nitroxoline Derivatives Impair Tumor Cell Invasion through Inhibition of Cathepsin B Activity

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ABSTRACT: Lysosomal cysteine peptidase cathepsin B (catB) is an important tumor-promoting factor involved in tumor progression and metastasis representing a relevant target for the development of new antitumor agents. In the present study, we synthesized 11 ruthenium compounds bearing either the clinical agent nitroxoline that was previously identified as potent selective reversible inhibitor of catB activity or its derivatives. We demonstrated that organoruthenation is a viable strategy for obtaining highly effective and specific inhibitors of catB endo- and exopeptidase activity, as shown using enzyme kinetics and microscale thermophoresis. Furthermore, we showed that the novel metallodrugs by catB inhibition significantly impair processes of tumor progression in in vitro cell based functional assays at low noncytotoxic concentrations. Generally, by using metallodrugs we observed an improvement in catB inhibition, a reduction of extracellular matrix degradation and tumor cell invasion in comparison to free ligands, and a correlation with the reactivity of the monodentate halide leaving ligand.

INTRODUCTION

Cathepsin B (EC 3.4.22.1; catB) is a lysosomal cysteine peptidase that belongs to clan CA of the papain family (C1). The proteolytic activity of this enzyme is crucial in the mechanisms of cancer progression. Specifically, it has been identified as an important tumor-promoting factor involved in extracellular matrix degradation, a process which enables tumor migration, invasion, metastasis, and angiogenesis. CatB is unique in its structure among cysteine cathepsins by possessing an occluding loop, a 20 amino acid insertion, which defines whether the enzyme acts as an endopeptidase or an exopeptidase.

At low pH, present within lysosomes, salt bridges hold the occluding loop in the closed conformation, attached on the body of the enzyme, and prevent access of protein substrates to the active site cleft and consequently catB acts as an exopeptidase. At higher pH the salt bridges are weakened, resulting in conformational change, which enables the access of protein substrates to the active site and endopeptidase activity of catB. The endopeptidase activity of catB, with the optimum at neutral pH, is mainly associated with its pathological role, including processes of cancer progression. However, we have demonstrated previously that, in addition to endopeptidase activity, catB exopeptidase activity also contributes to tumor progression. High pharmacological relevance of catB has been established in various tumor mouse models, validating this enzyme as a target for new chemotherapeutic strategies.

In 2011, we identified the antibacterial agent nitroxoline (nxH), a member of the 8-hydroxyquinolone (hq) family, as a potent reversible inhibitor of catB (Figure 1; left). Nitroxoline binds to the S2′ subsite of the catB active site cleft, for which active site mapping of catB with substrates and inhibitors revealed a preference for large aromatic residues. The crystal structure of the enzyme–inhibitor complex revealed that the interaction of the nitro group of nxH with two histidine residues (His110 and His 111) is crucial for binding into an active site, whereas the quinoline ring nitrogen N1 and the hydroxyl group at position 8 are not involved in direct interactions with the protein. Nitroxoline was found to potently impair tumor progression in both in vitro and in vivo models, and these effects correlated with catB inhibition.

This discovery was followed by a structure–activity relationship study of over 50 compounds with structural modifications at positions 2, 5, 7, and 8 and within the quinoline ring. In comparison with the parent nxH, the 7-aminomethyl-8-hydroxy-5-nitroquinoline derivatives (Figure 1; center top)
showed improved endopeptidase activity inhibition and selectivity against other cathepsins. Moreover, one compound from this class was highly effective in in vitro and in vivo cancer models by regulating catB activity.\textsuperscript{20}

Metallodrugs are very important parts of cancer chemotherapeutics as, for example, 7 out of the 10 most commonly used combination therapies include one of three globally used platinum-based agents: cisplatin, oxaliplatin, or carboplatin.\textsuperscript{21}

Figure 1. Summary of previous and present work: (left) discovery of nitroxoline (nxH) as a catB inhibitor; (top center) development of selective catB inhibitors based on a SAR study; (center bottom) discovery of an organoruthenium catB inhibitor; (right) organoruthenium-nitroxolinato complexes presented in this work.

Table 1. Structures of Synthesized Organoruthenium Complexes with Nitroxoline and Its Derivatives

| Ligand/Complex | 1a \textsuperscript{44} | 1b | 1c | 1d |
|----------------|-----------------|---|---|---|
| nxH; (L\textsuperscript{1}H) | (L\textsuperscript{2}H) | (L\textsuperscript{3}H) | (L\textsuperscript{4}H) | (L\textsuperscript{5}H) | (L\textsuperscript{6}H) |
Those agents, however, possess serious downsides as patients often develop resistance to platinum-based agents or display severe side effects. The importance of metals other than platinum is corroborated by the fact that compounds of gallium, copper, titanium, and, most prominently, three ruthenium compounds have entered clinical trials. In recent years, we have been intensely investigating the biological properties and anticancer potential of metal complexes of hydroxyquinolines. For instance, we synthesized an organoruthenium complex (Figure 1; center bottom) of clioquinol (cqH) and showed that the complex (Ru-cq) exhibits selective toxicity toward leukemic cell lines through a caspase-dependent mechanism of cell death. Moreover, we determined that the complex Ru-cq does not interact with DNA and shows proteasome-independent inhibition of the NFκB signaling pathway without affecting cell-cycle distribution. A more detailed study revealed that the complex is a low micromolar inhibitor of catB and impairs the degradation of extracellular matrix and tumor cell invasion at noncytotoxic concentrations, which revealed a specific anticancer mechanism not related to the general intrinsic toxicity.

Cathespin B has previously been investigated as a potential target for metalloinhibitors, most notably, compounds of gold, rhenium, and ruthenium. Despite intensive investigations no structural data are available for catB metalloinhibitors. It is, however, generally accepted that they act through direct metal binding to the Cys29 residue in the enzyme active site which is in close proximity to the nitroxoline binding site. The binding mode of metalloinhibitors was proposed on the basis of an active-site titration and protection-binding site. The binding mode of metalloinhibitors was proposed on the basis of an active-site titration and protection-binding site. The binding mode and overall structural properties of previously known organoruthenium complexes with 8-hydroxyquinolinato complexes.

To continue with the exploration of the chemical space of the 5-nitroquinoline scaffold, we decided to introduce metal-containing fragments to positions 1 and 8 of the nitroxoline ring (Figure 1; right). Herein, we present the synthesis, characterization, and biological evaluation of 10 new and 1 previously reported organoruthenium complex with the parent ligand nitroxoline and five nitroxoline-derived inhibitors of catB.

### RESULTS AND DISCUSSION

**Synthesis and Characterization.** The synthesis of the parent ruthenium nitroxoline complex 1a (Table 1) was reported earlier by Thai et al., but we have modified the method as described below to obtain simple high-yield procedures. In the case of complex 1a, we followed the previously reported simplified and high-yield synthetic route for the preparation of the clioquinolato complex with in-situ deprotonation of the ligand with sodium methoxide in a 1/1 CHCl3/MeOH mixture, removal of the byproduct NaCl from a DCM solution, and precipitation of the product with the addition of n-hexane. The bromido, iodoido, and azido analogues 1b–d were, however, synthesized using acetonitrile as a solvent to allow the removal of NaCl directly from the reaction mixture and facilitate precipitation by addition of n-hexane as, when precipitating from a DCM solution, we often observed the formation of oily products. In the case of the synthesis of complexes with 7-substituted nitroxolines, this approach was unsuccessful. It often resulted in mixtures of several ruthenium-containing species, most likely due to the coordinating ability of the fragments at position 7 of the hq ligand.

In the crude reaction mixture of complex 2a, we observed a few crystals of distinctly different color and morphology and were able to determine the crystal structure of the secondary species 2a*, which proved to be a dimer. In this compound, the new bridging diaionic ligand L2 was bound to one ruthenium ion through the N1 and O8 atoms and to the second ruthenium ion through the carboxylate oxygen, which replaced the dissociated chlorido ligand (see the Supporting Information). Attempts to isolate this compound in pure form have been unsuccessful as yet. In order to achieve even milder reaction conditions, we reacted the ruthenium precursors with the ligands without the addition of a base. The reactions resulted in pure compounds with high reaction yields where, interestingly, ligands L2H and L4H were bound in zwitterionic form with the deprotonated hydroxyl group and the protonated amine group on the substituent in position 7 resulting in PF6 salts of the corresponding cationic complexes 2a,b and 6a. On the other hand, ligands L3−L5 were bound in anionic form, resulting in neutral complexes. This was confirmed by both CHN elemental analysis and the presence of a sharp signal in the IR spectrum at approximately 815–835 cm⁻¹ corresponding to the vibrations of the hexafluorophosphate anion.

We were able to obtain crystals suitable for X-ray crystallography analysis of complexes 1a, 1c, and 2a’ by slow evaporation of 1/1 DCM/n-hexane solutions. The structures (Figure 2) confirm the binding mode and overall structural properties of previously known organoruthenium complexes with 8-hydroxyquinolinato complexes.

The solution stability of 1a was monitored by 1H NMR in DMSO-d6 and D2O. The spectra remained unchanged for 3 and 5 days (Figures S1 and S2), respectively, which conforms with the findings of Kubanik et al., where an in-depth study of solution stability was performed including pD-dependence experiments. Their study revealed the stability of ruthenium hydroxyquinolinato complexes in DMSO-d6 solutions, a quick aquation step with the substitution of halido ligand by D2O molecules in aqueous media, and the stability of the complexes in highly acidic media comparable to the stomach environment.

**Inhibition of catB Activity Determined by Enzyme Kinetics.** All of the prepared organoruthenium complexes were first evaluated in initial enzyme kinetic assay to determine their relative inhibition of catB endopeptidase and exopeptidase activities (Table 2). Relative inhibition is expressed as a percentage of the decrease in reaction velocity in the presence of an inhibitor in comparison to the reaction velocity in the absence of an inhibitor. For that purpose, the specific substrates Z-Arg-Arg-7-amido-4-methylcoumarin (Z-Arg-Arg-Dnp)-OH for catB endopeptidase and exopeptidase activities were used, respectively. CatB cleaves protein substrates at lower pH when the occluding loop is attached to the body of the enzyme active site which is in close proximity to the nitroxoline binding site. The binding mode of metalloinhibitors was proposed on the basis of an active-site titration and protection-binding site. The binding mode and overall structural properties of previously known organoruthenium complexes with 8-hydroxyquinolinato complexes.

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exopeptidase substrate (Abz)-Gly-Ile-Val-Ala-Lys(Dnp)-OH mimics the C-terminal end of a polypeptide chain.

Next, derivatives that exhibited relative inhibition of 20% or more were characterized in detail by determining their inhibition constants and modes of inhibition (Table 2). Four organoruthenium complexes with nitroxoline (1a–d) differed in the reactivity of the monodentate ligand, as the chlorido ligand in the parent Ru-nx complex (1a) was replaced by a bromido, iodido, or azido ligand in compounds 1b–d, respectively. These four monodentate ligands are known to possess increasingly slow substitution/aquation rates, which are known to affect the biological activity of organoruthenium compounds, although to a minor extent. According to the obtained constants of inhibition, the change in monodentate ligand affected the inhibitory properties of complexes against catB. Generally, the formation of organoruthenium complexes with nitroxoline and its derivatives resulted in increased inhibition of catB exopeptidase activity in comparison with the ligands alone (Table 2). In the case of nxH (L₃H) complexes, the most potent inhibitor of catB exopeptidase activity was 1d, with an 8-fold increase in inhibition in comparison to nxH. Moreover, a change in mode of inhibition for complexes 1d and the next most potent inhibitor of catB exopeptidase activity, 1a (5-fold increase of inhibition), was observed, exhibiting a mixed type of inhibition with a predominantly uncompetitive component, in comparison to the noncompetitive mode observed for nxH. On the other hand, inhibition of endopeptidase activity was affected to a lesser extent by ruthenium complexes in comparison to the inhibition by nxH alone. Here, only compound 1d with the azido group as the monodentate ligand the improved inhibition of catB endopeptidase activity in comparison with nxH. Similar to the case for nxH, which is a mixed type inhibitor with a predominantly uncompetitive component, complexes 1c,d with lower constants of inhibition showed an uncompetitive mode of inhibition, while 1a,b with slightly higher Kᵢ values showed a noncompetitive type of inhibition. A

Figure 2. (left to right) Crystal structures of organoruthenium complexes with nitroxoline 1a,c and 2a'. The ellipsoids are drawn at the 35% probability level, and the hydrogen atoms are omitted. Detailed crystallographic data are given in Table S1 in the Supporting Information.

Table 2. Relative Inhibition, Inhibition Constants, and Mechanisms of Inhibition of Cathepsin B Endopeptidase and Exopeptidase Activities

| Compound       | Z-RR-AMC | cathepsin B | Abz-GIVRAK(Dnp)-OH |
|----------------|----------|-------------|-------------------|
|                | Rᵢ (%)   | Kᵢ (%)    | Rᵢ (%) | Kᵢ (%)    | Kᵢ (%)    |
| nxH, L₃H      | 154.4 ± 26.7 | 39.5 ± 2.8 | 22.5 ± 4.7 | 126.2 ± 16.9 | 371.8 ± 16.1 |
| 1a             | 44.3 ± 1.7 | 77.8 ± 16.1 | 22.5 ± 4.7 | 126.2 ± 16.9 | 371.8 ± 16.1 |
| 1b             | 27.2 ± 2.0 | 94.7 ± 1.7 | 12.0 ± 7.4 | 224.6 ± 2.1  | 64.4 ± 5.2  |
| 1c             | 24.9 ± 2.3 | 55.9 ± 4.0 | 11.2 ± 15  | 224.6 ± 2.1  | 64.4 ± 5.2  |
| 1d             | 12.1 ± 1.6 | 21.0 ± 0.4 | 11.2 ± 15  | 224.6 ± 2.1  | 64.4 ± 5.2  |
| 1b             | 92.9 ± 1.9 | 66.5 ± 0.4 | 31.0 ± 6.6 | 216.7 ± 43.1 | 32.1 ± 16.3 |
| L₃H            |        |        | 347.8 ± 16.1 |        |
| 2a             | 21.6 ± 2.1 | n.d.    | 4.2 ± 7.1  | n.d.        |           |
| 2b             | 22.2 ± 1.1 | 140.8 ± 29.9 | 14.6 ± 0.5 | 129.8 ± 20.1 |           |
| L₃H            | 118.8 ± 4.2 | n.d.    | 7.3 ± 3.2  | n.d.        |           |
| 3a             | 17.2 ± 1.5 | 104.4 ± 38.7 | 2.8 ± 1.4  | 117.6 ± 5.6  |           |
| 3c             | 17.2 ± 0.3 | n.d.    | 7.3 ± 3.2  | n.d.        |           |
| L₃H            | 129.8 ± 3.5 | n.d.    | 116 ± 2.4  | n.d.        |           |
| 4a             | 22.3 ± 3.1 | 89.0 ± 2.9 | 24.8 ± 2.2 | 73.2 ± 2.0  | 149.9 ± 3.0 |
| L₃H            | 126 ± 40 | n.d.    | 172 ± 10  | n.d.        |           |
| 5a             | 18.7 ± 2.5 | n.d.    | 13.5 ± 6.0 | n.d.        |           |
| L₃H            | 117.8 ± 3.1 | n.d.    | 108.2 ± 13.4 | n.d.        |           |
| 6a             | 11.7 ± 0.4 | n.d.    | 0.4 ± 0.3  | n.d.        |           |

aThe dissociation constant for the dissociation of enzyme–inhibitor complex. bThe dissociation constant for dissociation of the enzyme–substrate–inhibitor complex. cMixed inhibition. dNoncompetitive inhibition. eUncompetitive inhibition. fCompetitive inhibition; n.d. = not determined. gData reported in ref 15. hData reported in ref 18.
similar effect on catB inhibition was observed also for ruthenium complexes with nitroxoline derivatives. Again, the formation of ruthenium complexes predominantly improved the inhibition of catB exopeptidase activity in comparison with free ligands \(L_2H^{-}\)−\(L_6H\) (at most 2.7-fold for \(2b\)) and only influenced the endopeptidase activity to a lesser extent (Table 2). This observation is also in line with our previous findings, where we showed that larger substituents are preferred for improved inhibition of catB exopeptidase activity.18,19

**Figure 3.** MST affinity analyses of interactions of organoruthenium complexes with cathepsin B: (A) nitroxoline-ruthenium complexes (compounds 1a–d) and (B) ruthenium complexes with nitroxoline derivatives (compounds 2b, 3a, and 4a). Data are presented as \(K_d\) values calculated from normalized fluorescence \(\left(F_{\text{norm}}\right)\) as a function of compound concentration from three independent experiments (mean ± STDEV). The ligand concentration is presented in mol/L. The inserts show MST traces used for calculation of \(F_{\text{norm}}\) for the construction of sigmoid dose–response curves.
microscale thermophoresis (MST). MST is highly sensitive biophysical method that enables the quantification of binding affinity between a ligand and its fluorescently labeled target on the basis of changes in directed movement of a fluorescently labeled molecule in a temperature gradient.\textsuperscript{49,50} To the best of our knowledge, we are aware of only another study where MST was used to study the interactions of metal complexes with their protein targets.\textsuperscript{51}

From dose–response curves and MST traces (Figure 3) we can confirm the binding of compounds 1a–d, 2b, 3a, and 4a to catB. The obtained average dissociation constants ($K_d$) for ruthenium complexes with nitroxoline (1a–d) are in the same concentration range as their constants of inhibition determined by a enzyme kinetic assay and only minor differences in $K_d$ values between complexes with different monodentate ligands can be seen (Figure 3A). In contrast, for ruthenium complexes with nitroxoline derivatives 2b, 3a, and 4a the average $K_d$ values were lower in comparison to the $K_d$ values for endopeptidase activity inhibition obtained using fluorescent substrates in a enzyme kinetics assay: i.e. 14.4-fold (6.2 ± 0.9 μM) for 4a, 7.5-fold (13.9 ± 1.2 μM) for 3a, and 3.6-fold (39.0 ± 5.8 μM) for 2b (Figure 3B). Among them, similarly as in the enzyme kinetic assay, the $K_d$ value was the lowest for 4a and the highest for 2b. The deviation between dissociation and inhibition constants can be explained by differences in experimental setup between two methods: namely, the MST is a binding study, whereas the enzyme kinetics measurements evaluate the ability of an enzyme to degrade a fluorescently labeled substrate.

**Effect on the Viability of Tumor Cells.** Next, we evaluated the complexes in cell-based in vitro assays of tumor invasion and degradation of the extracellular matrix (ECM). To be able to distinguish effects caused by specific mechanisms in functional assays and not only effects due to triggering cell cytotoxicity, we first assessed the cytotoxic effect of ruthenium complexes on MCF-10A neoT cells and selected the concentrations of compounds that did not decrease cell viability for further studies. MCF-10A neoT cells express high levels of proteolytically active catB\textsuperscript{17} and are therefore used as a model cell line for functional assays. Using the MTS assay, we evaluated the cytotoxic effect of ruthenium complexes after 72 h treatment in the concentration range from 250 nM to 5 μM (Figure 4). On the basis of the obtained results, a concentration of 1.25 μM was selected as noncytotoxic for ruthenium complexes 1a,b,d (Figure 4A) and 2b, 3a, and 4a (Figure 4B). Only ruthenium nitroxoline complex 1c bearing the iodido ligand was more cytotoxic; therefore, to avoid an apparent decrease in the tumor cell invasion as a result of the higher cytotoxicity of the compound, the concentration 500 nM was used in a tumor cell invasion assay for 1c (Figure 4A).

**The Ruthenium Complexes Impair Degradation of ECM by Tumor Cells.** During cancer progression, the degradation of ECM is one of the most crucial processes enabling tumor cell migration, invasion, and metastasis. In this process, catB was shown to substantially contribute, acting either extracellularly or intracellularly. Extracellularly, ECM is degraded by secreted and membrane-associated catB, while intracellular degradation of ECM takes place within lysosomes following endocytosis of partially degraded components of ECM.\textsuperscript{18,19} To investigate the effect of ruthenium complexes on the degradation of ECM, we used DQ-collagen type IV and MCF-10A neoT cells that were shown to degrade DQ-collagen type IV both intracellularly and extracellularly.\textsuperscript{17} Collagen type IV is a major component of the ECM that can be tagged with fluorescein, and the proteolytic cleavage gives rise to bright green fluorescence (Figure 5A,D). Degradation of DQ-collagen IV was quantified by flow cytometry for intracellular degradation and by spectrofluorimetry for extracellular degradation (Figure 5). Among the ruthenium complexes with the parent nitroxoline ligand (1a–d) we observed a general trend in both intra- and extracellular DQ-collagen IV degradation rates in dependence on the monodentate ligand (Cl$^-$ > Br$^-$ > I$^-$ > N$_3^-$ ≥ 1$^-$). Complexes 1a, 1b, 1d, and 1c at a concentration of 1.25 μM reduced intracellular degradation by 45 ± 2%, 40 ± 3%, 31 ± 2%, and 21 ± 2%, respectively (Figure 5B). A similar pattern was observed also for extracellular degradation that was reduced by 35 ± 2%, 26 ± 3%, 21 ± 3%, and 19 ± 2% for complexes 1a, 1b, 1d, and 1c at a concentration of 1.25 μM, respectively (Figure 5C). Despite the reduction in cell viability for complex 1c (Figure 5A) we included this concentration in the assays, as ECM degradation experiments were performed at shorter time points in comparison to viability assays. In addition, when intracellular degradation was monitored by flow cytometry, propidium iodide was added to exclude dead cells and only effects in viable cells were measured. At a lower concentration (0.5 μM) 1c displayed a less pronounced effect on DQ-collagen IV

![Figure 4](image-url)
degradation (Figure 5B,C). The most potent inhibitor of both intra- and extracellular DQ-collagen IV degradation was complex 1a, bearing the chlorido ligand as the leaving group.

Furthermore, ruthenium complexes with nitroxoline derivatives 2b, 3a, and 4a also significantly impaired DQ-collagen IV degradation, both intracellularly and extracellularly. The most potent inhibitor was 4a, which reduced intracellular and extracellular degradation by 68 ± 1% and 40 ± 1%, respectively (Figure 5E), whereas 3a and 2b reduced intracellular degradation by 36 ± 4% and 18 ± 5%, respectively, and extracellular degradation by 31 ± 1% and 17 ± 3%, respectively (Figure 5F). These results also confirm our previous observation that both intra- and extracellular DQ-collagen IV degradation is dependent on the monodentate ligand, as both complexes with nitroxoline derivatives bearing the chlorido ligand as the leaving group (3a and 4a) were more potent inhibitors in comparison to 2b bearing the bromido ligand. Moreover, the results obtained for ruthenium complexes with nitroxoline derivatives also point out the importance of the main ligand in the ruthenium complexes and its potency for catB inhibition, as compounds 1a, 3a and 4a, all bearing the chlorido ligand as the leaving group, have different potencies for inhibition of ECM degradation, where a higher...
The Ruthenium Complexes Reduce Tumor Cell Invasion at Noncytotoxic Concentrations. To further characterize the antitumor characteristics of selected complexes, their effect on tumor cell invasion was evaluated. For this purpose, we continuously monitored the invasion of MCF-10A neoT cells through Matrigel, a model of ECM, using the xCELLigence system (Figure 6A,C). The system follows tumor cell invasion during the whole course of the experiment by continuously measuring the impedance, expressed as cell index (CI), across the microelectrodes integrated in the membrane separating the top and bottom compartments of the CIM (cell invasion and migration) plate 16. As shown in Figure 6A,B, low noncytotoxic concentrations of 1a–d which bear different monodentate ligands impaired tumor cell invasion to different extents. The most potent was complex 1a, which significantly reduced tumor invasion of MCF-10A neoT cells by 82 ± 3%, followed by 1b with a 59 ± 13% reduction in invasion (both at a concentration of 1.25 μM), and 1c with a reduction of 48 ± 13% at 0.5 μM (Figure 6B). The invasion of MCF-10A neoT cells in the presence of 1d was not significantly impaired (Figure 6B). In line with the data obtained for degradation of ECM, these data show that complexation of nitroxoline with an organoruthenium moiety further improves in vitro inhibition of tumor cell invasion. In comparison to ECM degradation using DQ-collagen IV, tumor cell invasion is a more complex process involving the coordinated action of several peptidases. Therefore, inhibition of one of them could have an effect on other peptidases and their contribution to the tumor invasion. That could be an explanation for the differences in some of the compounds, such as 1d, between the ECM degradation assay and the invasion assay. Moreover, compensational mechanisms observed between related cathepsins could be differently affected by the complexes. The process of tumor invasion also involves the action of other tumor-promoting molecules that could also serve as therapeutic targets. In addition to affecting catB activity, nxH was identified to impair tumor progression by inhibition of other molecular targets, such as methionine aminopeptidase type 2, sirtuin, Foark head box protein 4 (BRD4) member of the BET family. Additionally, nxH inhibits tumor progression by inducing apoptosis. Therefore, the possible effect of complexes 1a–d on other targets involved in tumor invasion cannot be excluded. In addition, ruthenium complexes with nitroxoline derivatives 2b, 3a, and 4a also significantly impaired invasion of MCF-10A neoT cells (Figure 6C,D). In this group, compound 2b was the best inhibitor with 86 ± 8% inhibition of cell invasion, whereas 3a and 4a decreased this process by 54 ± 8% and 38 ± 8%, respectively (Figure 6D). Again, differences between the ECM degradation assay and the cell invasion assay can be observed, probably as a result of the aforementioned processes. Also, nitroxoline derivatives with different substituents can affect the selectivity for catB affecting the activity of other cathepsins in addition to catB.
Taken together, we have demonstrated here that new ruthenium complexes in noncytotoxic 1.25 μM concentrations significantly decreased tumor cell invasion by inhibition of tumor-promoting peptidase catB. In addition to catB, the compounds may affect also other specific targets involved in cell invasion.

**CONCLUSIONS**

By synthesizing 11 ruthenium compounds bearing either the clinical agent nitroxoline (nxH) or its potent cathepsin B (catB) inhibiting derivatives, we have demonstrated that organorhuthenation of the lead nxH scaffold is a viable strategy for obtaining highly effective catB inhibitors, the agents that efficiently reduce tumor cell invasion. Using enzyme kinetics and MST, we showed that the novel metallodrugs inhibit both endo- and exopeptidase activity of catB and significantly impair the processes of tumor progression such as degradation of ECM and tumor cell invasion.

The present study provides us with several insights into the influence of the chemical structure on the pharmacological properties of this class of compounds. As a general trend, we observed an improvement in catB inhibition, lowering of ECM properties of this class of compounds. As a general trend, we observed an improvement in catB inhibition, lowering of ECM and tumor cell invasion.

**EXPERIMENTAL SECTION**

**Materials and Methods.** [(η⁶-p-cymene)RuCl(μ-Cl)]_2 (P1) was purchased from Strem Chemicals, nitroxoline (nxH), [(η⁶-p-cymene)-Ru(μ-η⁶)]_2 (P3), and the solvents were obtained from Sigma-Aldrich. All of the materials were used as received. The bromide precursor [(η⁶-p-cymene)RuBr(μ-Br)]_2 (P2) and [(η⁶-p-cymene)RuCl(μ-η⁶)]_2 (P4) were synthesized by reacting precursor P1 with excess KBr and NaN₃, respectively, according to published procedures.⁵¹,⁶² Nitroxoline-based ligands were prepared according to the published procedure.⁵³ H NMR spectra were recorded on a Bruker Avance III 500 spectrometer at room temperature and 500.10 MHz by using TMS as an internal standard. Infrared spectra were recorded with a PerkinElmer Spectrum 100 FTIR spectrometer, equipped with a Specac Golden Gate Diamond ATR as a solid sample support. UV–vis spectra were collected on a PerkinElmer LAMBDA 750 UV/vis/near-IR spectrophotometer. Elemental analyses were recorded using a PerkinElmer 2400 II instrument (CHN), and HRMS were measured on an Agilent 6224 Accurate Mass TOF LC/MS instrument. X-ray diffraction data were collected on an Oxford Diffraction SuperNova diffractometer with a Mo/Cu microfocus X-ray source (Kr radiation, λMo = 0.71073 Å, λCu = 1.54184 Å) with mirror optics and an Atlas detector at 150(2) K. The structures were solved in Olex² graphical user interface⁶³ by direct methods implemented in SHELXT and refined by a full-matrix least-squares procedure based on F² using SHELXL. All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were placed at calculated positions and treated using appropriate riding models. The crystal structures have been submitted to the CCDC and have been allocated the deposition numbers 1909464—1909466.

**Syntheses and Characterization.** [Ru(η⁶-p-cymene)Cl(nxH)] (1a). The synthesis and characterization of complex 1a were previously reported.⁶⁴ Here, we report an improved reliable high-yield synthesis procedure. A 40.0 mg portion of the ruthenium chloride precursor (P1, 0.0650 mmol), 24.9 mg of nxH (1.00 equiv, 0.130 mmol), and 7.0 mg of NaOMe (0.130 mmol) were dissolved in 20 mL of a 1/1 mixture of CHCl₃ and MeOH. The solution was refluxed for 2 h and the solvent evaporated. The crude product was redissolved in DCM and the solution filtered to remove the precipitated NaCl. The filtrate was concentrated on a rotary evaporator and precipitated by the addition of n-hexane. The orange precipitate was collected by filtration and washed with n-hexane (η = 90%).

H NMR (500 MHz, CDCl₃): δ 9.51 (d, J = 8.8 Hz, 1H, C²H); 9.01 (d, J = 4.6 Hz, 1H, C¹H); 8.56 (d, J = 9.3 Hz, 1H, C¹H); 7.63 (dd, J = 8.9, 4.8 Hz, 1H, C¹H); 6.90 (d, J = 9.3 Hz, 1H, C¹H); 5.68 (d, J = 5.9 Hz, 1H, Ar-H-1); 5.57 (d, J = 5.8 Hz, 1H, Ar-H-5); 5.50 (d, J = 5.9 Hz, 1H, Ar-H-6); 5.39 (d, J = 5.8 Hz, 1H, Ar-H-4); 2.81 (sept, J = 6.8 Hz, 1H, Ar-CH(CH₃)-2); 2.33 (3H, 3H, Ar-CH(CH₃)-1); 1.20 (dd, J = 6.9, 2.7 Hz, 6H, Ar-CH(CH₃)-3, 5). Selected IR resonances (cm⁻¹, ATR): 2969, 1595, 1565, 1510, 1481, 1464, 1385, 1287, 1278, 1189, 1149, 860, 820, 788, 742. Anal. Calc'd for C₂₂H₁₆Cl₂InO₂Ru: C, 39.06; H, 3.72; N, 5.06. Found: C, 39.06; H, 3.73; N, 5.05. UV/vis (λ, nm (ε; L mol⁻¹ cm⁻¹)) = 1 × 10⁻³ mol L⁻¹, MeOH: 268 (15000), 350 (6500), 430 (14100), 450 (13900). ESI-HRMS (CH₃CN) m/z (observed [M – Cl]⁻, [M + H]⁺, [M + H]⁺ (expected)): 425.0439 (425.0439) 461.0198 (461.0206), 500.9703 (500.9703), 531.9228 (531.9228).

[6Ru(η⁶-p-cymene)Br(nxH)] (1b). A 40.0 mg portion of the ruthenium bromide precursor (P2, 0.0500 mmol) and 19.0 mg of nxH (1.00 equiv, 0.100 mmol) were suspended in 20 mL of acetone and stirred at room temperature for 3 h. The precipitate was filtered off, the deep orange filtrate was concentrated on a rotary evaporator, and the product was precipitated by the addition of n-hexane. The orange precipitate was collected by filtration and washed with n-hexane (η = 90%).

H NMR (500 MHz, CDCl₃): δ 9.51 (d, J = 8.7 Hz, 1H, C²H); 8.98 (s, 1H, C¹H); 8.56 (d, J = 9.3 Hz, 1H, C¹H); 7.62 (d, J = 4.9 Hz, 1H, C¹H); 6.90 (d, J = 9.3 Hz, 1H, C¹H); 5.64 (d, J = 5.3 Hz, 1H, Ar-H-4); 5.54 (dd, J = 22.1, 5.1 Hz, 2H, Ar-H-4); 5.40 (d, J = 4.9 Hz, 1H, Ar-H-6); 2.88 (sept, J = 13.5, 6.7 Hz, 1H, Ar-CH(CH₃)-2); 2.33 (3H, 3H, Ar-CH(CH₃)-1); 1.22 (d, J = 6.8 Hz, 6H, Ar-CH(CH₃)-1, 5). Selected IR resonances (cm⁻¹, ATR): 2969, 1595, 1564, 1564, 1505, 1476, 1464, 1412, 1384, 1271, 1180, 1142, 1103, 810, 781, 745. Anal. Calc'd for C₂₂H₁₆Br₂InO₂Ru: C, 42.55; H, 3.80; N, 5.55. Found: C, 45.16; H, 3.66; N, 5.43. UV/vis (λ, nm (ε; L mol⁻¹ cm⁻¹)) = 1 × 10⁻³ mol L⁻¹, CHCl₃: 276 (18900), 372 (9900), 455 (20900), 478 (20200). ESI-HRMS (CH₃CN) m/z (observed [M – Br]⁻, [M + H]⁺ (expected)): 419.0466 (419.0466), 456.0198 (456.0206), 493.9703 (493.9703).
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n-hexane. The brown precipitate was collected by filtration and washed with n-hexane (n = 85%).

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 9.59 (dd, $J = 8.9, 1.3$ Hz, 1H, C'H$^2$), 8.99 (dd, $J = 4.9, 1.3$ Hz, 1H, C'H$^4$), 8.60 (dd, $J = 9.3$ Hz, 1H, C'H$^1$), 7.70 (dd, $J = 9.0, 4.9$ Hz, 1H, C'H$^4$), 6.93 (dd, $J = 9.3$ Hz, 1H, C'H$^3$), 5.67 (dd, $J = 6.0$ Hz, 1H, Ar-H cym), 5.57 (dd, $J = 5.8$ Hz, 1H, Ar-H cym), 5.57 (dd, $J = 5.9$ Hz, 1H, Ar-H cym), 2.81 (dt, $J = 14.0, 7.0$ Hz, 1H, Ar-CH$_2$ cym), 2.35 (s, 3H, Ar-CH$_3$ cym), 1.23 (d, $J = 7.0$ Hz, 6H, Ar-CH$_2$(CH$_3$)$_2$ cym). Selected IR resonances (cm$^{-1}$); ATR: 2961, 2027, 1595, 1565, 1507, 1475, 1465, 1415, 1274, 1181, 1164, 1104, 812, 793, 746. Anal. Calc. for C$_{6}$H$_{15}$N$_{2}$O$_{4}$Ru: C, 48.49; H, 4.75; N, 7.07. Found: C, 48.19; H, 4.35; N, 7.07. UV/vis ($\lambda$, nm; $L$ mol$^{-1}$ cm$^{-1}$) = 1 $\times$ 10$^{-4}$ mol L$^{-1}$, MeOH: 269 (12600), 344 (5000), 343 (11000). ESI-HRMS (CH$_3$CN) m/z (observed [M - Cl]) (expected): 560.884 (560.0890).

$^{[Ru]_2(p$-cymene)(Cl)(L$^2$)P$_2$] (2a). A 30.0 mg portion of the ruthenium dichloride precursor (P1, 0.0494 mmol) was filtered off, 32.5 mg of L$^2$ (1.00 equiv, 0.098 mmol), and 20 mg of KPF$_6$ (1.10 equiv, 1.1 mmol) were dissolved in 20 mL of acetone, and the solution was stirred for 2 h at room temperature. KCl and excess KPF$_6$ were removed by filtration. The filtrate was concentrated on a rotary evaporator and precipitated by the addition of n-hexane. The pale orange precipitate was collected by filtration and washed with n-hexane (n = 85%).

$^1$H NMR (500 MHz, acetone-d$_6$): $\delta$ 9.42 (dd, $J = 5.0, 1.3$ Hz, 1H), 9.25 (dd, $J = 8.9, 1.3$ Hz, 1H), 8.56 (dd, $J = 18.4$ Hz, 1H), 7.81 (dd, $J = 8.9, 5.0$ Hz, 1H), 5.88 (dd, $J = 12.4, 6.2$ Hz, 2H), 5.64 (dd, $J = 5.9$, 4.3 Hz, 2H)$_1$, 4.84 (dd, $J = 27.3$, 13.3 Hz, 4.51 (t, $J = 13.5$ Hz, 1H), 3.72 (dd, $J = 86.6, 12.9$ Hz, 2H), 3.25 (t, $J = 12.9$ Hz, 2H), 2.81 (td, $J = 8.6$, 7.8, 6.0 Hz, 1H), 2.64 (td, $J = 1.2$, 3.7 Hz, 1H), 2.17 (s, 3H), 1.96 (s, 2H), 1.22-1.09 (m, 7H). Selected IR resonances (cm$^{-1}$); ATR: 1716, 1597, 1562, 1518, 1466, 1410, 1287, 1197, 1136, 820. Anal. Calc. for C$_{14}$H$_{29}$Cl$_2$Ni$_2$P$_2$Ru: C, 31.86; H, 4.05; N, 5.63. Found: C, 31.70; H, 3.92; N, 5.42. UV/vis ($\lambda$, nm; $L$ mol$^{-1}$ cm$^{-1}$) = 1 $\times$ 10$^{-4}$ mol L$^{-1}$, MeOH: 269 (11750), 345 (47900), 476 (9970), ESI-HRMS (CH$_3$CN) m/z (observed [M - PF$_2$]) (expected): 602.0988 (602.09).
mol L⁻¹, MeOH: 271 (15000), 350 (7500), 456 (13000). ESI-HRMS (CH₃CN) m/z observed [M + H⁺]⁺: 543.0730 (543.0737). [Ru(npy)_2-p-cymene]Cl[(L²H²)]PF₆ (6a). A 30.0 mg portion of the ruthenium chloride precursor (P1, 0.049 mmol), 28 mg of L¹H¹ (1.00 equiv, 0.098 mmol), and 20 mg of KPF₆ (1.10 equiv, 1.1 mmol) were dissolved in 20 mL of acetone, and the solution was stirred for 2 h at room temperature. KCl and excess KPF₆ were removed by filtration. The filtrate was concentrated on a rotary evaporator and precipitated by the addition of n-hexane. The orange precipitate was collected by filtration and washed with n-hexane (n = 90%).

1H NMR (500 MHz, acetone-d₆): 9.55 (d, J = 5.0, 1.2 Hz, 1H), 9.40 (d, J = 9.0, 1.1 Hz, 1H), 8.71 (s, 1H), 7.95 (ddd, J = 9.0, 4.9, 0.9 Hz, 6.04 (m, 5.2H), 5.78 (t, J = 6.3 Hz, 2H), 5.05 (q, J = 4.9, 1.4 Hz, 1H), 4.66–4.59 (m, 1H), 3.89–3.81 (m, 1H), 3.80–3.72 (m, 1.2 Hz, 1H), 3.56 (d, J = 10.7 Hz, 1H), 3.45 (dq, J = 13.6, 7.6, 6.8 Hz, 1H), 2.95 (dt, J = 14.5, 7.3 Hz, 1H), 2.32 (s, 3H), 2.10 (d, J = 1.2 Hz, 3H), 1.36–1.23 (m, 7H). Selected IR resonances (cm⁻¹, ATR): 2971, 1739, 1592, 1541, 1464, 1366, 1217, 1135, 815, 751. Anal. Calcd for C₂₉H₂₇ClF₅NO₇PrCu: C, 41.90; H, 4.10; N, 6.11. Found: C, 41.52; H, 3.92; N, 5.94. UV/vis (λ max, nm): 344 (4170), 444 (9200). ESI-HRMS (CH₃CN) m/z observed [M – PF₆]⁻: 544.0937 (544.0900).

**Enzyme Kinetics.** Human recombinant catB was prepared as reported.⁵⁵ The catB endopeptidase and exopeptidase assay 100 mM phosphate buffer (pH 6.0) and 200 mM acetate buffer (pH 5.0) were respectively used. Each contained 0.1% PEG 8000 (Sigma-Aldrich, St. Louis, MO, USA), 5 mM cysteine, and 1.5 mM EDTA. Prior to the assay, the enzyme was activated in the assay buffer for 5 min at 37 °C. **Determination of Relative Inhibition.** The effect of inhibitors on catB endopeptidase and exopeptidase activity was determined using the substrates Z-Arg-Arg-AMC and Abz-Gly-Ile-Val-Arg-Ala-Lys(Dnp)-OH (Aldrich, St. Louis, MO, USA), 5 mM cysteine, and 1.5 mM EDTA. To prevent false-positive inhibition due to the formation of complexes with catB was evaluated using microscale thermophoresis (MST) on a Monolith NT.115 Spico Red apparatus (NanoTemper Technologies, Munich, Germany). MST is a biophysical method that allows quantitative analysis of wide range of bimolecular interactions in free solution. It is based on the change in motion of fluorescently labeled molecules in a temperature gradient following the ligand binding.⁶⁵,⁶⁶

For MST detection, recombinant catB was fluorescently labeled using Monolith NT Protein Labeling Kit RED-NHS (NanoTemper Technologies) with the red fluorescent dye NT-647-NHS (NanoTemper Technologies) according to the manufacturer's instructions. Sixteen-step serial dilutions of inhibitors were prepared in 100 mM phosphate buffer (pH 6.0) supplemented with 0.1% PEG 8000, 5 mM cysteine, 1.5 mM EDTA, and 0.05% Tween 20 starting with the final concentration of 1000 μM for each compound. The labeled catB was diluted in assay buffer to a final assay concentration of 5 nM, activated for 5 min at 37 °C prior to the assay, and added to the serial dilutions of inhibitors in a 1:1 volumetric ratio. All assay mixtures contained 5% (v/v) DMSO. The samples were loaded into Monolith NT.115 Standard Treated Capillaries (NanoTemper Technologies). The experiments were performed using medium MST power (40%) and 60% excitation power. Measurements were repeated at least twice. Binding affinities expressed as constants of dissociation (Kₐ) were obtained by fitting normalized fluorescence (Fₛₑₜₑ) calculated from MST traces as the ratio between fluorescence after thermodiffusion and the initial fluorescence against increasing inhibitor concentrations. Data were analyzed using MO Affinity Analysis Software (NanoTemper Technologies).

**Cell Culture.** MCF-10A neoT, a c-Ha-ras oncogene transfected human breast epithelial cell line, was provided by Boston, MA, USA) was added and cells were incubated for an additional 2 h at 37 °C. Propidium iodide (BD Biosciences) was used to exclude dead cells, and green fluorescence arising from DQ-collagen IV (10 μg/mL) in 100 μL of medium containing 0.25, 0.5, 1.25, 2.5, or 5 μM of the respective compound or DMSO (0.05%). After 72 h treatment, 10 μL of MTS (Promega, Madison, WI, USA) was added to the wells and the following incubation absorbance of formazan was measured at 492 nm on a Tecan Safire 2 apparatus. The cell viability (%) was expressed as the ratio between absorbance obtained in the presence of compounds versus DMSO. All assays were performed in quadruplicate and repeated at least two times.

**DQ-Collagen IV Degradation Assay.** The effect of ruthenium complexes with nitroxoline and its derivatives on the cell viability of the MCF-10A neoT cells was assessed using an MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2′H-tetrazolium) colorimetric assay. Into wells of a 96-well microplate were seeded 1 × 10⁴ cells and incubated overnight to attach. The medium was replaced with 200 μL of medium containing 0.25, 0.5, 0.5, 1.25, 2.5, or 5 μM of the respective compound or DMSO (0.05%). After 72 h treatment, 10 μL of MTS (Promega, Madison, WI, USA) was added to the wells and the following incubation absorbance of formazan was measured at 492 nm on a Tecan Safire 2 apparatus. The cell viability (%) was expressed as the ratio between absorbance obtained in the presence of compounds versus DMSO. All assays were performed in quadruplicate and repeated at least two times.
µL) was transferred into empty wells of a 96-well black microplate, where the fluorescence intensity was continuously monitored for 2 h at 515 ± 5 nm with excitation at 495 ± 5 nm on a ‘Tecan Safire’ apparatus. The inhibition of extracellular DQ-collagen IV degradation was expressed as the ratio between the average 2 h fluorescence obtained in the presence of compounds versus DMSO.

**Real-Time Invasion Assay.** A real-time tumor cell invasion assay to monitor the effect of ruthenium complexes with nitroxoline and its derivatives on MCF-10A neoT cells was performed on the xCELLigence Real Time Cell Analyzer (RTCA; ACEA Biosciences Inc., San Diego, CA, USA). Prior to the assay, the cells were serum-starved for 24 h. First, on the CIM-plate 16 (ACEA Biosciences Inc.) the down sides of the microporous PET membrane were coated with 0.3 µg of fibronectin from bovine plasma (Calbiochem, Darmstadt, Germany) for 30 min at room temperature. Then, the upper chambers of CIM-plate 16 wells were coated with 20 µL of Matrigel (1 mg/mL; BD Biosciences, Franklin Lakes, NJ, USA) in serum-free medium (SFM) and allowed to gel for 30 min at 37 °C. The compound (1.25 or 0.5 µM for compound 1c) in the complete medium (180 µL) was added to the lower chambers and afterward the top and bottom parts of the CIM-plate 16 were assembled together. To the upper chambers was added the compound (1.25 or 0.5 µM for compound 1c) in 60 µL of SFM. DMSO (0.05%) was used as a control. The CIM-plate 16 was allowed to equilibrate for 1 h at 37 °C. Finally, after equilibration, MCF-10A neoT cells (3 × 10⁴ cells/well) in 80 µL of SFM were seeded in the top chambers of CIM-plate 16. The xCELLigence system measured impedance data, reported as cell index (CI), in real time every 15 min during the entire course of the experiment for 72 h. Obtained data were analyzed using the RTCA Software (Roche). The relative invasion (%) was expressed as a percentage relative to the control cells treated with DMSO.

**Statistical Analysis.** Data were analyzed using the GraphPad Prism 6.0 software package and are presented as mean ± SEM unless stated otherwise. Results were compared by Student’s t test (nonparametric, two-tailed). Differences were considered significant at P < 0.05.

**ASSOCIATED CONTENT**

1. Supporting Information
   The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.inorgchem.9b01882.

Crystallographic data and NMR solution stability studies of compound 1a in DMSO-d₄ and D₂O (PDF)

**Accession Codes**

CCDC 1909464–1909466 contain the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 360333.

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**Notes**

The authors declare no competing financial interest.

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