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Highly cytotoxic Cu(II) terpyridine complexes as chemotherapeutic agents†

Kaixin Ni,‡ Nicolás Montesdeoca‡ and Johannes Karges *

Cancer is considered as the biggest medicinal challenge worldwide. During a typical treatment, the tumorous tissue is removed in a surgical procedure and the patient further treated by chemotherapy. One of the most frequently applied drugs are platinum complexes. Despite their clinical success, these compounds are associated with severe side effects and low therapeutic efficiency. To overcome these limitations, herein, the synthesis and biological evaluation of Cu(II) terpyridine complexes as chemotherapeutic drug candidates is suggested. The compounds were found to be highly cytotoxic in the nanomolar range against various cancer cell lines. Mechanistic insights revealed that the compounds primarily accumulated in the cytoplasm and generated reactive oxygen species in this organelle, triggering cell death by apoptosis. Based on their high therapeutic effect, these metal complexes could serve as a starting point for further drug development.

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Introduction

The last decades, cancer remains the leading cause of death worldwide.[1] Statistics from the World Health Organization indicated that nearly 10 million people died from cancer in 2020, and it is predicted that the number of new cancer diagnoses per year is expected to rise to 29.5 million and the number of cancer-related deaths to 16.4 million by 2040.[2] To date, platinum complexes (*i.e.*, cisplatin, oxaliplatin, carboplatin) are among the most commonly used chemotherapeutic agents worldwide.[3] Despite their frequent clinical use, these compounds are associated with severe side effect such as nerve and kidney damage, nausea, vomiting, or bone marrow suppression.[4] Insights into the mechanism of action revealed that the metal complexes coordinate to the nucleobases of the DNA, triggering cell death of the cancer cells.[5] However, due to various biological barriers only a small fraction of the metal complex reaches its target. A clinical study has reported that approximately 95% of the administered dose binds to human serum albumin and is therefore therapeutically deactivated.[6] Further analyses indicated that less than 1% of the administered doses accumulated in the tumor and less than 0.01% binds to the nuclear DNA, causing the desired therapeutic effect.[7] These results strongly suggest that the clinically used platinum complexes are associated with a poor therapeutic efficiency and that there is a need for the development of new compounds with an improved therapeutic efficiency.

Copper complexes as anticancer agents have received increasing attraction due their high bioavailability and biocompatibility.[8] The ability of copper to change its redox state between copper(I) and copper(II) under physiological conditions allows for electron transfer reactions inside the cancer cells and ultimately the generation of reactive oxygen species.[9] Reactive oxygen species are able to induce oxidative stress inside the cancer cells, causing damage to biomolecules such as lipids, proteins, and DNA and therefore triggering cell death.[9b,10] Capitalizing on this, considerable research effort has been focused on developing copper-containing compounds with anticancer properties.[8–11] Several copper(II) polypyridine complexes have demonstrated to localize in the nucleus of cancerous cells and interact with DNA. In this localization, these compounds are able to generate reactive oxygen species, causing oxidative cleavage of the phosphodiester bond of the DNA and ultimately triggering cell death.[12] Our own group has recently reported on terminally functionalized copper(II) terpyridine complexes with a high cytotoxic effect against drug resistant colon or lung cancer cells.[11b,13] Recently variously functionalized copper(II) 4′-phenyl-2,2′:6′,2″-terpyridine complexes have been reported as highly active anticancer agents that interact through a multimodal mechanism including cell cycle arrest, mitochondria misfunctioning and cell apoptosis. Promisingly, the compounds demonstrated a strong tumor growth inhibition effect in a tumor-bearing mouse model.[14] Based on the generally promising properties of copper(II) complexes, the Casiopeinas® complexes with the

Faculty of Chemistry and Biochemistry, Ruhr-University Bochum, Universitätsstrasse 150, 44780 Bochum, Germany. E-mail: johannes.karges@ruhr-uni-bochum.de; https://www.kargesgroup.ruhr-uni-bochum.de+49 2343224187

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‡The authors have contributed equally to this study.

molecular formula $[Cu(NN)(OO)]NO_3$ or $[Cu(NN)(NO)]NO_3$ (NN = 2,2′-bipyridines or 1,10-phenanthrolines, OO = acetylacetonate or salicylaldehyde, NO = amino acid or peptide) are currently studied in clinical trials in Mexico.[15] These specific metal complexes were found with a high cytotoxic effect against various cancer cell lines, inducing cell death through apoptosis by catalytic redox cycling.[16]

Based on these promising findings, herein, a structure–activity relationship study of differently methoxy functionalized copper(II) terpyridine complexes including Cu(4′-(4′-*ortho*-methoxy-phenyl)-2,2′:6′,2″-terpyridine)$(Cl)_2$ (**Cu1**), Cu(4′-(4′-*meta*-methoxy-phenyl)-2,2′:6′,2″-terpyridine)$(Cl)_2$ (**Cu2**), and Cu(4′-(4′-*para*-methoxy-phenyl)-2,2′:6′,2″-terpyridine)$(Cl)_2$ (**Cu3**) was performed. The compounds exhibited a high cytotoxicity at nanomolar concentrations against diverse cancer cell lines. Mechanistic investigations revealed that these compounds predominantly accumulated in the cytoplasm, inducing the generation of reactive oxygen species within this organelle. The reactive oxygen species production led to cell death through apoptosis. Based on their high therapeutic effects, these metal complexes could be considered as a promising starting point for subsequent drug development.

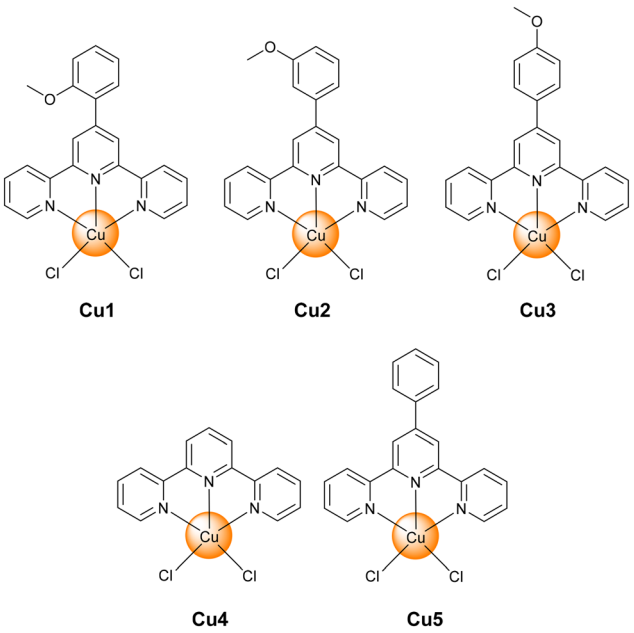

Fig. 1 Chemical structures of Cu(II) terpyridine complexes **Cu1–Cu3** investigated in this study. The metal complexes **Cu4** [11b] and **Cu5** [13] have been previously reported and are used here for comparison with previous studies.

Results and discussion

The terpyridine ligands 4′-(4′-*ortho*-methoxy-phenyl)-2,2′:6′,2″-terpyridine,[17] 4′-(4′-*meta*-methoxy-phenyl)-2,2′:6′,2″-terpyridine,[18] and 4′-(4′-*para*-methoxy-phenyl)-2,2′:6′,2″-terpyridine were synthesized according to the previous published reports.[19] The differently methoxy functionalized benzaldehydes, 2-acetylpyridine, and potassium hydroxide were dissolved in ethanol and stirred at room temperature for several hours. After this time, ammonia was added and the mixture stirred at room temperature overnight to generate the tridentate terpyridine ligand scaffold.[19c] The desired 4′-(4′-substituted-phenyl)-2,2′:6′,2″-terpyridine copper(II) complexes Cu(4′-(4′-*ortho*-methoxy-phenyl)-2,2′:6′,2″-terpyridine)$(Cl)_2$ (**Cu1**), Cu(4′-(4′-*meta*-methoxy-phenyl)-2,2′:6′,2″-terpyridine)$(Cl)_2$ (**Cu2**), and Cu(4′-(4′-*para*-methoxy-phenyl)-2,2′:6′,2″-terpyridine)$(Cl)_2$ (**Cu3**) (Fig. 1) were synthesized upon stirring of equimolar amounts of the terpyridine ligand and copper(II) chloride in a mixture of dry methanol and dichloromethane at room temperature overnight. The generated precipitate was collected, washed, and recrystallized from methanol. The identity of the compound was verified by high resolution electrospray ionization mass spectroscopy (Fig. S1†) and the purity was confirmed by elemental analysis.

The cytotoxicity of the metal complexes **Cu1–Cu3** against cancerous human breast adenocarcinoma (MCF-7), colon adenocarcinoma (HT-29), mouse colorectal carcinoma (CT-26) as well as and non-cancerous human fibroblasts (GM-5657) cells was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Promisingly, all compounds exhibited a highly cytotoxicity in the low micromolar down to nanomolar range (IC_{50} = 236–3620 nM) against all cell lines. Importantly, the organic ligands 4′-(4′-*ortho*-methoxy-phenyl)-2,2′:6′,2″-terpyridine, 4′-(4′-*meta*-methoxy-phenyl)-2,2′:6′,2″-terpyridine, and 4′-(4′-*para*-methoxy-phenyl)-2,2′:6′,2″-terpyridine were found to be non-toxic (>20 μM) in all tested cell lines. The metal complexes were not able to differentiate between cancerous and non-cancerous cells. **Cu1** was 63-times more cytotoxic against MCF-7 cells, **Cu3** was 29-times more cytotoxic against MCF-7 cells, and **Cu2** was 116-times more cytotoxic against CT-26 cells than cisplatin (overview: Table 1, drug response curves: Fig. S2–S4†). These findings suggest that the cytotoxic effect of the metal complex is dependent on the position of the functionalization of the methoxy substituent. The observed cytotoxic effect was in the same range as for previously reported copper(II) terpyridine complexes against (cisplatin-resistant) cervix adenocarcinoma cells.[12a] The herein reported Cu(II) complexes were found to be significantly more cytotoxic than the previously reported metal complexes **Cu4** [11b,d] and **Cu5**.[13] Within the herein studied series of compounds, **Cu2** was found with the highest cytotoxicity against MCF-7 (0.687 ± 0.125 μM), HT-29 (0.537 ± 0.117 μM), or CT-26 (0.236 ± 0.059 μM) cells. Based on the strongest therapeutic effect, further experiments were performed with **Cu2** against CT-26 cells. Besides the treatment for 48 h, the cytotoxicity of the metal complex was further studied upon treatment for 4 h. With this incubation time, **Cu2** was found to be cytotoxic in the very low micromolar range against CT-26 (1.04 ± 0.08 μM) cells.

To understand the difference in cytotoxicity of the metal complexes, the cellular uptake of **Cu1–Cu3** upon incubation for 4 h was investigated. The results showed no statistically sig-

Table 1 IC_{50} values (in μM) of **Cu1–Cu3** and the anticancer drug cisplatin against cancerous human breast adenocarcinoma (MCF-7), colon adenocarcinoma (HT-29), mouse colorectal carcinoma (CT-26) cells and non-cancerous human fibroblasts (GM-5657). Terpy-*ortho*-OMe = 4′-(4′-*ortho*-methoxy-phenyl)-2,2′:6′,2″-terpyridine, Terpy-*meta*-OMe = 4′-(4′-*meta*-methoxy-phenyl)-2,2′:6′,2″-terpyridine, Terpy-*para*-OMe = 4′-(4′-*para*-methoxy-phenyl)-2,2′:6′,2″-terpyridine. The metal complexes **Cu4** [11b,d] and **Cu5** [13] have been previously reported and are used here for comparison with previous studies

	MCF-7	HT-29	CT-26	GM-5657
Cu1	0.542 ± 0.136	0.964 ± 0.214	0.630 ± 0.113	0.841 ± 0.127
Cu2	0.687 ± 0.125	0.537 ± 0.117	0.236 ± 0.059	0.740 ± 0.089
Cu3	1.159 ± 0.206	3.620 ± 0.472	1.247 ± 0.358	2.761 ± 0.492
Terpy-*ortho*-OMe	>20	>20	>20	>20
Terpy-*meta*-OMe	>20	>20	>20	>20
Terpy-*para*-OMe	>20	>20	>20	>20
Cisplatin	34.5 ± 1.2	54.3 ± 8.6	27.5 ± 2.4	42.7 ± 9.2
Cu4	3.2 ± 0.6	2.7 ± 1.1	2.3 ± 0.7	3.4 ± 0.5
Cu5	1.1 ± 0.3	1.5 ± 0.6	1.3 ± 0.4	2.1 ± 0.4

nificant differences (Fig. S5†), ruling out cellular uptake efficiency as the primary reason. Based on its superior therapeutic effect, further studies were performed with **Cu2**.

The stability of a compound is essential for its use in a biological setting. The stability of **Cu2** upon incubation in Dulbecco's modified Eagle's cell media was studied upon monitoring of the absorption profile over a period of 48 h (Fig. S6†). No significant changes were observed, indicative of the stability of the metal complex under physiological conditions.

Previous studies have indicated that copper(II) terpyridine complexes could undergo redox cycling within a biological setting.[20] The ability of **Cu2** to interact with the cellular reducing agents glutathione or ascorbate was studied upon constant monitoring of the absorption spectra of the metal complex in an aqueous solution. As no significant changes of the absorption spectrum were observed upon titration of glutathione (Fig. S7†), the change in oxidation state of the metal complex was ruled out. Contrary, the titration of ascorbate to **Cu2** showed drastic change in the intensity of the peak centred at approximately 270 nm (Fig. S8†), indicative of the reduction of the Cu(II) centre to Cu(I).

Previous studies have indicated that copper(II) terpyridine complexes could generate reactive oxygen species.[20] The ability of the metal complex to generate reactive oxygen species in CT-26 cells was studied by fluorescence microscopy using the reactive oxygen species specific dye 2′,7′-dichlorodihydrofluorescein diacetate. This dye is non-emissive in human cells but gets readily oxidized in the presence of reactive oxygen species, generating a strongly green fluorescent signal. As expected, no fluorescence signals were observed in the cancerous cell incubated with 2′,7′-dichlorodihydrofluorescein diacetate (5 μM) for 30 min (Fig. S7†). The cancer cells were treated with **Cu2** (1 μM) for 4 h and incubated with 2′,7′-dichlorodihydrofluorescein diacetate (5 μM) for 30 min. The cancer cells treated with the metal complex showed a strong green emission, indicative of generation of reactive oxygen species inside the cancer cells (Fig. 2).

For an understanding of the observed cytotoxicity, the biodistribution of **Cu2** was determined. The cancer cells were incubated with the metal complex (10 μM) for 4 h. After this time, the cell organelles were separately extracted using a specific nucleus extraction kit, a mitochondria extraction kit, a lysosome extraction kit, and a cytoplasm extraction kit, and

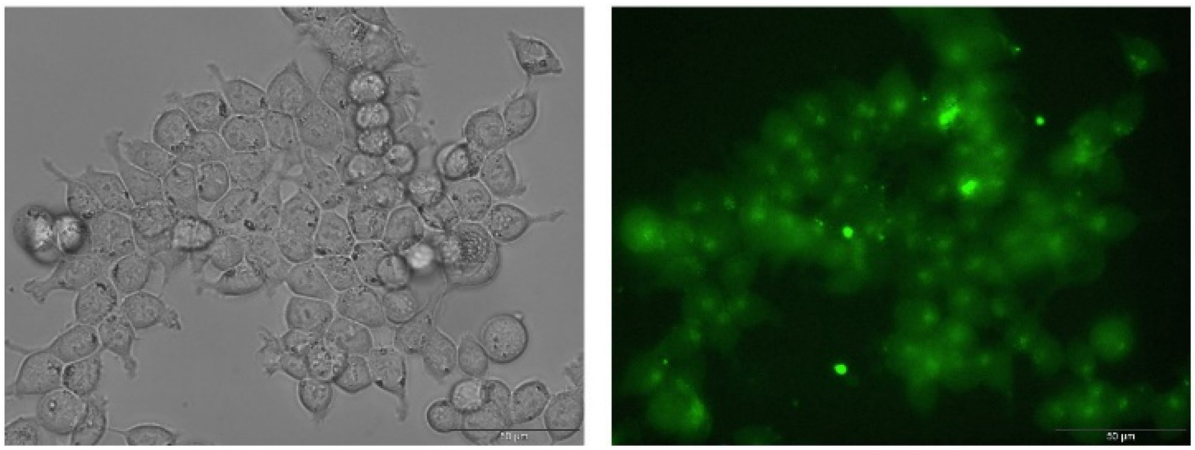

Fig. 2 Left: Brightfield and right: fluorescence microscopy images of CT-26 cells upon incubation with the reactive oxygen species specific dye 2′,7′-dichlorodihydrofluorescein diacetate and treatment with **Cu2**. Scale bar = 50 μm, λ_{ex} = 460–490 nm, λ_{em} = 517–527 nm.

digested in concentrated nitric acid. The metal content in each organelle was determined by inductively coupled plasma optical emission spectroscopy and the naturally occurring copper content subtracted. While small amounts of **Cu2** were found in the nucleus, mitochondria, and the lysosomes, the metal complex primarily accumulated in the cytoplasm of the cancer cells (Fig. 3). Notably, small quantities of a compound inside a specific cell organelle could cause a significant therapeutic effect and therefore cannot be neglected in the assessment of the biological mechanism of action.

For an understanding of the therapeutic effect, the cell death mechanism of the metal complex was studied upon pre-incubation of the CT-26 cells with specific cell death inhibitors 3-methyladenine (autophagy inhibitor), Z-VAD-FMK (apoptosis inhibitor), cycloheximide (paraptosis inhibitor), and necrostatin-1 (necrosis inhibitor), and afterwards treated with **Cu2**. As the pre-incubation with 3-methyladenine, cycloheximide, and necrostatin-1 did not significantly influence the cell survival, these cell death mechanisms were ruled out. Contrary, the incubation with Z-VAD-FMK enhanced the survival of the cells (Fig. 4), indicative that the cell death mechanism of **Cu2** is primarily driven by apoptosis.

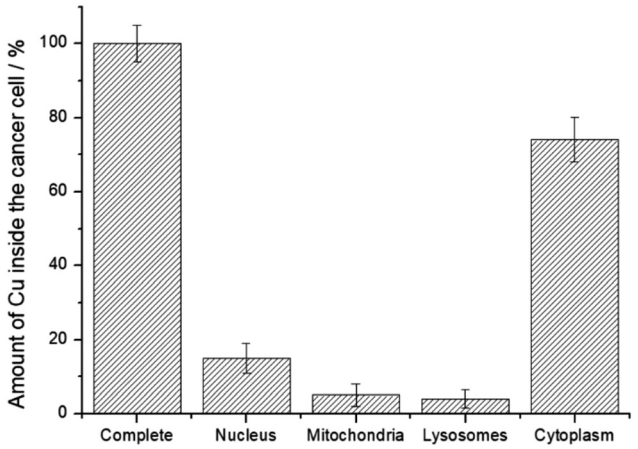

Fig. 3 Subcellular localization of **Cu2** (10 μM) upon incubation in CT-26 cells for 4 h and determination of the metal content by inductively coupled plasma optical emission spectroscopy.

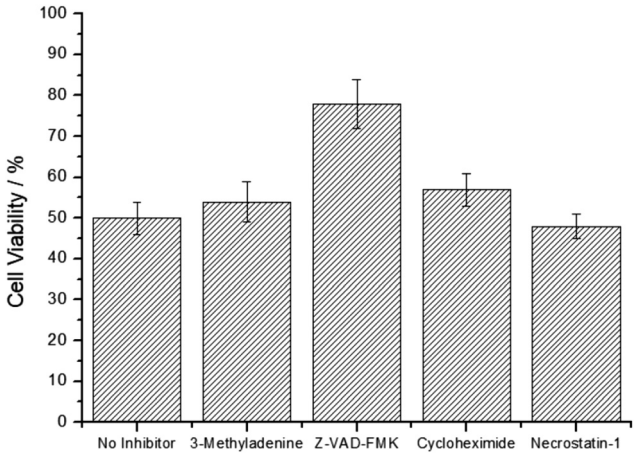

Fig. 4 Determination of the cell death mechanism of **Cu2** in CT-26 cells upon treatment with the IC_{50} concentration in the presence of different cell death inhibitors. Autophagy inhibitor: 3-methyladenine (100 μM), apoptosis inhibitor: Z-VAD-FMK (20 μM), paraptosis inhibitor: cycloheximide (0.1 μM), necrosis inhibitor: necrostatin-1 (60 μM).

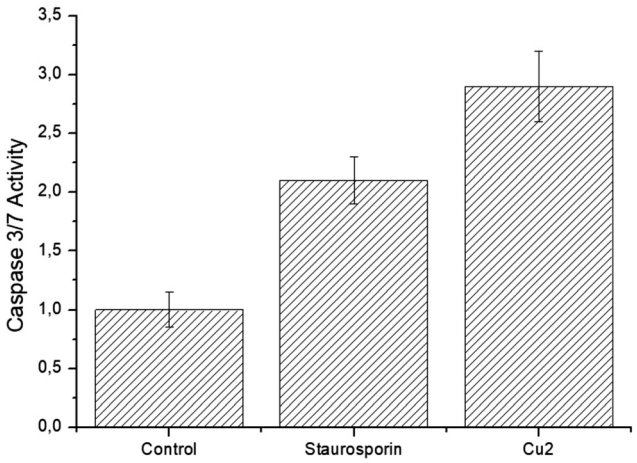

Fig. 5 Caspase 3/7 activity in CT-26 cells upon treatment with **Cu2** (1 μM) for 4 h.

For a deeper insight into the cell death mechanism, the dependency of the cell death of **Cu2** on the caspase 3/7 pathway was assessed using a caspase 3/7 glo assay. These caspases are well characterized executes of apoptotic cell death. Staurosporine was used as a well-known positive control substance. Upon treatment of the cancerous cells with **Cu2**, the caspase 3/7 activity was strongly elevated (Fig. 5). These findings suggest that the metal complex triggers cell death by apoptosis using the caspase 3/7 pathway.

Conclusions

In summary, this study reports on the chemical synthesis and biological evaluation of differently methoxy functionalized copper(II) terpyridine complexes as potential chemotherapeutic agents for anticancer therapy. The compounds were synthesized upon coordination of copper(II) ions with a tridentate terpyridine ligand and characterized by high resolution electrospray ionization mass spectroscopy and elemental analysis. The biological evaluation against human breast adenocarcinoma, colon adenocarcinoma, and mouse colorectal carcinoma cells demonstrated a high cytotoxic effect in the nanomolar range. Mechanistic insights of the lead compound revealed that the metal complex primarily accumulated in the cytoplasm. Within this organelle, the copper(II) complex efficiently generated reactive oxygen species, that induced cell death by apoptosis. Based on their high cytotoxic effect, these metal complexes could represent a starting point for further drug development. Previous studies have indicated that anti-

cancer agents that specifically localize in a cell organelle are associated with an enhanced therapeutic effect. Future work could focus on chemically modifying the lead compound with cell organelle targeting moieties. The reported metal complexes were unable to differentiate between cancerous and healthy cells. Future studies will focus on the chemical modification of the structural scaffold to generate a conjugate with tumor selectivity.

Conflicts of interest

The author declares no competing interests.

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