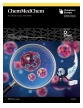
ChemMedChem

Research Article
doi.org/10.1002/cmdc.202200701

Chemistry Europe
European Chemical Societies Publishing

www.chemmedchem.org



Terpyridine Glycoconjugates and Their Metal Complexes: Antiproliferative Activity and Proteasome Inhibition

Roberta Panebianco,[a] Maurizio Viale,[b] Fabrizio Loiacono,[c] Valeria Lanza,[d] Danilo Milardi,[d] and Graziella Vecchio*[a]

Metal terpyridine complexes have gained substantial interest in many application fields, such as catalysis and supramolecular chemistry. In recent years, the biological activity of terpyridine and its metal complexes has aroused considerable regard. On this basis, we synthesised new terpyridine derivatives of trehalose and glucose to improve the water solubility of terpyridine ligands and target them in cancer cells through glucose transporters. Glucose derivative and its copper(II) and iron(II) complexes showed antiproliferative activity. Interestingly, trehalose residue reduced the cytotoxicity of terpyridine. Moreover, we tested the ability of parent terpyridine ligands and their copper complexes to inhibit proteasome activity as an antineoplastic mechanism.

Introduction

Terpyridines are heterocyclic compounds comprising three pyridine rings. Particularly 2,2′:6,2″-terpyridine (tpy) is the most studied isomer.[1] Due to free-rotatable C–C-bonds between pyridine rings, the tpy can serve as a tridentate pincer ligand for a wide range of transition and lanthanide metals.[2] Tpy is a strong σ-donor and a very good π-acceptor. Metal tpy complexes have gained substantial interest in many application fields such as catalysis, supramolecular and bioinorganic chemistry.[3–10]

In recent years, tpy and its metal complexes have been studied as potential metallodrugs.[8,11–15] It was shown that tpy derivatives and their metal complexes have higher *in vitro* antiproliferative activity in tumour cells than cisplatin.[16–21]

Despite the interest in tpy chemistry and its applications, there are few examples of tpy conjugates with biomolecules.[22] The functionalisation of the tpy-based systems may be exploited to increase water solubility and drug selectivity in the cancer environment. Only for some Pt tpy complexes, sugar moieties have been explored as a targeting unit.[23]

Several examples of targeted ligands, such as hydroxyquinoline, deferiprone, polypyridine, and cyclam ligands, have proved the effectiveness of bioconjugation.[24] Their functionalisation has produced systems that may be selectively recognised and internalised in cells. This may produce a selective accumulation in the tumour site, thus decreasing systemic side effects.

In many cases, conjugation with glycoside units has been explored for enhancing selective cellular uptake.[25,26] Particularly, glucose conjugates can exploit the Warburg effect of cancer cells and cross the membrane through GLUT transporters overexpressed in tumour cells.[25]

Glycoconjugates have been shown to be a successful strategy for tuning the selectivity of the ligand and its metal complexes.[27–29] On the other hand, functionalisation with biomolecules has been used to reduce the cytotoxicity of metal complexes.[30]

On this basis, we designed glucose (Glc) and trehalose (Tre) conjugates and synthesised their Cu(II) and Fe(II) complexes (Figure 1).

Copper(II) and iron(II) complexes of parent tpy ligands have been characterised and the stability constants for ML_2 species are $\log\beta = 19.1$ for M=Cu(II) and $\log\beta = 20.7$ for M=Fe(II).[31]

Sugar moieties confer water solubility to the tpy ligands. In this way, it is possible to exploit the coordination ability of tpy in aqueous solutions. We compared glycoconjugates with 4′-Chloro 2,2′:6′,2″-terpyridine (TpyCl) and 3-([2,2′:6′,2″-terpyridin]-4′-yloxy)propan-1-amine ($TpyNH_2$) free ligand (Figure 1S) and their Cu(II) and Fe(II) complexes.

We also studied the ability of parent tpy ligands to inhibit the proteasome, a well-known target for many antineoplastic therapeutic strategies.[32] Indeed, the proteasome has increased activity in cancer cells, and many studies have addressed it as a target for developing novel drugs.[33–35] The 26S proteasome is a multi-subunit enzymatic assembly constituted of two main subcomplexes: the 20S proteasome, also known as the catalytic

[a] R. Panebianco, Prof. G. Vecchio
Dipartimento di Scienze Chimiche
Università degli Studi di Catania,
Viale A. Doria 6, 95125, Catania (Italy)
E-mail: Gr.vecchio@unict.it
[b] Dr. M. Viale
U.O.C. Bioterapie
IRCCS Ospedale Policlinico San Martino
L.go R. Benzi 10, 16132, Genova (Italy)
[c] Dr. F. Loiacono
U.O.C. Immunologia
IRCCS Ospedale Policlinico San Martino
L.go R. Benzi 10, 16132, Genova (Italy)
[d] Dr. V. Lanza, Dr. D. Milardi
Istituto di Cristallografia
Consiglio Nazionale delle Ricerche, CNR
Via Paolo Gaifami 9, 95126, Catania (Italy)

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cmdc.202200701

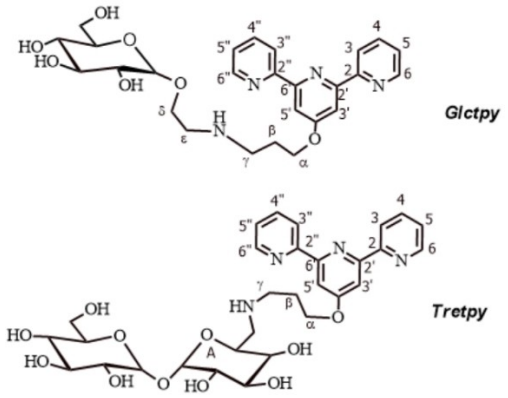


Figure 1. Schematic structures of Tpy glycoconjugates.

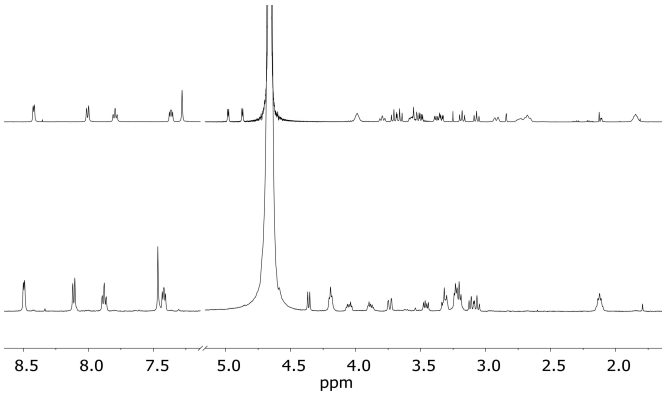


Figure 3. ^{1}H NMR spectra of Tretpy (top) and Glctpy (bottom) (500 MHz, D_2O).

particle (CP) and the 19S assembly, a regulatory particle (RP) that may bind to one or both sides of the 20S particle.[36,37] The 20S proteasome exhibits multiple peptidase activities (i.e., chymotrypsin (CT)-, trypsin (T)- and caspase (PGPH)-like).[38] Several metal complexes with known cytotoxicity have been proven to inhibit proteasome activity.[39–42] The proteasome inhibition capacity of some Cu(II) complexes of 8-hydroxyquinolines have been recently reported.[29,43]

The inhibition of the proteasomal chymotrypsin-like activity but not the trypsin-like activity is associated with the induction of apoptosis in cancer cells.[34,41,44] Therefore, we addressed this specific proteasome activity. Particularly, we studied the proteasome inhibition ability of parent tpy ligands (Figure 1S) and their copper(II) complexes.

Results and Discussion

Sugar conjugates of tpy were synthesised starting from precursor sugar derivatives (Figure 2).

Acetylated glucose derivative was isolated and characterised after the hydrolysis of the acetyl groups.

Glctpy and Tretpy were also characterised by NMR in D_2O (Figure 3, 2S–12S).

In the ^{1}H NMR spectrum of Tretpy, the signals of glucose rings appear different due to functionalisation. The glucose functionalised ring (A) signals are slightly downfield shifted compared to the unfunctionalised ring protons, except for diastereotopic H-6 protons at 2.73 and 2.88 ppm. The γ protons of the propylenic linker resonate at 2.61 and 2.71 ppm, while ten protons of the tpy unit are in the aromatic region.

^{1}H NMR spectra of Glctpy and Tretpy iron(II) complexes have also been recorded. Iron(II) complexes are low spin d^6 and diamagnetic. ^{1}H NMR spectrum of $[Fe(Glctpy)_2]^{2+}$ system is reported in Figure 4. The complexation showed evident modifications of the chemical shifts. The ligand and the metal complex signals can be distinguished in the spectra at a substoichiometric M/L ratio (Figure 15S). Because the complexation stabilises the cis-cis conformation of the tpy ring, aromatic protons are shifted upon complexation.

Metal coordination causes a significant downfield shift of tpy moiety protons except for the H-6, H-6", H-5 and H-5". H-6 and H-6" are upfield shifted compared to the free tpy ligand and this suggests the formation of $[Fe(Glctpy)_2]^{2+}$ species at M/L 1/2 molar ratio. This trend is due to the disposition of the two ligands perpendicular to each other, as reported for tpy and

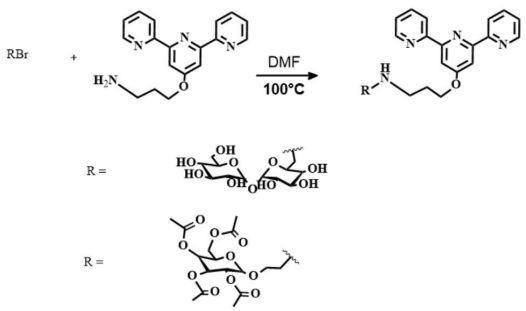


Figure 2. Synthetic scheme of Glctpy and Tretpy.

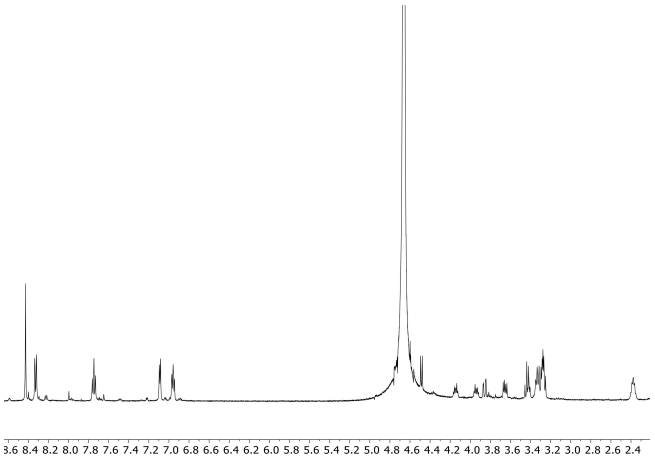


Figure 4. ^{1}H NMR spectra of $[Fe(Glctpy)_2]^{2+}$ M/L 1:2 (500 MHz, D_2O, pD = 7.0).

bipyridine complexes.[45,46] Interestingly, the complexation determines an evident modification of the glucose proton resonances.

UV-Vis spectra of ligands and their metal complexes were carried out (Figure 5 and 16S). Ligands spectra show a band at 280 nm with a shoulder at 310 nm due to π-π* transitions, as found for free tpy. UV-Vis spectra of Fe(II) or Cu(II) complexes showed a band at 313 nm due to the conformational change from the trans-trans to cis-cis conformation of tpy in the metal complexes, as reported elsewhere.[47] Additionally, Fe(II) complexes spectra show a characteristic band at about 560 nm due to the metal-ligand charge transfer (MLCT).

Antiproliferative activity

The antiproliferative activity of Glctpy, Tretpy and their metal complexes were studied in A2780, SKHep1, A549 and MDA-MB-453 cancer cells (Table 1). TpyCl and its metal complexe antiproliferative activities were also studied. $TpyNH_2$ and its metal complexes were previously studied[48] and are reported in Table 1 for comparison. Glctpy shows a good antiproliferative effect in all cell lines. As for $[Fe(Glctpy)_2]^{2+}$ or $[Cu(Glctpy)_2]^{2+}$ systems, the antiproliferative activity decreased compared to the free ligand in all cell lines. This behaviour is particularly evident in the case of the Cu(II) complex. On the contrary, Tretpy showed an IC_{50} value >30 μM in all cell lines. In the case of Tretpy, metal complexes were more active than the ligand in A2780 and SKHep1 cells. In this case, the copper complex had higher antiproliferative activity in A2780 cells.

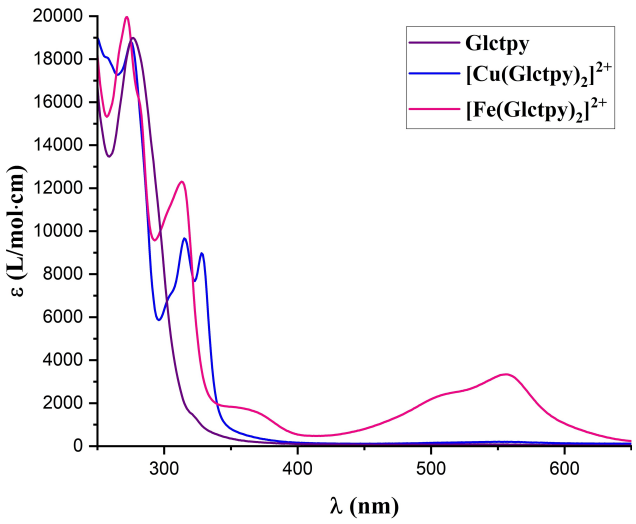


Figure 5. UV-Vis spectra of Glctpy and Fe(II) or Cu(II) complexes (M/L 1:2 molar ratio).

The comparison with $TpyNH_2$ and TpyCl showed that the sugar moiety did not increase the antiproliferative activity in all cases.

We investigated GLUT involvement in the uptake of metal complexes of sugar derivatives. Various studies suggested that pre-treatment of cells with $CoCl_2$ improves the expression of GLUT1 in the cell membrane.[49,50] We pre-treated MDA-MB-453 cells with 50 and 100 μM $CoCl_2$ to study the sensitivity of these cells to Glctpy and its metal complexes that may be transported by GLUT1.

After many experiments, we did not observe any increase of GLUT1 in the cell membrane, but rather we observed a decrease of GLUT1 by measuring MFI (Mean Fluorescence Intensity) after treatment with 100 μM $CoCl_2$.

As shown in Table 2, after treatment with $CoCl_2$, the cells expressing GLUT1 decreased by about 8.6% (not significant) and 35.6% ($p=0.05$) in terms of positive cells and by about 26.1% (not significant) and 43.5% ($p=0.028$) in terms of MFI. Similarly, cells expressing GLUT2 did not show a significant decrease of MFI after treatment with 100 μM $CoCl_2$ (26.5%, $p=0.028$) and an increase of GLUT2 in cells pre-treated with 50 μM $CoCl_2$ both in terms of frequency of positive cells (8.7%, $p=0.014$) and MFI (37.1%, $p=0.014$).

We also analysed the antiproliferative activity of Glctpy, $[Fe(Glctpy)_2]^{2+}$ and $[Cu(Glctpy)_2]^{2+}$ in MDA-MB-453 cells pre-treated with $CoCl_2$. We did not observe a general increase in the antiproliferative activity of Glctpy compounds. In the case of MDA-MB-453 pre-treated with 50 μM $CoCl_2$ and treated with $[Fe(Glctpy)_2]^{2+}$ and $[Cu(Glctpy)_2]^{2+}$, we observed a higher antiproliferative activity. On the contrary, pre-treatment with 100 μM $CoCl_2$ caused a significant decrease in the antiproliferative activity of Glctpy (Table 3). Values of antiproliferative activity suggested that GLUT1 could be involved in the uptake of Glctpy.

Table 1. IC_{50} values (μM) of Glctpy, Tretpy, TpyCl and their Cu(II) and Fe(II) complexes M/L (1:2). TpyNH2 systems were reported for comparison.[48]

	A2780	SKHep1	A549	MDA-MB-453
Tretpy	>30	>30	>30	>30
$[Fe(Tretpy)_2]^{2+}$	16.9±6.2	2.37±0.77	>30	>30
$[Cu(Tretpy)_2]^{2+}$	3.88±2.51	3.97±0.60	>30	>30
Glctpy	0.97±0.32	2.99±1.45	1.87±0.12	5.10±1.30
$[Fe(Glctpy)_2]^{2+}$	5.13±2.42	2.92±0.61	9.76±0.26	16.5±4.3
$[Cu(Glctpy)_2]^{2+}$	11.0±3.3	14.5±4.9	>30	>30
$TpyNH_2$	0.51	0.50	0.18	0.72
$[Fe(TpyNH_2)_2]^{2+}$	1.21	1.86	0.50	3.95
$[Cu(TpyNH_2)_2]^{2+}$	0.29	1.42	0.41	5.62
TpyCl	0.31±0.08	0.64±0.26	0.42±0.19	1.40±0.58
$[Fe(TpyCl)_2]^{2+}$	0.26±0.011	0.32±0.14[a] $p<0.05$	0.28±0.02	1.33±0.33
$[Cu(TpyCl)_2]^{2+}$	0.22±0.04[a] $p<0.05$	0.45±0.14	0.26±0.05	1.47±0.70

Table 2. Immunofluorescence experiments. Percentage of MDA-MB-453 expressing GLUT1 and GLUT2 after the treatment for 72 hours with $CoCl_2$.

Frequency of positive cells			
Glucose Transporter	Control	50 μM $CoCl_2$	100 μM $CoCl_2$
GLUT1	45.2 ± 8.2	41.3 ± 10.7	29.1 ± 1.9[a]
GLUT2	88.3 ± 3.7	96.0 ± 0.2	88.1 ± 1.5
MFI[b]			
Glucose Transporter	Control	50 μM $CoCl_2$	100 μM $CoCl_2$
GLUT1	4.6 ± 1.1	3.4 ± 1.6	2.6 ± 0.5[c]
GLUT2	11.3 ± 1.0	15.5 ± 1.1	8.3 ± 0.4[c]

[a] $p = 0.05$, as calculated by the Mann-Whitney test ($n = 3–4$). [b] MFI was calculated as MFI treated/MFI blank (only 2° antibody). [c] $p = 0.028$, as calculated by the Mann-Whitney test ($n = 3–4$).

Table 3. IC_{50} values (μM) of Glctpy and its Cu(II) and Fe(II) complexes with or without previous treatment for 72 h with different concentrations of $CoCl_2$.

Cell line/pre-treatment	Glctpy	$[Fe(Glctpy)_2]^{2+}$	$[Cu(Glctpy)_2]^{2+}$
MDA-MB-453	5.10 ± 1.30	16.5 ± 4.3	> 30
MDA-MB-453 + 50 μM $CoCl_2$	5.42 ± 0.70	10.7 ± 0.3[a]	18.4 ± 1.6[a]
MDA-MB-453 + 100 μM $CoCl_2$	9.22 ± 0.63[b]	18.0 ± 1.3	> 30

[a] $p = 0.001$, values evaluated versus the not pre-treated MDA-MB-453 by the Mann-Whitney test ($n = 3–9$). [b] $p = 0.006$, values evaluated versus the not pre-treated MDA-MB-453 by the Mann-Whitney test ($n = 3–8$).

GLUTs did not seem to be involved in the uptake of metal complexes containing sugar derivatives, as found for similar systems.[23] For the complexes remain uncertain, the mechanism of transport into the cells.

The uptake of Glctpy and Tretpy may be different. In particular, the passive transport of Tretpy could be strongly reduced because of the sugar unit hydrophilicity, thus reducing its antiproliferative activity.

LogP values analysis can support this hypothesis: trehalose derivative has the lowest calculated logP value −1.18, while Glctpy has logP 0.41, $TpyNH_2$ logP 2.15, and TpyCl logP 3.20.

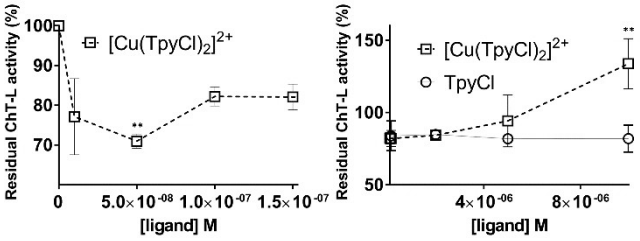


Figure 6. Residual ChT-L activity of TpyCl and its (1:2) copper (II) complex Left) Normalised percentages of residual activity ChT-L activity vs ligand concentration (0–150 nM); Right) Normalised ChT-L activity vs ligand concentration (150 nM–10 μM). Three replicates were run for each data point. According to the Dunnett's multiple comparison test, ChT-L activity at 50 nM and 10 μM was significantly different to the control (**$p > 0,05$).

Proteasome inhibition assay

We evaluated the ability of TpyCl and $TpyNH_2$ ligands to inhibit the chymotrypsin-like activity of the purified 20S proteasome. The purified 20S proteasome (1.5 nM) was incubated with increasing concentrations (0.5 nM–5.0 μM) of $TpyNH_2$ and TpyCl and their copper (II) complexes in 50 mM TRIS buffer (pH = 7.4). The results indicated that TpyCl was ineffective in inhibiting the chymotrypsin-like (ChT-L) activity of the proteasome, whereas $[Cu(TpyCl)_2]^{2+}$ was a mild 20S inhibitor (30% of inhibition at a concentration of about 50 nM). As reported in Figure 6, the Cu(II) complex exhibits an inhibition profile with a minimum of 50 nM, as observed for other proteasome inhibitors.[51–54] Based on those early accounts, this behaviour suggests that the modulatory effects may be due to the occurrence of two different main mechanisms: i) at high concentration (micromolar range), the promotion of substrate entry into the catalytic chamber by inducing the opening of the α-ring gate (gate openers), and ii) at lower concentrations, the inhibition of substrate binding to the catalytic pockets and degradation induced by allosteric interactions.[54,55]

Indeed, this phenomenon is typically explained as a mechanism where the inhibitor binds with high affinity to a first allosteric site of the enzyme, recognising a second orthosteric/allosteric site with a reduced affinity.[53,55–58] Conversely, $[Cu(TpyNH_2)_2]^{2+}$ does not affect the ChT-L activity. Intriguingly, the ligand $TpyNH_2$ significantly enhances the ChT-L activities of the 20S proteasome in the low micromolar range.

Of note, there is increasing interest in developing proteasome enhancers due to their promising effect in treating protein misfolding disorders. Although far from the purpose of the present note, this molecule deserves future investigations aimed at unveiling its potential use as a drug candidate for treating diseases related to abnormal proteinopathies[58,59] whereas the free ligand behaves as an activator of human 20S proteasome (Figure 7).

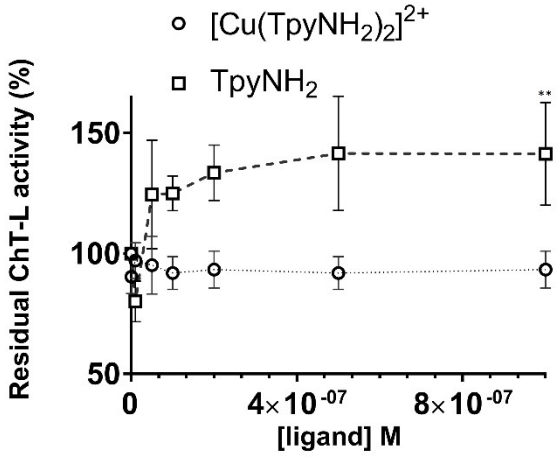


Figure 7. Normalised percentages of residual 20S ChT-L activity vs ligand $TpyNH_2$ and its (1:2) copper (II) complex $[Cu(TpyNH_2)_2]^{2+}$, in the concentration range 0–500 nM. According to Dunnett's multiple comparison test, residual ChT-L activity at 1 μM was significantly different to the control (**$p > 0{,}05$).

Conclusion

We synthesised new glycoconjugates of tpy ligand to exploit the role of the sugar unit in the improvement of water solubility and targeting of cancer cells. We found that the Cu(II) and Fe(II) ternary complexes of tpy derivatives showed antiproliferative activity (μM range). We found that the sugar units did not improve the cytotoxicity of the ligands or their metal complexes compared to parent tpy ligands.

Immunofluorescence experiments showed that the glucose conjugate could pass through GLUT1, although data suggest that its complexes cannot. Interestingly, the functionalisation with trehalose significantly reduced the toxicity of tpy, and this may suggest a strategy to exploit tpy properties in cells. We assumed that the polarity of the disaccharide might affect cellular uptake.

Finally, we investigated the proteasome inhibition ability of parent terpyridine as an antineoplastic mechanism. To the best of our knowledge, this is the first study of terpyridine derivatives as proteasome modulators.

Our results demonstrated that $[Cu(TpyCl)_2]^{2+}$ exhibits an inhibition profile with a minimum value of 50 nM. Remarkably, the $TpyNH_2$ compound shows the opposite effect. Indeed, it enhances the chymotrypsin-like activities of the 20S proteasome in the low micromolar range.

Experimental Section

Chemicals

Commercially available reagents were used directly unless otherwise noted. 4′-Chloro 2,2′:6′,2″-terpyridine (TpyCl) was purchased from TCI (TOKYO CHEMICAL INDUSTRY CO., Tokyo, Japan). 3-([2,2′:6′,2″-terpyridin]-4′-yloxy)propan-1-amine ($TpyNH_2$), bromoethyl-per-O-acetyl-β-glucoside (AcGlcBr) and 6-deoxy-6-bromo-α,α′-trehalose (TreBr) were synthesised as reported elsewhere.[48,60] TLC (Thin layer chromatography) was carried out on silica gel plates (Merck 60-F254). Carbohydrate derivatives were detected on TLC with the anisaldehyde test. LogP values were calculated with ChemDraw. Human 20S proteasome purified from erythrocytes was purchased from Boston Biochem, Inc. (Cambridge, MA). Multiple batches of proteasomes were used and performed reproducibly. The model peptide substrate releasing fluorescent 7-amino-4-methylcoumarin (AMC) reporter group for the ChT-L peptidase was succinyl-LeuLeuValTyr-AMC SucLLVY-AMC (Bachem Bioscience Inc.). The purity of Glctpy and Tretpy (>97%) was checked by HPLC using a reversed-phase C18 column and a linear gradient $H_2O \rightarrow CH_3CN$ as the eluent.

NMR spectroscopy

^1H and ^{13}C NMR spectra were recorded at 25 °C with a Varian UNITY PLUS-500 spectrometer at 499.9 and 125.7 MHz, respectively, using standard pulse programs from the Varian library. 2D experiments (COSY, TOCSY, HSQC, and NOESY) were acquired using 1 K data points, 256 increments. The functionalised ring in Tretpy was labelled A.

UV-Vis spectroscopy

UV-Vis spectra were recorded with a VersaWave microvolume UV/Vis spectrophotometer. The metal complexes were prepared by mixing a metal ion solution with the ligand in a 1:2 M/L molar ratio. Freshly prepared $FeSO_4$ solution in 2 mM H_2SO_4 was used.

Synthesis of Glctpy

$TpyNH_2$ (357 mg, 1.16 mmol) and AcGlcBr (256 mg, 0.56 mmol) were dissolved in dry DMF (4 ml). The reaction mixture was stirred at 90 °C for 48 h. DMF was evaporated under a vacuum at 40 °C. NH_3 solution (37%, 1 ml) was added to the solid reaction mixture under stirring for 4 h to hydrolyse the acetyl groups. The final product was isolated by CM Sephadex C-25 (NH_4^+ form) column eluted with a linear gradient H_2O-NH_4HCO_3 (0→0.2 M).

ESI MS m/z 513.238 $[M+H]^+$, m/z 513.213

^1H NMR (500 MHz, D_2O) δ (ppm) = 8.50 (2H, d, H-6, H-6″ tpy); 8.11 (2H, d, H-3, H-3″ tpy); 7.87 (2H, t, H-4, H-4″ tpy); 7.46 (2H, s, H-3′, H-5′ tpy): 7.41 (2H, t, H-5 and H-5″ tpy); 4.35 (1H, d, H-1 Glc); 4.18 (2H, t, α); 4.04 (1H, m, δ); 3.87 (1H, m, δ′); 3.73 (1H, d, H-6 Glc); 3.46 (1H, q, H-6′ Glc); 3.31 (H, m, H-5 Glc); 3.23 (2H, m, ε), 3.20 (2H, m, γ); 3.10 (1H, t, J = 9.3 Hz, H-2 Glc), 3.07 (1H, t, J = 8.2 Hz, H-4 Glc); 2.11 (2H, p, J = 6.0 Hz, β).

$^{13}C\{^1H\}$ NMR (125 MHz, D_2O) δ(ppm) = 166.9 (C4′); 156.0 (C-2 and C-2″) 154.0 (C-2′ and C-6′), 149.2 (C-6, C-6″), 138.3 (C-4, C-4″), 125.2 (C-5, C-5″) 122.2 (C-5′ C-3′ tpy), 108.0 (C-3, C-3″ tpy), 102.3 (C-1 Glc) 76.4 (C-5 Glc), 73.0 (C-2 Glc), 69.2 (C-4 Glc), 65.9 (α), 65.5 (δ), 61.0 (C-6 Glc), 48.3 (ε), 45.8 (γ) 26.1 (β).

Synthesis of Tretpy

TpyNH2 (121 mg, 0.4 mmol) and TreBr (160 mg, 0.4 mmol) were dissolved in dry DMF (1.5 ml). The reaction mixture was stirred at 100 °C. After 48 h, DMF was evaporated under a vacuum at 40 °C. The product was isolated by CM Sephadex C-25 ($NH4^+$ form) column eluted with a linear gradient H_2O-NH_4HCO_3 (0→0.2 M).

ESI MS m/z 631.257 $[M+H]^+$, m/z 631.243

^{1}H NMR (500 MHz, D_2O) δ(ppm) = 8,.42 (2H, d, H-6, H-6'' tpy); 8.00 (2H, d, H-3, H-3'' tpy); 7.80 (2H, t, H-4, H-4'' tpy); 7.35 (2H, t, H-5 and H-5'' tpy); 7.27 (2H, s, H-3', H-5' tpy); 4.96 (1H, d, J = 3.9 Hz, H-1 Tre A ring); 4.87 (1H, d, J = 3.9 Hz, H-1 Tre); 3,97 (2H, m, α CH2), 3.79 (1H, t, *J* = 8.0 Hz, H-5 Tre A ring) 3.71 (1H, t, *J* = 9.5 Hz, H-3 Tre A ring) 3.65 (1H, t, *J* = 9.6 Hz, H-3 Tre) 3.59–3.52 (2H, m, H-5 and H-6 Tre), 3.49 (1H, dd, *J* = 9.9, 3.9 Hz, H-2 Tre A ring) 3.38 (dd, *J* = 12.5, 5.8 Hz, H-6 Tre) (3.34 (1H, dd, *J* = 10.0, 3.8 Hz, H-2 Tre); 3.18 (1H, t, *J* = 9.5 Hz, H-4 Tre A ring) 3.08 (1H, t, *J* = 9.4 Hz, H-4 Tre) 2.89 (1H, d, *J* = 11.4 Hz, H-6' Tre A), 2.65 (3H, m, H-6 Tre A, γ CH_2); 1,82 (2H, t, β CH_2).

^{13}C{^{1}H} NMR (125 MHz, D_2O) δ(ppm) = 166.7 (C4'), 156.7 (C-2 and C-2''), 154,5 (C-2' and C-6'), 148.7 (C-6, C-6''), 138.3 (C-4, C-4''), 125.0 (C-5, C-5'') 122.2 (C-3, C-3'' tpy), 108.7 (C-3', C-5' tpy), 93.8 (C-1 Tre A ring), 93.0 (C-1 Tre), 72.6 (C-3 Tre A ring), 72.4 (C-3 Tre), 71.5 (C-2 tre A ring), 71.3 (C-2 Tre), 71.9 (C-4 Tre A ring) 69.7 (C-5 Tre A ring), 69.6 (C-4 Tre), 67.3 (α CH2),, 60.6 (C-6 Tre), 49.4 (C-6 Tre A ring) 45.9 (γ CH_2) 27.3 (β CH_2).

Synthesis of Glctpy and Tretpy metal complexes

Cu(II) and Fe(II) complexes were synthesised by adding $Cu(NO_3)_2$ or $FeSO_4$ water solution to the ligand water solution (20% DMSO) in a 1:2 M/L molar ratio. Stock solutions (1 mM) were diluted in phosphate buffer pH 7.4.

$[Cu(Glctpy)_2]^{2+}$: UV-Vis spectrum λ nm (ε, L $M^{-1}cm^{-1}$): 249 (sh, 19073), 257 (sh, 18049), 275 (18806), 302 (sh, 6848), 315 (9667), 328 (8942)

$[Fe(Glctpy)_2]^{2+}$: UV-Vis spectrum λ nm (ε, L $M^{-1}cm^{-1}$): 241 (sh, 21230), 272 (19939), 281 (sh), 312 (12348), 363 (1725), 510 (2433), 557 (3340)

$[Cu(Tretpy)_2]^{2+}$: UV-Vis spectrum λ nm (ε, L $M^{-1}cm^{-1}$): 249 (sh, 20973), 257 (sh, 19072), 275 (18332), 302 (sh, 6232), 315 (8715), 328 (7969)

$[Fe(Tretpy)_2]^{2+}$: UV-Vis spectrum λ nm (ε, L $M^{-1}cm^{-1}$): 243 (sh, 17130), 272 (15917), 281 (sh), 313 (12204), 362 (2764), 505 (2865), 556 (3618).

Proteasome activities assays

ChT-L proteasome activities were assayed in 50 μL of 25 mM TRIS buffer (pH 7.4) at 37 °C in the presence of the fluorogenic substrate (50 μM). Human 20S (2 nM) was incubated with Tpy compounds or its copper (II) complexes 1:2 (M/L ratio) for 45 min before enzymatic assays.[51] The compounds were tested at concentrations ranging from 0.5 nM to 5000 nM. The fluorescence signal activated by peptide cleavage was recorded at 440 nm (excitation at 360 nm) for 45 min by a Varioskan (Thermo©) plate reader in 384 multiwell plates. Three replicates were performed for each data point. Data were reported as normalised percentages of residual ChT-L activity.

Evaluation of the antiproliferative activity of ligands and complexes

Human cell lines A2780 (ovary, adenocarcinoma), A549 (lung, carcinoma), MDA-MB-453 (breast, carcinoma), and SKHep1 (liver, carcinoma) were plated in 180 μl into flat-bottomed 96-well microliter plates at the opportune concentration in complete media (RPMI 1640 or DMEM) added with 10% fetal calf serum (FCS). After 6–8 h, cells were administered with 20 μl containing five concentrations of tpy derivatives or their complexes with Cu(II) or Fe(II) diluted in water plus 0.75% DMSO. Plates were then processed as described elsewhere.[13] The compound concentrations inhibiting 50% cell growth (IC_{50}) were calculated based on the analysis of the concentration-response curves. Each experiment was repeated 3–11 times. In another series of experiments, MDA-MB-453 cells were pre-treated with 100 and 50 μM $CoCl_2$ to let cells overexpress GLUT1.[61,62] Once treated, cells were washed with fresh culture medium, resuspended in a complete medium, counted, and administered with our complexes as described before. Each experiment was repeated 4 times.

Immunofluorescence study of GLUT1 and GLUT2 expression

MDA-MB-453 cells were treated for 72 hours with 100 and 50 μM $CoCl_2$ for 24 h. Part of the cells was harvested and washed twice with PBS plus 2% fetal calf serum. We then pelleted 2.0×10^5 cells that were incubated at 22 °C for 30 min with 50 μl (1:1000) of either anti-GLUT1 monoclonal antibody (SPM498, (Ab40084) Abcam, Cambridge, UK), previous fixation with methanol (10 min) and incubation in PBS plus 10% normal goat serum and 0.3 M glycine, or anti-GLUT2 monoclonal antibody (5D1 (Ab85715), Abcam), previous fixation with 3.7% paraformaldehyde (10 min) and permeabilisation of membranes with PBS plus 0.1% Tween 20 for 10 min followed by incubation in PBS plus 10% normal goat serum and 0.3 M glycine. Then cells were washed two times with PBS plus 2% fetal calf serum and incubated again with 50 μl PE-labelled goat anti-mouse IgG2a (Southern Biotech, Birmingham, AL, USA). After being washed two times, cells were evaluated by flow cytometry (Cytoflex-S, Beckman Coulter, Milano, Italy) and analysed by FlowJo software v10.8 (BD Biosciences, Ashland, OR, USA). Results were expressed as the frequency of positive cells and MFI calculated as MFI treated/MFI negative control (only PE-labelled goat anti-mouse IgG2a).

Statistical analysis

The Mann-Whitney test for non-parametric data was used for statistical analysis.

Acknowledgements

The authors thank Università degli Studi di Catania (Piano di incentivi per la ricerca di Ateneo 2020/2022 Pia.ce.ri.) and the Italian Ministry of Health (Ricerca Corrente, 2022-2772566, recipient Maurizio Viale) for the financial support. Open Access funding provided by Università degli Studi di Catania within the CRUI-CARE Agreement.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Copper · Cytotoxicity · Glucose · Iron · Trehalose

[1] C. Bazzicalupi, A. Bencini, A. Bianchi, A. Danesi, E. Faggi, C. Giorgi, S. Santarelli, B. Valtancoli, *Coord. Chem. Rev.* **2008**, *252*, 1052–1068.
[2] E. U. Mughal, M. Mirzaei, A. Sadiq, S. Fatima, A. Naseem, N. Naeem, N. Fatima, S. Kausar, A. A. Altaf, M. N. Zafar, B. A. Khan, *R. Soc. Open Sci.* **2020**, *7*, 201208.
[3] C. Wei, Y. He, X. Shi, Z. Song, *Coord. Chem. Rev.* **2019**, *385*, 1–19.
[4] C. E. Housecroft, E. C. Constable, *Chem. Commun.* **2020**, *56*, 10786–10794.
[5] G. Krieger, B. Tieke, G. Krieger, B. Tieke, *Macromol. Chem. Phys.* **2017**, *218*, 1700052.
[6] H. Hofmeier, J. Pahnke, C. H. Weidl, U. S. Schubert, *Biomacromolecules* **2004**, *5*, 2055–2064.
[7] R. Dobrawa, P. Ballester, C. R. Saha-Möiler, F. Würthner, in: *ACS Symp. Ser.* **2006**, *vol. 928*, pp. 43–62.
[8] Z. Ma, L. Wei, E. C. B. A. Alegria, L. M. D. R. S. Martins, M. F. C. Guedes da Silva, A. J. L. Pombeiro, *Dalt Trans.* **2014**, *43*, 4048–4058.
[9] J. Costa, R. Ruloff, L. Burai, L. Helm, A. E. Merbach, *J. Am. Chem. Soc.* **2005**, *127*, 5147–5157.
[10] A. Winter, U. S. Schubert, *ChemCatChem.* **2020**, *12*, 2890–2941.
[11] K. Malarz, D. Zych, M. Kuczak, R. Musioł, A. Mrozek-Wilczkiewicz, *Eur. J. Med. Chem.* **2020**, *189*, 112039.
[12] K. Malarz, D. Zych, R. Gawecki, M. Kuczak, R. Musioł, A. Mrozek-Wilczkiewicz, *Eur. J. Med. Chem.* **2021**, *212*, 113032.
[13] M. Savic, A. Arsenijevic, J. Milovanovic, B. Stojanovic, V. Stankovic, A. R. Simovic, D. Lazic, N. Arsenijevic, M. Milovanovic, *Molecules* **2020**, *25*, 4699.
[14] C. Li, F. Xu, Y. Zhao, W. Zheng, W. Zeng, Q. Luo, Z. Wang, K. Wu, J. Du, F. Wang, *Front. Chem.* **2020**, *8*, 210.
[15] M. Khosravifarsani, S. Ait-Mohand, B. Paquette, L. Sanche, B. Guérin, *Nanomaterials* **2021**, *11*, 2154.
[16] X. Liang, J. Jiang, X. Xue, L. Huang, X. Ding, D. Nong, H. Chen, L. Pan, Z. Ma, *Dalt Trans.* **2019**, *48*, 10488–10504.
[17] J. Karges, O. Blacque, M. Jakubaszek, B. Goud, P. Goldner, G. Gasser, *J. Inorg. Biochem.* **2019**, *198*, 110752.
[18] L. Huang, R. Liu, J. Li, X. Liang, Q. Lan, X. Shi, L. Pan, H. Chen, Z. Ma, *J. Inorg. Biochem.* **2019**, *201*, 110790.
[19] S. Jain, K. Bhar, A. K. Sharma, S. Kumar, S. Tapryal, S. Bandyopadhyaya, C. C. Mandal, *Dalt Trans.* **2020**, *49*, 4100–4113.
[20] J. Jiang, J. Li, C. Liu, R. Liu, X. Liang, Y. Zhou, L. Pan, H. Chen, Z. Ma, *J. Biol. Inorg. Chem.* **2020**, *25*, 311–324.
[21] Q. P. Qin, Z. F. Wang, S. L. Wang, D. M. Luo, B. Q. Zou, P. F. Yao, M. X. Tan, H. Liang, *Eur. J. Med. Chem.* **2019**, *170*, 195–202.
[22] V. Ramu, S. Gautam, A. Garai, P. Kondaiah, A. R. Chakravarty, *Inorg. Chem.* **2018**, *57*, 1717–1726.
[23] D. L. Ma, T. Y. T. Shum, F. Zhang, C. M. Che, M. Yang, *Chem. Commun.* **2005**, 4675–4677.
[24] L. Holden, C. S. Burke, D. Cullinane, T. E. Keyes, *RSC Chem Biol.* **2021**, *2*, 1021.
[25] E. C. Calvaresi, P. J. Hergenrother, *Chem. Sci.* **2013**, *4*, 2319–2333.
[26] T. Storr, M. Merkel, G. X. Song-Zhao, L. E. Scott, D. E. Green, M. L. Bowen, K. H. Thompson, B. O. Patrick, H. J. Schugar, C. Orvig, *J. Am. Chem. Soc.* **2007**, *129*, 7453–7463.
[27] S. S. Mullapudi, D. Mitra, M. Li, E. T. Kang, E. Chiong, K. G. Neoh, *Mol. Syst. Des. Eng.* **2020**, *5*, 772–791.
[28] A. Pettenuzzo, K. Vezzù, M. L. Di Paolo, E. Fotopoulou, L. Marchiò, L. D. Via, L. Ronconi, *Dalt Trans.* **2021**, *50*, 8963–8979.
[29] V. Oliveri, M. Viale, G. Caron, C. Aiello, R. Gangemi, G. Vecchio, *J Chem Soc Dalt Trans.* **2013**, *42*, 2023–2034.
[30] M. X. Zhao, M. Zhao, E. Z. Zeng, Y. Li, J. M. Li, Q. Cao, C. P. Tan, L. N. Ji, Z. W. Mao, *J. Inorg. Biochem.* **2014**, *137*, 31–39.
[31] R. M. Smith, A. E. Martell, *Critical Stability Constants, Second Supplement* Springer US, **1989**, page 280.
[32] S. V. Rajkumar, P. G. Richardson, T. Hideshima, K. C. Anderson, *J. Clin. Oncol.* **2005**, *23*, 630–639.
[33] Y. Li, *IUBMB Life* **2020**, *72*, 1900–1908
[34] K. G. Daniel, D. Chen, B. Yan, Q. P. Dou, *Front. Biosci.* **2007**, *12*, 135–44.
[35] A. M. Santoro, I. Monaco, F. Attanasio, V. Lanza, G. Pappalardo, M. F. Tomasello, A. Cunsolo, E. Rizzarelli, A. De Luigi, M. Salmona, D. Milardi, *Sci Reports* **2016**, *6*, 1–10.
[36] J. A. M. Bard, E. A. Goodall, E. R. Greene, E. Jonsson, K. C. Dong, A. Martin, *Annu. Rev. Biochem.* **2018**, *87*, 697–724.
[37] D. Voges, P. Zwickl, W. Baumeister, *Annu. Rev. Biochem.* **1999**, *68*, 1015–1068.
[38] L. Borissenko, M. Groll, *Chem. Rev.* **2007**, *107*, 687–717.
[39] C. N. Verani, *J. Inorg. Biochem.* **2012**, *106*, 59–67.
[40] Shagufta, I. Ahmad, *Inorg. Chim. Acta* **2020**, *506*, 119521..
[41] S. Zhai, L. Yang, Q. C. Cui, Y. Sun, Q. P. Dou, B. Yan, *J. Biol. Inorg. Chem.* **2010**, *15*, 259–69.
[42] Z. Zhang, H. Wang, M. Yan, H. Wang, C. Zhang, *Mol Med Rep.* **2017**, *15*, 3–11.
[43] V. Oliveri, G. Vecchio, *Eur. J. Med. Chem.* **2016**, *120*, 252–274.
[44] A. M. Ruschak, M. Slassi, L. E. Kay, A. D. Schimmer, *J. Natl. Cancer Inst.* **2011**, *103*, 1007–1017.
[45] H. Elsbernd, J. K. Beattie, *J. Inorg. Nucl. Chem.* **1972**, *34*, 771–774.
[46] S. Castellano, H. Günther, S. Ebersole, *J. Phys. Chem.* **1965**, *69*, 4166–4176.
[47] R. Dobrawa, P. Ballester, C. R. Saha-Möiler, F. Würthner, *ACS Symp. Ser.* **2006**, *928*, 43–62.
[48] R. Panebianco, M. Viale, N. Bertola, F. Bellia, G. Vecchio, *Dalt Trans.* **2022**, *51*, 5000–5003.
[49] P. Wang, L. Li, Z. Zhang, Q. Kan, S. Chen, F. Gao, *Mol Med Rep.* **2016**, *13*, 2909–2917.
[50] D.-Y. Hwang, F. Ismail-Beigi, *Arch. Biochem. Biophys.* **2002**, *399*, 206–211.
[51] M. Persico, S. García-Viñuales, A. M. Santoro, V. Lanza, G. R. Tundo, D. Sbardella, M. Coletta, V. Romanucci, A. Zarrelli, G. Di Fabio, C. Fattorusso, D. Milardi, *Bioorg. Med. Chem.* **2022**, *66*, 116813.
[52] A. M. Santoro, V. Lanza, F. Bellia, D. Sbardella, G. R. Tundo, A. Cannizzo, G. Grasso, M. Arizzi, V. G. Nicoletti, S. Alcaro, G. Costa, A. Pietropaolo, G. Malgieri, G. D'Abrosca, R. Fattorusso, S. García-Viñuales, I. M. M. Ahmed, M. Coletta, D. Milardi, *ChemMedChem.* **2020**, *15*, 302–316.
[53] E. Jankowska, M. Gaczynska, P. Osmulski, E. Sikorska, R. Rostankowski, S. Madabhushi, M. Tokmina-Lukaszewska, F. Kasprzykowski, *Biopolymers* **2010**, *93*, 481–495.
[54] D. Sbardella, G. R. Tundo, A. Coletta, J. Marcoux, E. I. Koufogeorgou, C. Ciaccio, A. M. Santoro, D. Milardi, G. Grasso, P. Cozza, M. P. Bousquet-Dubouch, S. Marini, M. Coletta, *Cell. Mol. Life Sci.* **2018**, *75*, 3441–3456.
[55] P. A. Osmulski, P. Karpowicz, E. Jankowska, J. Bohmann, A. M. Pickering, M. Gaczynska, *Molecules* **2020**, *25*.
[56] A. F. Kisselev, A. Callard, A. L. Goldberg, *J. Biol. Chem.* **2006**, *281*, 8582–8590.
[57] E. Njomen, P. A. Osmulski, C. L. Jones, M. Gaczynska, J. J. Tepe, *Biochemistry* **2018**, *57*, 4214–4224.
[58] D. E. George, J. J. Tepe, *Biomol. Eng.* **2021**, *11*, 1789.
[59] A. M. Santoro, V. Lanza, F. Bellia, D. Sbardella, G. R. Tundo, A. Cannizzo, G. Grasso, M. Arizzi, V. G. Nicoletti, S. Alcaro, G. Costa, A. Pietropaolo, G. Malgieri, G. D'Abrosca, R. Fattorusso, S. García-Viñuales, I. M. M. Ahmed, M. Coletta, D. Milardi, *ChemMedChem.* **2020**, *15*, 302–316.
[60] V. Lanza, F. Bellia, R. D'Agata, G. Grasso, E. Rizzarelli, G. Vecchio, *J. Inorg. Biochem.* **2011**, *105*, 181–188.
[61] D. Y. Hwang, F. Ismail-Beigi, *Arch. Biochem. Biophys.* **2002**, *399*, 206–211.
[62] A. M. Basavaraju, N. Shivanna, C. Yadavalli, P. K. Garlapati, A. K. Raghavan, *Biol. Trace Elem. Res.* **2021**, *199*, 1345–1355.

Manuscript received: December 26, 2022
Revised manuscript received: February 10, 2023
Accepted manuscript online: February 11, 2023
Version of record online: February 27, 2023