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Discovery of a potent and highly specific β_2 proteasome inhibitor from a library of copper complexes

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ABSTRACT

We reported the synthesis, characterization and biological activity of several copper(II) Schiff base complexes, which exhibit high proteasome inhibitory activities with particular selectivity of β_2 subunit. Structure–activity relationships information obtained from complex Na_2[***Cu***(***a4s1***)] demonstrated that distinct bonding modes in β_2 and β_5 subunits determines its selectivity and potent inhibition for β_2 subunit.

Metal complexes have been widely used in clinic application since the great discovery of cisplatin by Rosenberg in the late 1960s.[1–5] However, severe side effects like nephrotoxicity, ototoxicity restrict their use as clinic treatment,[6–8] thus development of new metal complexes with low cytotoxicity, low drug-resistance, high selectivity as well as potent efficacy is highly demanded. Among them, copper, as an essential intracellular cofactor for many enzymes, is playing vital roles in many physiological processes. It has gained rapid growing interests and their complexes have been applied as antitumor drug candidates.[9–13] Up to date, several copper complexes such as some copper–Phenanthroline complexes have been propelled into pre-clinical research as antitumor drug candidates, which demonstrated considerable antitumor potential and relatively lower side effects compared with some platinum drugs. A typically proposed mechanism regarding the antitumor activity is that copper complexes are able to generate reactive oxygen species (ROS) which trigger DNA degradation and apoptosis of cancer cells.[14] Recent Letters demonstrated copper complexes also interface cell processes via enzyme inhibition.[15–17] For example, Dou demonstrated some copper Schiff base complexes which have significant antitumor activity, associated with proteasome inhibition. The detailed mechanism is probably cytotoxic effect raised by ROS which is induced by these copper Schiff base complexes.[15] Nevertheless, in this Letter, we focus on screening copper Schiff base complexes as selective proteasome β_2 inhibitors.

The Ubiquitin–Proteasome pathway (UPP), as the major pathway of intracellular protein degradation in eukaryotic cells, is essential for some key cellular functions, such as cell cycling, signal transduction, antigen processing for appropriate immune responses, and apoptosis.[18–20] It also plays a pivotal role in the origin, evolution and transfer of malignant carcinoma.[21] It has been reported that tumor cells are more sensitive to proteasome inhibitors than normal cells and thus proteasomes are recognized as promising antitumor drug targets. Recently, many proteasome inhibitors have been explored extensively as potential antitumor agents and several successful hits including bortezomib, carfilzomib, and marizomib, were approved by FDA in 2003, 2012, and 2014, respectively, for the treatment of multiple myeloma.[22–24]

It is well known 26S proteasome is a large multicatalytic protein complex, consisting of two 19S regulatory particles and one 20S catalytic core. Crystal structure (PDB:3MG6) shows that the active sites of the 20S proteasome are mainly located on the β_1, β_2 and β_5 subunits, which have caspase-like (C-L), trypsin-like (T-L), and chymotrypsin-like (ChT-L) activities, respectively. Recent studies on the function of these subunits demonstrated that

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inhibition of β_5 subunit could achieve therapeutic effects.[25–31] However, co-inhibition of proteasome β_1 or β_2 subunit have attracted much attention, since inhibition of β_1 or β_2 subunit is also important in some cancer cell lines.[32] So far the effect of inhibition of β_1 or β_2 subunit has not been fully elucidated mainly due to the lack of specific inhibitor. Therefore, exploration of selective proteasome β_1 or β_2 inhibitors may provide us more information to study the mechanism and further develop novel drug candidates, as well as avoiding side effects. Herein we report the copper Schiff base complexes as selective proteasome β_2 inhibitors with valuable structure–activity relationship.

The Schiff base contains a C=N double bond as a functional group prepared by condensation of an aldehyde or ketone with a primary amine. As ligand, Schiff base coordinates metals in various states by the lone pair of the nitrogen atom of the C=N moiety and other electron-rich functional groups.[33] In the previous Letters, various copper Schiff base complexes have been designed with different substituted groups. The synthetic method has also been explored and optimized.[34–36] In this work, we hypothesized that modification of the Schiff base ligand would change the docking mode of the inhibitor in the active site of proteasome. As shown in Figure 1, various diamines (***a***) and salicylaldehydes (***s***) were employed to construct tridentate and tetradentate Schiff base ligands by typical methods.

Before assessment of the copper Schiff base complexes, we prepared various Schiff base ligands and measured their activity toward proteasome inhibition (Table 1). Different diamines and salicylaldehydes were chosen to construct a library for further study of structure–activity relationships (Scheme 1). It is revealed that the synthesized Schiff base ligands showed no selectivity toward all three β subunits though some have inhibitory activities. To increase the water solubility of the ligands, sulfonate groups were introduced to the salicylaldehyde moiety (***s3***). However, all the Schiff base ligands containing ***s3*** showed no inhibitory activity except ***a5s3*** which exhibited IC_{50} 10 μM for all β subunits. ***a2s3***, ***a3s3***, ***a4s3***, ***a6s3***, ***a8s3***, ***a9s3*** and the tridentate ligand $\boldsymbol{a2(s3)_{1/2}}$ showed negligible activities. Moreover, tetradentate ligands including ***a1s1***, ***a4s1***, ***a6s1***, ***a7s1*** showed no activities neither. To identify the diamine moiety, we used the 5-carboxylic acid substituted diamine ***a4*** to build Schiff base ligands with disubstituted 3,5-dichloro and 3,5-dibromo salicylaldehydes (***s4***, ***s5***). To our delight, ***a4s4***, ***a4s5*** showed obvious improvement of proteasome inhibitory activity around 1 μM despite ***a4s3*** was incompetent. Bulky diamine moiety binaphthyl diamine ***a9*** was used to replace ***a4***, but the activity disappeared. Furthermore, when the salicylaldehyde moiety ***s5*** was fixed, ***a3s5*** was prepared to compare with ***a4s5***. Although both of them have activity, ***a3s5*** showed higher IC_{50} approximately 6 times to ***a4s5***. Therefore, both the diamine and the salicylaldehyde are crucial, and precise collaboration of these two moieties is essential for the rational design of metal Schiff base complexes as proteasome inhibitors.

Table 1
Schiff base ligands as proteasome inhibitors[a]

Ligand	IC_{50}[b] (μM)	Ligand	IC_{50} (μM)
a1s1	NI[c]	***a4s4***	$\beta_1 = 1.26 \pm 0.02$ $\beta_2 = 1.19 \pm 0.01$ $\beta_5 = 1.29 \pm 0.08$
$\boldsymbol{a2(s3)_{1/2}}$	NI	***a4s5***	$\beta_1 = 1.65 \pm 0.06$ $\beta_2 = 1.71 \pm 0.08$ $\beta_5 = 1.82 \pm 0.18$
a2s3	NI	***a5s3***	$\beta_1 = 9.38 \pm 0.27$ $\beta_2 = 12.47 \pm 0.29$ $\beta_5 = 10.24 \pm 0.30$
a3s2	$\beta_1 = 7.35 \pm 0.16$ $\beta_2 = 8.20 \pm 0.18$ $\beta_5 = 8.02 \pm 0.14$	***a6s1***	NI
a3s3	NI	***a6s3***	NI
a3s5	$\beta_1 = 9.52 \pm 0.27$ $\beta_2 = 12.32 \pm 0.28$ $\beta_5 = 10.05 \pm 0.29$	***a7s1***	NI
a4s1	NI	***a8s3***	NI
a4s3	NI	***a9s3***	NI
Bortezomib[d]	4.2 ± 0.34		

[a] Values represent the mean ± SD of three independent experiments, each based on four biological replicates.
[b] Percent inhibition at 25 μg mL^{-1}.
[c] NI: no inhibition.
[d] Positive control.

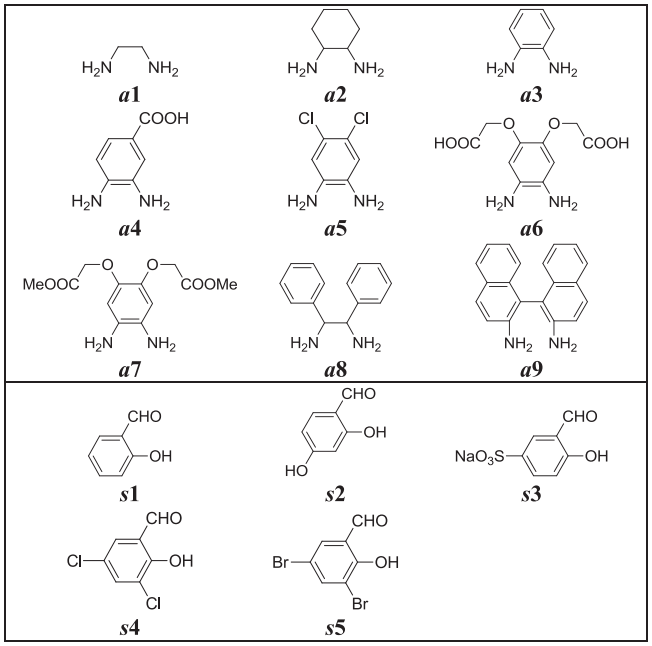
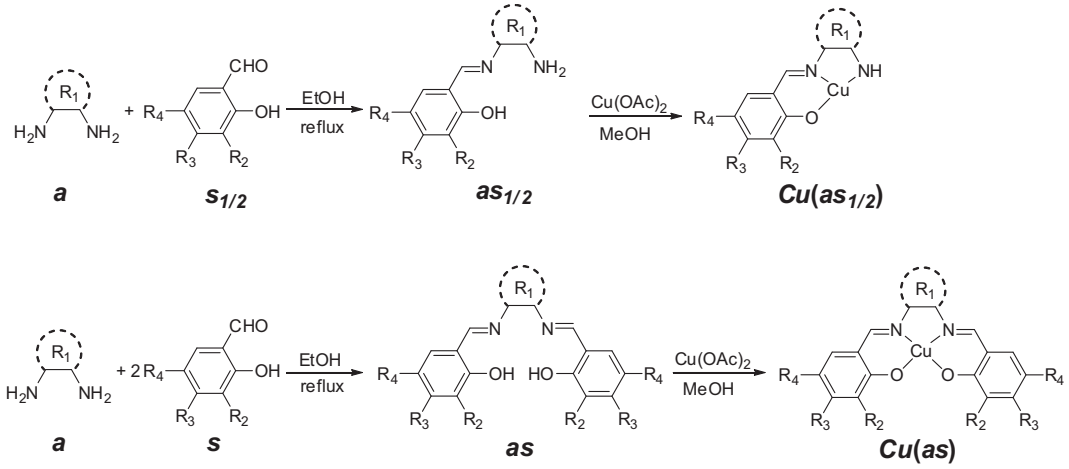


Figure 1. Different diamines and salicylaldehydes used in this work.

Then we synthesized series of copper(II) and platinum(II) complexes in different solvents with modest to good yields (Scheme 1). To confirm the accurate structure of the copper(II) complex, proper diffusion of ethanol into the aqueous solution of Na_2[***Cu***(***a2s3***)] afforded brownish red crystals. Before this, crystal structure of Na_2[***Cu***(***a2s3***)] with half-hydrolyzed C=N bond has been reported by Rogez and co-workers.[37] In this work, we obtained the intact single crystal of Na_2[***Cu***(***a2s3***)] which crystallizes in the triclinic P1 space group. The X-ray diffraction reveals a complete Schiff base ligand in the space lattice consists of NNOO tetradentate coordination with a central copper ion structure. It is also clear to see the two deprotonated sulfonate groups on the salicylaldehydes bonding sodium ions (Fig. 2).

Although complexation of copper ion with Schiff base ligand might change the structure significantly, the primary results of proteasome inhibition of the Schiff base ligands are still valuable. We considered that complexation of copper ion with Schiff base ligand might change the activity by alteration of the molecular configuration and further the bonding mode in the active site of proteasome. Before the evaluation of copper(II) complexes, we examined the fluorescence spectrum of our compounds including all the Schiff base ligands and copper(II) complexes in the presence of the reaction buffer, all of them showed negligible fluorescence intensity under the test condition of proteasome inhibition experiment. Furthermore, we tested the fluorescence of Schiff base ligand with addition of magnesium ion in HEPEs buffer but no obvious change of fluorescence spectrum has been observed which indicated that magnesium ion has no influence to our



Scheme 1. Synthetic routes of tridentate and tetradentate copper(II) Schiff base complexes.

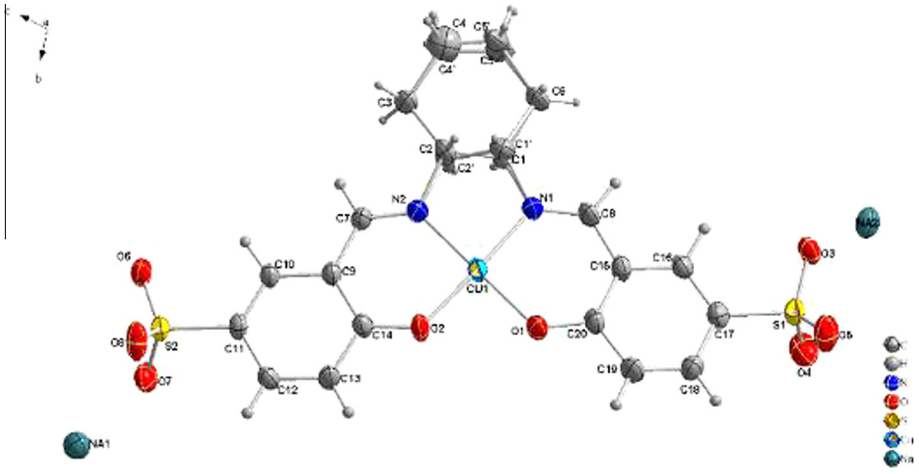


Figure 2. An ORTEP view (50% ellipsoids) of $Na_2[\boldsymbol{Cu}(\boldsymbol{a2s3})]$ (CCDC 847754).

experiments.[38] Then we did control experimental to eliminate the possible quenching and we found the copper(II) complexes does not quench the final fluorescent readout. All of these preliminary experiments showed the possible deviation of our results interrupted by photoluminescence does not exist thus we initiated our biological evaluation of these copper(II) complexes.

As shown in Table 2, although the Schiff base ligands and their corresponding copper complexes exhibited nonparallel relationship on the proteasome inhibition, we still found encouraging results that $Na_2[\boldsymbol{Cu}(\boldsymbol{a3s3})]$, $Na_2[\boldsymbol{Cu}(\boldsymbol{a4s4})]$, $Na_2[\boldsymbol{Cu}(\boldsymbol{a4s5})]$ and $Na_2[\boldsymbol{Cu}(\boldsymbol{a9s3})]$ showed obvious proteasome β_2 inhibitory activities with negligible effect on β_1 and β_5 subunits. It is noteworthy that $Na_2[\boldsymbol{Cu}(\boldsymbol{a4s1})]$ showed submicromolar IC_{50} toward β_2 subunit. Besides, $Na_2[\boldsymbol{Cu}(\boldsymbol{a2s3})]$ also showed β_2 inhibitory activity approximate to $Na_2[\boldsymbol{Cu}(\boldsymbol{a4s1})]$. Parallel to the results of Schiff base ligands, 5-carboxylic acid substituted diamine ***a4*** played positive role in proteasome β_2 inhibition. All copper complexes containing ***a4*** moiety exhibited moderate to excellent activities. In detail, ***s3*** still played negative role similar to the results obtained from Schiff base ligands. In spite of the negative effect of ***s3*** involved in the specific type of ***a4*** family, it reversed the effect in $Na_2[\boldsymbol{Cu}(\boldsymbol{a2s3})]$ and $Na_2[\boldsymbol{Cu}(\boldsymbol{a9s3})]$. Structure differentiation affecting the interaction between copper complexes and their targets is accountable for the proteasome inhibition though the details are not clear.

Table 2
Copper Schiff base complexes as proteasome β_2 inhibitors[a]

Complex	IC_{50}[b] (μM)	Complex	IC_{50} (μM)
$Na_2[\boldsymbol{Cu}(\boldsymbol{a1s1})]$	NI[c]	$Na_2[\boldsymbol{Cu}(\boldsymbol{a4s5})]$	7.65 ± 0.24
$Na_2[\boldsymbol{Cu}[\boldsymbol{a2}(\boldsymbol{s3})_{1/2}]\}$	NI	$Na_2[\boldsymbol{Cu}(\boldsymbol{a5s3})]$	NI
$Na_2[\boldsymbol{Cu}(\boldsymbol{a2s3})]$	**1.08 ± 0.09**	$Na_2[\boldsymbol{Cu}(\boldsymbol{a6s1})]$	NI
$Na_2[\boldsymbol{Cu}(\boldsymbol{a3s2})]$	NI	$Na_2[\boldsymbol{Cu}(\boldsymbol{a6s3})]$	NI
$Na_2[\boldsymbol{Cu}(\boldsymbol{a3s3})]$	7.01 ± 0.14	$Na_2[\boldsymbol{Cu}(\boldsymbol{a7s1})]$	NI
$Na_2[\boldsymbol{Cu}(\boldsymbol{a4s1})]$	**0.89 ± 0.02**	$Na_2[\boldsymbol{Cu}(\boldsymbol{a8s3})]$	NI
$Na_2[\boldsymbol{Cu}(\boldsymbol{a4s3})]$	23.94 ± 0.32	$Na_2[\boldsymbol{Cu}(\boldsymbol{a9s3})]$	6.78 ± 0.18
$Na_2[\boldsymbol{Cu}(\boldsymbol{a4s4})]$	3.77 ± 0.21	Bortezomib[d]	4.2 ± 0.34

[a] Values represent the mean ± SD of three independent experiments, each based on four biological replicates.
[b] Percent inhibition at 25 $\mu g \cdot mL^{-1}$.
[c] NI: no inhibition.
[d] Positive control.

To reveal the structure–activity relationships, AutoDock was often used in our previous study as one of the powerful tools.[39] Herein, Autodock 4.2 was used to predict of the bonding mode between the potent copper complexes and different proteasome β subunits by docking $\boldsymbol{Cu}(\boldsymbol{a4s1})$ into β_2 and β_5 subunits (PDB: 5cgi) of proteasome respectively. As shown in Figure 3a and b, the 3,4-diaminobenzoic acid moiety stretched into the pocket of β_2, generating hydrogen bonds between O**H**(Thr1)-CO**O**H, C=**O** (Arg19)-COO**H**, N**H**(Lys33)-C**O**OH. Meanwhile, the salicylaldehyde groups are buried in the gap, with the two oxygen atoms as part of the hydrogen bond network together with Gln22. In another case, the binding mode of $\boldsymbol{Cu}(\boldsymbol{a4s1})$ in β_5 subunit is distinguished.

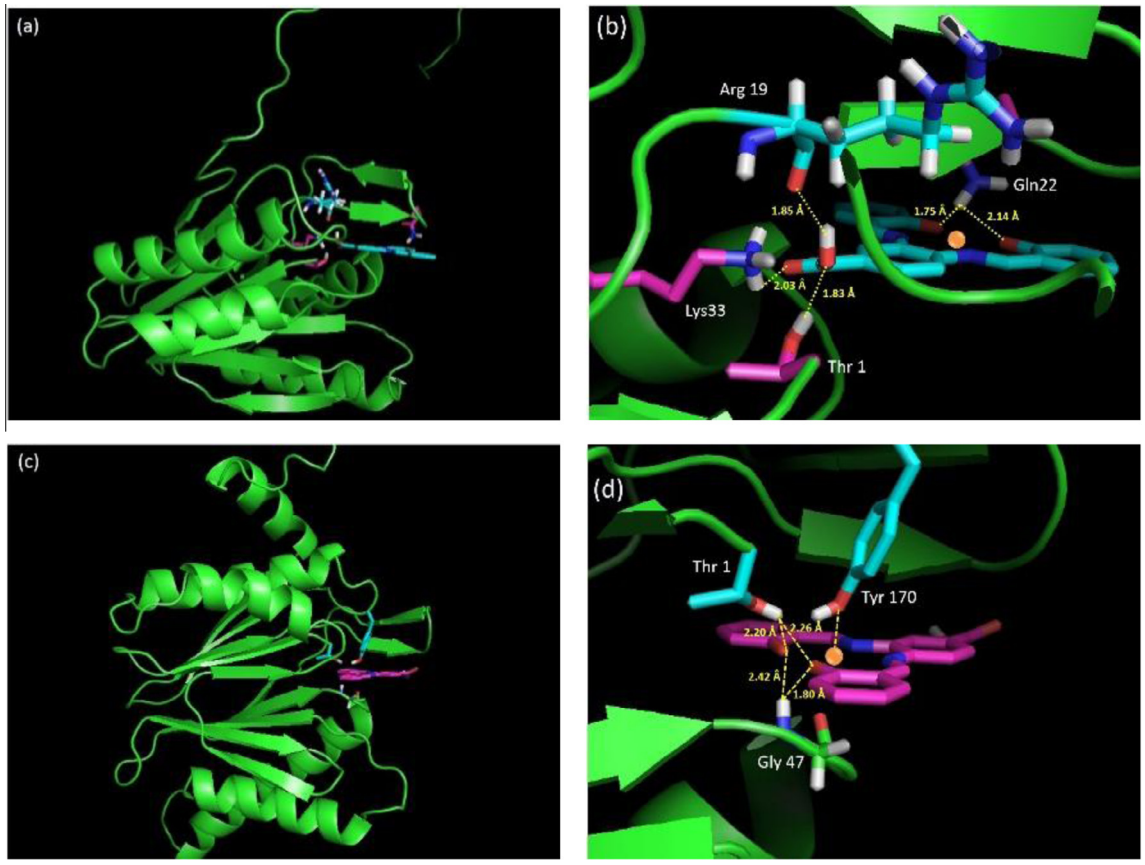


Figure 3. (a) and (b) Conformation of ***Cu***(***a4s1***) in proteasome β_2 subunit. (c) (d) ***Cu***(***a4s1***) in proteasome β_5 subunit.

As shown in Figure 3c and d, the spatial orientation of ***Cu***(***a4s1***) in β_5 subunit is opposite. The hydrogen bonds formed by nearest residues of amino acids including Thr1, Gly47 with two phenoxy oxygen atoms of salicylaldehydes [O**H**(Thr1)-**O**, O**H**(Gly47)-**O**]. Besides, the phenoxy group of Tyr170 might has interaction with the copper center.

Comparing the bonding mode of ***Cu***(***a4s1***) in β_2 and β_5 subunits, it is observed clearly that the buried depth and spatial orientation of inhibitor in each pocket, the amount and pattern of hydrogen bonds are quite different. Due to the carboxylic group, the spatial orientation of ***Cu***(***a4s1***) in β_2 and β_5 subunits are opposite while ***Cu***(***a4s1***) embedded deeper in β_2 than in β_5. Moreover, the amount of hydrogen bonds between ***Cu***(***a4s1***) and target in β_2 subunit is more than that in β_5 subunit. All of these differences might contribute to the selective proteasome inhibition of copper(II) complexes.

In summary, we demonstrated the design, synthesis and biological evaluation of series of copper(II) Schiff base complexes as proteasome inhibitors. Consequently we discovered several copper(II) Schiff base complexes which exhibited highly inhibitory activities toward proteasome with selective β_2 subunit inhibition. Together, some valuable structure–activity relationships information has been obtained in our present study which may lead further optimization for copper complexes as antitumor drug candidates. Mechanistic study and other evaluation of these copper Schiff base complexes as antitumor agents is now underway in our laboratory.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2016.10.043.

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