Trivalent metal ions based on inorganic compounds with *in vitro* inhibitory activity of matrix metalloproteinase 13

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1.Introduction

Matrix metalloproteinases (matrixins or MMPs) represent a family of zinc metalloproteinases that is capable of degrading and remodeling structural proteins in the extracellular matrix such as gelatin, elastins, collagens, matrix glycoproteins and proteoglycan. MMPs play a major role in almost all aspects of cell behavior and belong to the metzincin superfamily of metalloproteinases. The latter is characterized by the presence of a divalent zinc atom (Zn^{2+}) in the active center of the enzyme. The typical structure of MMPs consists of a signal peptide (SP), a catalytic domain and a haemopexin-like domain [1]. The Zn^{2+} binding site in the catalytic core domain of all MMPs features a HEXXHXXGXXH amino acid, and the individual sequences bear high similarity in their 3D structures. The pocket in the core domain is hydrophobic in nature, but variable in depth depending on the MMPs, representing a determining factor of substrate specificity of MMPs. In fact, the S1' pocket in the catalytic domains of MMPs has been reported to be particularly important. The depth and the length of the S1' pocket and amino acids all represent critical foundations for the design and synthesis of matrix metalloproteinase inhibitors (MMPIs)[2-6].

MMPs mainly regulate the synthesis and secretion of cytokines, growth factors, hormone receptors, and cell adhesion molecules. Moreover, MMPs are also found to be critically involved in processes including tissue remodeling, angiogenesis, arthritis, cardiovascular disease, stroke, multiple sclerosis, neurodegenerative diseases, and allergies[7,8]. In tumor progression, MMPs play a key role not only in invasion processes, angiogenesis, and metastasis, but also in cancer cell transformation, growth,

apoptosis, signal transduction and immune regulation[9,10]. It has been shown before that the secretion of MMPs by microvascular endothelial cells represents a critical step in the formation of new blood vessels and most of the MMPs may initiate and promote angiogenesis[11, 12]. In recent years, research groups around the globe have focussed on finding suitable inhibitors for MMPs to treat cancer or other related serious health conditions[13-18]. The activities of MMPs can be regulated by endogenous inhibitors, such as tissue inhibitors of metalloproteinase (MMPIs)[19-21]. In general, MMPIs can be classified as follows: natural MMPIs secreted by tissues, synthetic MMPIs, MMPIs screened from natural products and MMPIs screened from phage. Originally, the first generation of MMPIs, used as peptides or derivatives, has been designed to simulate the substrate of MMPs. Inhibition is achieved by competing chelation of the central zinc ion (Zn^{2+}) in MMP by ligands present in the inhibitors, e.g. hydroxylamine, carboxyl, thiols, etc[22]. In recent years, synthetic MMPIs have been used in clinical trials for MMP inhibition in the treatment of progressive lung cancer. However, due to a variety of side effects caused by the use of synthetic small molecule inhibitors in clinical applications, research has now focussed on finding MMPIs derived from natural products[23-25]. Anti-MMPs properties have been reported from the components of various natural products. For example, epigallocatechin gallate (EGCG) extracted from green tea exhibits a potent MMPI activity[26,27]. The Korean monkshood root (*Guanbaifu* in Chinese), is commonly used in the treatment of many diseases such as arthralgia, headache, convulsive epilepsy, coronary heart disease, ischemic arrhythmia, pyocutaneous disease and

anemogenous phlegm. It has been demonstrated to exhibit a strong inhibitory effect on different MMPs, such as MMP-2 and MMP-9[28-30]. The active compound featuring an inhibitory effect on MMPs extracted from the Korean monkshood root was identified as aluminum ammonium sulfate dodecahydrate ($\text{NH}_4\text{Al}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$). Further experiments have confirmed that the aluminum(III) ion (Al^{3+}) is responsible for the inhibitory activity. This was the first time that an inorganic substrate, i.e. aluminum ammonium sulfate, has been demonstrated to exhibit MMP inhibitory activity, potent enough to interfere with MMP related cellular processes[31].

Human matrix metalloproteinase 13 (MMP-13), also known as collagenase-3, represents a key member of the MMP family and has been identified as an important target for the treatment of osteoarthritis (OA)[32-35]. Until now, MMP-13 inhibitors have been identified mainly via high-throughput screening (HTS) in the synthetic compound library. Hydroxamic acid is a typical inhibitor for MMP-13 and the reaction mechanism for this activity has been suggested to be due to a functional group that is able to coordinate the central zinc(II) ion to two oxygen atoms via a bidentate ligand interaction[36-39]. In our previous work, Al^{3+} has been shown to exhibit strong inhibitory effects on both MMP-2 and MMP-9 using a HT1080 cell line[31]. We are now interested to determine whether Al^{3+} or other metal ions would exhibit similar effects on the catalytic domain in MMP-13 (cdMMP-13) *in vitro*. Therefore, we further characterized the cdMMP-13 inhibitory activity using different metal ions and analyzed the corresponding active components. In this work, we obtained the target protein by refolding the recombinant histidine-tagged catalytic

domain of MMP-13[40]. Circular dichroism (CD) analysis confirmed that cdMMP-13, desalted with the metal ions, increases the stability of the secondary structure of the protein. By comparison of the inhibitory activity of compounds containing different valence metal ions with a positive control inhibitor (i.e. CL-82198) on cdMMP-13, the half maximal inhibitory concentration value (IC_{50}) showed that trivalent metal compounds in particular exhibit a strong cdMMP-13 inhibition. Compared to other compounds, potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$) has been shown to exhibit the best inhibitory effect[36]. Furthermore, the binding event between $K_3[Fe(CN)_6]$ and cdMMP-13 has been confirmed by isothermal titration calorimetry (ITC) and an endothelial cell tube formation test provided evidence for the notion that this interaction may have a potential effect on anti-angiogenesis.

2. Materials and Methods

2.1. Materials

Yeast extract and tryptone were purchased from OXOID (Basingstoke, UK). Isopropyl β -D-1-thiogalactopyranoside (IPTG), kanamycin sulfate, urea, imidazole, acrylamide, tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), N,N-methylenebisacrylamide and glycine were purchased from Sangon Biotech (Shanghai, China). The markers for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Thermo Scientific (Waltham, MA, USA). Brij-35 was purchased from Amresco (Solon, OH, USA). CL-82198 was purchased from Biovision (San Francisco, CA, USA). DQ-gelatin was purchased from Molecular Probes (Eugene, OR, USA). The Escherichia coli (E. coli) strain BL21, to express the

His-tagged cdMMP-13 catalytic domain, was provided from Xuexun Fang (Jilin University, China). Other reagents used in this series of experiments were of analytical grade and were purchased from Kermel (Tianjin, China). Purified water was prepared using a Milli-Q apparatus (Millipore, Bedford, MA, USA).

2.2. Cloning, expression and purification of cdMMP-13

The cdMMP-13 protein was recombinantly produced in the *E. coli* strain BL21 (DE3) with a N-terminal affinity tag using the plasmid pET22b. A detailed method for cloning, expression and purification of cdMMP-13 was carried out as previously reported. cdMMP-13 was cultured in an LB medium containing kanamycin at 37°C. When OD₆₀₀ reached 0.8, IPTG was added to the culture medium to reach a final concentration for induction of 1 mM. The induced culture was further incubated for 4 hours at 37°C. The cells were harvested by centrifugation ($8000 \times g$ for 30 minutes at 4°C) and resuspended in 50 mL of lysis buffer (pH 7.5, 20 mM Tris). The inclusion bodies were recovered by centrifugation at 12,000 g, rinsed with washing buffer I (20 mM Tris, 1 M urea, 100 mM NaCl, and 1% Triton-100) and washing buffer II (10 times diluted washing buffer I). Finally, the inclusion bodies were solubilized in the solubilization buffer consisting of 8 M urea in 20 mM Tris (pH 7.5) for 12 hours at 4°C and were clarified by centrifugation at $12,000 \times g$ for 30 minutes at 4°C. The clarified supernatant included the target protein.

The clarified supernatant was purified using a HisTrap FF 5mL column. The column was equilibrated with binding buffer consisting of 8 M urea in 20 mM Tris (pH 7.5) and 75 mM imidazole [41]. The column was loaded with 1 mL of the clarified supernatant and was washed with the binding buffer to remove the contaminating proteins. The target protein was then eluted by the elution buffer consisting of 8 M

urea in 20 mM Tris (pH 7.5) and 300 mM imidazole. All steps were performed at a flow rate of 1 mL/min. The fractions containing the target protein were pooled for renaturation. The overall purity of the purified protein was confirmed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein concentration was determined by the Bradford method using bovine serum albumin as a protein standard.

2.3. Refolding of cdMMP-13 using SEC

Direct refolding was performed using a HisTrap desalted 5 mL column[42]. The column was equilibrated with the refolding buffer (pH 7.5, 20 mM Tris, 20 mM CaCl₂, 20 μ mol ZnCl₂). The purified target protein was loaded, and a flow rate of 1.0 mL/min was maintained. The fractions containing cdMMP-13 were detected using a detection wavelength of 280 nm and were subsequently pooled for bioactivity determination. The purity of the purified protein was confirmed by 15% SDS-PAGE and the protein concentration was determined by the Bradford method using bovine serum albumin as a protein standard.

2.4. Circular dichroism

The secondary structures of the refolded cdMMP-13 with or without metal ions were compared by circular dichroism (CD) studies[43, 44]. All measurements were carried out at room temperature under a constant flow of nitrogen. The concentration of the target protein was diluted to 0.2 mg/mL. A quartz cell of 0.01 cm path length was rinsed with buffer. Each CD spectrum was scanned at 50 nm/min with a 1 nm slit width and constant times of 0.125 seconds. The data was collected at wavelengths ranging from 190 to 250 nm.

2.5. Enzymatic Activity Assay

The activities of the refolded cdMMP-13 were measured using the quenched fluorescein conjugate-dye-quenched (DQ) gelatin[45]. The assays were conducted in 20 mM Tris (pH 7.5) containing 200 mM NaCl, 10 mM CaCl₂, 20 mM ZnSO₄ and 0.05% Brij-35. After addition of the samples into Tris buffer, incubation at 37°C for 30 minutes followed. Upon addition of 4 mg/mL DQ-gelatin substrate, the reactions were immediately measured by a fluorescence microplate reader (FLX-800, Bio-Tek) with an excitation wavelength of 320 nm and an emission wavelength of 405 nm for 8 minutes. An enhanced fluorescence intensity indicated that cdMMP-13 forms an active enzyme, which in turn provides evidence for that notion that protein refolding has occurred.

2.6. Study on compound inhibition

The refolded cdMMP-13 was added to the enzyme reaction buffer after the compounds have been serially diluted, resulting in a final concentration of cdMMP-13 of 9 µg/mL. The control sample and cdMMP-13 were then incubated at 37°C for 30 minutes. The enzyme substrate reaction buffer was diluted with the mother liquor to 20 µM. The samples in 96-well plates were incubated in each 50 µL well and the substrate was added to each well 55-60 µL to a row different from the samples[46]. The total volume of the reaction system was 100 µL and the final protein concentration was 4.5 µg/mL, with a final concentration of 10 µg/mL. The changes in fluorescence intensity were recorded afterwards. The excitation wavelength used was 320 nm, and the emission intensity was measured at 405 nm. The initial slope of the change in fluorescence intensity is the initial rate of the enzymatic reaction. The average value of the initial rate of the enzymatic reaction (v) in different inhibitor

concentrations ([I]) has been obtained with the protein having the same substrate concentration. The enzymatic reaction rate (v_0) without inhibitor is 100%.

All calculations were carried out according to the following equation:

$$act\% = \frac{v}{v_0} \bullet 100\%$$

The relative activity of the sample (act%) at different inhibitor concentrations could be obtained. The inhibitor concentration at which the activity of the protein is 50%, is the half maximal inhibitory concentration (IC_{50}). CL-82198 served as the positive control drug and has already been reported to be an efficient MMP-13 inhibitor[47].

2.7. Isothermal titration calorimetry (ITC)

ITC experiments allow for the measurement of the heat associated with a binding reaction. From one experiment, the observed enthalpy, reaction stoichiometry, and equilibrium constant for the binding reaction can be directly obtained, allowing for calculation of the Gibbs free energy and the change in entropy by using the following two equations: $\Delta G^\circ = -RT\ln K$ and $\Delta G^\circ = \Delta H - T\Delta S$. Entropy data presented herein is reported in terms of $-T\Delta S$ to allow for a direct comparison to the enthalpy and Gibbs free energy (ΔG°) terms.

ITC of cdMMP-13 with the inhibitor was carried out using a ITC200 calorimeter (MicroCal ITC200, GE Healthcare). The protein was extensively dialyzed in a refolding buffer 20 mM Tris (pH 7.5) containing 20 mM $CaCl_2$ and 20 μ M $ZnCl_2$. The titration involved 20 injections of $K_3[Fe(CN)_6]$ (injection volume: 100 μ L; concentration: 500 μ M) into the protein (volume: 400 μ L; concentration: 50 μ M). The binding stoichiometry (N), affinity constant (K_a) and the other thermodynamic

parameters, enthalpy (ΔH) and entropy (ΔS), of the binding event were analyzed using Microcal Origin 8.0 software (Origin Lab Corporation), provided with the instrument.

2.8. Tube formation assay

The generation of blood vessels surrounding the cells has been observed by a three-dimensional culture technique. Pipette tips and 96 well cell culture plates were pre-cooled at 4°C. The Matrigel matrix was removed from the freezer and was allowed to melt at 4°C overnight. Under strictly sterile conditions, the Matrigel matrix was diluted with serum free DMEM at a ratio of 1:3, and was then coated with 50 μ l in 96 well cell culture plates and incubated at 37°C for 20 minutes. The rest of the procedure was similar to the one described for the cell migration assay. The mean cell number was calculated for each group. The cells were collected, washed with cold PBS and re-suspended at a concentration of 5×10^4 cells/mL for apoptosis analysis. HepG2 and SMMC-7721 were placed onto Matrigel™-coated chamber slides, and were incubated with or without $K_3[Fe(CN)_6]$ and $AlCl_3$ for 24 hours. Images of the structures were taken using a confocal microscope (Olympus).

3. Results and Discussion

3.1. Expression and purification of cdMMP-13

The recombinant cdMMP-13 has been expressed and purified using the optimized conditions. The denatured his-cdMMP-13 was eluted (peak 4, cf. Fig. 1) and was analyzed by SDS-PAGE to give a single band with a protein size of about 23 kDa (Fig. 3). The target protein was obtained in 98% purity and was used for the refolding studies.

3.2. Optimization of refolding buffer

In the refolding buffer without metal ions (pH 7.5, 20 mM Tris), the recombinant cdMMP-13 could be obtained with a purity of about 90%. It has been reported that ionic interactions can be reduced by addition of proper metal ion concentrations. This method proves to be helpful to wash out the contaminant proteins bound to the matrix by nonspecific interactions [48, 49]. Consequently, the concentrations of the metal ions in the refolding buffer (pH 7.5, 20 mM Tris, 20 mM CaCl_2 , 20 μmol ZnCl_2) were optimized in an effort to obtain the target protein with higher purities and concentrations. As shown in Figure 3, the target protein moved as a single band with a size of about 23 kDa on the SDS-PAGE. The chromatograms, obtained from the refolding buffer with or without metal ions, are shown in Figure 2.

3.3. Secondary structures of the enzymes determined by circular dichroism

A CD spectrum was recorded and used to monitor the secondary structure of cdMMP-13 with or without metal ions at equilibrium. The corresponding spectra of the cdMMP-13 are indicative of α + β structures. The cdMMP-13 with metal ions exhibit secondary protein structures of 27.6% α -helix, 21.4% β -pleated sheet, 19.8% β -turn, and 31.2% random coil. Without metal ions, the secondary protein structures consist of 22.8% α -helix, 19.9% β -pleated sheet, 22.1% β -turn, and 35.2% random coil (cf. Fig. 4). The spectra between the target protein with and without the metal ions were found to be obvious, whereas a higher α -helix is found in the presence of metal ions. Therefore, it can be concluded that the presence of metal ions in the refolding buffer results in an efficient enhancement of protein stability.

3.4. Enzymatic Activity Assay

The activity of the refolded cdMMP-13 was determined using the quenched fluorescence signalin conjugate-DQ gelatin. DQ-gelatin could be hydrolyzed by refolded cdMMP-13 to result in the production of fluorescent peptides, which can be excited and monitored by a fluorescence spectrophotometer. Thus, the increasing rates of the fluorescence intensity reflected the proteolysis rates of the samples (cf. Fig. 5). The resulus from the refolded cdMMP-13 showed that fluorescence intensity increased with time, while the fluorescence intensity of purified cdMMP-13 and the blank control resulted in no fluorescence changes. Taken in concert, these results indicate that the enzymatic activity of cdMMP-13 recovered upon refolding.

3.5. Inhibitory activity of metal ions to cdMMP-13

The inhibitory activities of different valence metal compounds show that trivalent metal ions are able to inhibit the protein activity (cf. Table 2). The curves of the inhibitory activity of AlCl_3 , FeCl_3 and $\text{K}_3[\text{Fe}(\text{CN})_6]$ to cdMMP-13 demonstrate that uponincreasing the concentration of the compound, the activity of cdMMP-13 decreases accordingly. By comparison of the inhibitory activity of different valence metal compounds with the positive control drug (CL-82198), the half-effect values (IC_{50}) were determined as 4.6 μM , 1.3 μM and 3.4 μM , respectively (cf. Fig. 6). The results obtained show that trivalent metals,particularly $\text{K}_3[\text{Fe}(\text{CN})_6]$, exhibit a strong inhibitory effect on cdMMP-13. Therefore, further studies currently carried out in our laboratory mainly focus on $\text{K}_3[\text{Fe}(\text{CN})_6]$ as an inhibitor for cdMMP-13.

3.6. Thermodynamics and modeling of substrate binding

The thermodynamics of the binding event of $\text{K}_3[\text{Fe}(\text{CN})_6]$ to cdMMP-13 werestudied by ITC (cf. Fig. 7). For the binding reaction of $\text{K}_3[\text{Fe}(\text{CN})_6]$ to

cdMMP-13, the heat was baseline corrected and the isotherms were fitted for a one site binding to yield an average equilibrium constant (K) of $1.41(\pm 2.7) \times 10^5$, consistent with previously reported $1/K_d$ values. This K value results in a favorable Gibbs free energy, where a value for ΔG° could be determined at approximately $-9.9(\pm 0.2)$ kcal/mol. A single site binding model was found for $K_3[Fe(CN)_6]$ and the thermodynamic parameters for the binding event were: N (protein: substrate stoichiometry) = 0.78 ± 0.02 , ΔH (binding enthalpy) = 1.94 ± 0.32 kcal/mol and ΔS (entropy change) = 40.2 cal/mol K. The free energy change (ΔG) was found to be -9.99 kcal/mol (cf. Table 3). A positive enthalpy value is characteristic of hydrogen bonding and van der Waals interactions, whereas a positive entropy value is indicative of hydrophobic interactions between the protein and the ligand. Likely, the presence of the iron cyanide complexes in the binding pocket enhances the affinity. This ΔH binding event can then be used to calculate the $-T\Delta S$ binding term. Our ITC experiments were performed in buffers selected for their specific pH ranges and negligible heats of interaction with the protein itself. All experiments are baseline corrected and background subtracted. The background can consist of dilution heats of the $K_3[Fe(CN)_6]$ and the cdMMP-13 as well as residual heats from nonspecific interactions of $K_3[Fe(CN)_6]$ and the cdMMP-13 when the binding reaction is completed.

3.7. Inhibition of HUVEC capillary-like tube formation by compounds

Studies on the inhibition of capillary tube formation by $AlCl_3$, $K_3[Fe(CN)_6]$ and the positive control drug (CL-82198) were carried out. A tube formation assay was used to assess the ability of these compounds to inhibit tube formation by HUVECs in Matrigel™. The effects of $K_3[Fe(CN)_6]$ and $AlCl_3$ on the formation of capillary-like structures are shown in Figure 8. This test provides evidence for the anti-angiogenesis

potential of $K_3[Fe(CN)_6]$ and $AlCl_3$ and the result is found to be similar to CL-82198. Angiogenesis is dependent on the ability of endothelial cells (ECs) to proliferate. A colorimetric MTT cell proliferation assay was used to determine the effects of $AlCl_3$, $K_3[Fe(CN)_6]$ and CL-82198 on HUVEC proliferation. $AlCl_3$, $K_3[Fe(CN)_6]$ and CL-82198 all demonstrated a similar anti-proliferative activity towards HUVECs, with IC_{50} values of 4.6 μM , 1.3 μM and 3.4 μM , respectively (cf. Fig. 6). Therefore, it can be concluded that $K_3[Fe(CN)_6]$ may be useful as a new cdMMP-13 inhibitor.

Generally MMP-13 inhibition is occurred by competing chelation of Zn^{2+} in MMP-13 and dependent on the zinc binding affinity of chelating groups present in the inhibitors. Examples for such groups include hydroxylamine, carboxyl, SH, etc. In the protein, Zn^{2+} is mainly chelated by hydroxylamine-functionalities via combination of the peptide of the substrate analogs and at the same time through the substrate peptide analogs in combination with the catalytic domains of MMP-13. Strong Zn^{2+} chelating agents such as hydroxamate, as a MMPI type, have been developed and they all exhibit an ideal inhibitory activity towards MMPs. The protein-Zn structure with Zn^{2+} was obtained from MMP-13, and computational simulation studies of the protein with Fe^{3+} have been carried out. We combined the protein crystal structure with the predicted modeled binding mode and the protein-Zn was used as the template protein. Unfavorable contacts were removed through energy minimization of the MMP-13 bound to Fe^{3+} . From inspection of Figure 9 it can be determined that zinc(II) features a three-fold coordination sphere bound to three histidine units. When Fe^{3+} replaces Zn^{2+} and binds to MMP-13, electron-attracting groups are introduced in the same position and the electron densities of the active center change. As a consequence, the electron density increases, leading to an increased inhibition ability. This observation suggests that iron(III) potentially replaces zinc(II) in Zn-MMP-13 under these

conditions. Compared with Al^{3+} , Fe^{3+} and $\text{Fe}(\text{CN})_6^{3-}$ both feature superior inhibitory activities, mostly due to multi-interactions with the binding site in the activity center. As a consequence, the substrate could not interact with the target protein efficient inhibitory activities.

Oncogenic fusion proteins are a characteristic feature of many human leukemias and they often play a key role in driving the development and progression of the disease. Arsenic, an toxin and ancient drug used in traditional Chinese medicine, has attracted considerable interest around the world, most notably due to the fact that it shows substantial anticancer activity in patients suffering from acute promyelocytic leukemia (APL). Scientists have demonstrated that arsenic is able to bind to cysteine groups present in zinc fingers located within the RBCC domain of PML-RAR α and PML. Furthermore, it was found that this binding event supports the development of APL. In summary, PML as a direct target of As_2O_3 has been identified, potentially providing a therapeutic approach for leukemia[50]. Inorganic compounds, such as semimetals, metal ions and metal complexes which bind to the zinc finger protein, have emerged as a new research paradigm for anticancer treatment. However, in the extracts, the active ingredients of traditional Chinese medicine not only involve inorganic compounds but also metal ions. The effect of the latter has often been ignored and as such, the structural and molecular concept explaining the high sensitivity of MMP-13 towards $\text{K}_3[\text{Fe}(\text{CN})_6]$ merits further investigations.

4. Conclusions

Potassium hexacyanoferrate(III), $\text{K}_3[\text{Fe}(\text{CN})_6]$, as a new MMP-13 specific inhibitor has been studied. Moreover, we refolded the target protein, exhibiting a higher bioactivity, using size exclusion chromatography (SEC). The refolding buffer

with metal ions results in a higher α -helix than those without metal ions, did not affect the secondary structures of the enzymes and provided the target protein in a higher purity. Further studies show that trivalent metal ions, present in $K_3[Fe(CN)_6]$, pose a potential inhibitory effect on MMP-13 and the IC_{50} ($1.3\mu M$) was found to be better than the positive control drug (CL-82198) ($3.4\mu M$). Through isothermal calorimetry studies it was found that the trivalent iron(III) core in $K_3[Fe(CN)_6]$ is able to bind to MMP-13 and endothelial cell tube formation tests show that this interaction exhibits anti-angiogenesis potential. In general, trivalent metal compounds feature crucial advantages in terms of simple structures, low toxicities and high binding constants with the target protein. Thus, the identification of the zinc finger protein as a direct target of small molecules opens a new paradigm for the optimization of such systems in future cancer therapies.

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Table 1 Secondary structures of cdMMP-13 from CDspectroscopy.

	α (%)	β (%)	Turn (%)	Random (%)
With metal ions	27.6 ± 3.8	21.4 ± 4.1	19.8 ± 4.2	31.2 ± 3.5
Without metal ions	22.8 ± 4.0	19.9 ± 3.5	22.1 ± 3.8	35.2 ± 3.1

Table 2 The inhibitory activity of compounds to cdMMP-13.

compounds	IC ₅₀ (μmol/L)	compounds	IC ₅₀ (μmol/L)
NaCl	-	PrCl ₃	9.1 ± 0.08
KCl	-	SmCl ₃	4.9 ± 0.16
CaCl ₂	-	EuCl ₃	5.4 ± 0.22
CaSO ₄	-	TmCl ₃	9.7 ± 0.17
MgSO ₄	-	HoCl ₃	6.1 ± 0.19
MgCl ₂	-	ErCl ₃	9.3 ± 0.13
FeCl ₂	-	AsCl ₃	12.44 ± 0.22
CuCl ₂	-	CL-82198	3.4 ± 0.17
CeCl ₂	4.2 ± 0.05	FeCl ₃	4.3 ± 0.16
CrCl ₃	10 ± 0.17	AlCl ₃	4.6 ± 0.2
LaCl ₃	4.5 ± 0.14	K ₃ [Fe(CN) ₆]	1.3 ± 0.13

Table 3 Dissociation constants of the interaction between $K_3[Fe(CN)_6]$ and cdMMP-13.

	n	$K_d(\mu M)$	$\Delta H(Kcal\ mol^{-1})$	$\Delta S(cal\ K^{-1}mol^{-1})$
$K_3[Fe(CN)_6]$	0.78 ± 0.02	16.1 ± 1.2	1.94 ± 0.32	40.2

Figure Legends

Fig.S1. Chromatogram of the purification of cdMMP-13. Peaks 1, 2, and 3 are the washed-out peaks; Peak 4 is the target protein peak.

Fig.1. Chromatogram of the refolding of cdMMP-13.(A)Refolding buffer without metal ions (20mM Tris/HCl, pH 7.5);(B) Refolding buffer with metal ions (20mM CaCl₂, 20μmol ZnCl₂, 20 mMTris/HCl, pH 7.5).Peak 1 is the target protein peak; Peak 2 is the small molecule peak.

Fig.S2. SDS-PAGE of purified and refolded cdMMP-13. Lane 1-3: purified cdMMP-13; Lane 4-5: refolded cdMMP-13 with metal ions. The target protein gave a single band with a size of about 23kDa.

Fig.2. CD spectra of thecdMMP-13 desalted with (red line) or without (blue line) metal ions.

Fig. S3. Bioactivity detection of refolded cdMMP-13.

Fig.3. Inhibition curve of different compounds.(A), (B), (C) are experimental drugs (AlCl_3 , FeCl_3

and $\text{K}_3[\text{Fe}(\text{CN})_6]$);(D) is the positive control drug(CL-82198);(e) is the structure of CL-82198.

Fig.4. The ITC results between recombinant protein cdMMP-13 with $K_3[Fe(CN)_6]$.

Fig.5. Inhibition of capillary tube formation by AlCl_3 , $\text{K}_3[\text{Fe}(\text{CN})_6]$ and CL-82198. A tube formation assay was used to assess the ability of AlCl_3 , $\text{K}_3[\text{Fe}(\text{CN})_6]$ and CL-82198 to inhibit tube formation by HUVECs in Matrigel™. (A) is the control; (B) and (C) are 25 μM , 50 μM of AlCl_3 ; (D) and (E) are 25 μM , 50 μM of $\text{K}_3[\text{Fe}(\text{CN})_6]$; (F) and (G) are 25 μM , 50 μM of CL-82198.

Fig.6. Predicted binding mode of the Al^{3+} , Zn^{2+} and Fe^{3+} with protein. Al^{3+} , Zn^{2+} and Fe^{3+} are shown in white, gray and red color, respectively (A, B and C). The histidines binding to ion is shown in lines model.