Lead Poisoning

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Potent Cyclic Tetrapeptide for Lead Detoxification

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Abstract: *Lead (Pb) is a ubiquitous poisonous metal, affecting the health of vast populations worldwide. Medications to treat Pb poisoning suffer from various limitations and are often toxic owing to insufficient metal selectivity. Here, we report a cyclic tetrapeptide that selectively binds Pb and eradicates its toxic effect on the cellular level, with superior potency than state-of-the-art drugs. The Pb-peptide complex is remarkably strong and was characterized experimentally and computationally. Accompanied by the lack of toxicity and enhanced stability of this peptide, these qualities indicate its merit as a potential remedy for Pb poisoning.*

Lead (Pb) is a non-essential, toxic metal considered the most harmful metal to human health.[1,2] Pb poisoning is responsible for 1 million deaths worldwide annually.[3] Above 3% of the children in the United States of America are found to have dangerous blood Pb levels ($\geq 5\ \mu g\,dL^{-1}$),[4] while globally, every third child is poisoned by Pb.[5] Under physiological conditions, Pb predominantly occurs as Pb^{2+} ions that interact with various proteins, primarily with the thiol groups of cysteine and the carboxylate of aspartic or glutamic acid residues.[6–8] This metal binding reshapes the conformation of proteins, resulting in their diminished function.[7,8] Pb^{2+} ions also substitute several essential metal ions in metalloproteins, mainly calcium (Ca^{2+}) and zinc (Zn^{2+}) ions, causing metalloprotein dysfunction. As a result, Pb damages both organ operation and cellular processes.[8–10]

Chelation therapy is the current treatment for Pb poisoning.[11,12] An ideal chelating agent (CA)[11–13] possesses several essential characteristics: a) low toxicity of the *apo* and *holo* species, b) high metal selectivity, c) water solubility before and after complexation, d) formation of a depletable complex, and e) ability to penetrate tissues and cell membranes.[8,14,15] The CAs most commonly used against Pb poisoning are *ethylenediaminetetraacetic acid* (EDTA) and *dimercaptosuccinic acid* (DMSA; Figure 1A).[12–15] These drugs accomplish some of the requirements stated above,[11,13,15] yet, although being the primary treatment for Pb poisoning, they suffer from significant drawbacks. Most importantly, due to their low metal selectivity, they expel essential metals from the body during treatment.[12,16] They are also suspected of redistributing Pb^{2+} ions to various organs, including the brain.[16,17] Lastly, EDTA cannot cross cellular membranes, limiting its use to extracellular targets. As a result, these CAs are only approved for medicinal use in extremely high levels of toxic metals. However, they are not approved for use in pregnant women[18–20] and are only rarely applied in pediatric cases, even though these segments are the most affected populations.[15]

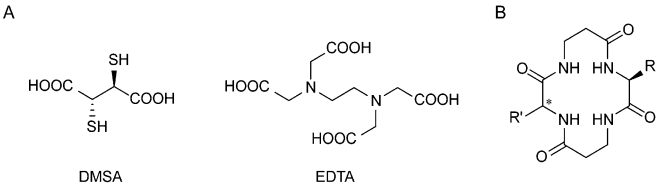

Figure 1. A) The benchmark drugs DMSA and EDTA for treating Pb poisoning, B) peptide scaffold designed for complexation with Pb^{2+} ions.

As opposed to the commonly used drugs, which are small molecules, organisms evolved detoxification systems based on peptides and short proteins to overcome metal poisoning.[21–24] Taking inspiration from nature, we envisioned that synthetic peptides could serve as ligands for Pb^{2+} ions[25–28] and fulfill all of the desired criteria for an effective CA.[25]

Herein we present a cyclic tetrapeptide that binds Pb^{2+} ions with high affinity and selectivity over other essential metal ions. Moreover, we show that the peptide can recover Pb-exposed human cells with a potency that outperforms the benchmark CAs.

We envisioned that a short, head-to-tail cyclic peptide[29] with at least two metal-binding moieties would be able to discriminate between Pb^{2+} and other essential ions. A specific cavity size and variations in the nature of the metal-binding groups should allow for preferential binding of the soft-borderline, large metal ion. Following this guideline, a scaffold bearing the sequence cyc-[Xaa-βAla-Zaa-βAla] (Xaa and Zaa depict for any α-AA; Figure 1B) was crafted. βAla was expected to facilitate the challenging intramolecular cyclization of the tetrapeptide and enhance proteolytic stability.

We started our studies by synthesizing nine cyclic tetrapeptides (Scheme 1, Table 1, **1**–**9**).[30] The sequences of peptides **1**–**8** were chosen based on the amino acids found to interact with Pb^{2+} ions in characterized peptides and proteins.[6–10,31] Peptide **2** was designed to study the effect of the amino acid configuration, where one of the LCys residues of **1** was replaced with DCys. Lastly, **9** was examined as a negative control to other hetero-bifunctionalized peptides since Phe was not expected to interact with the metal ion but sterically

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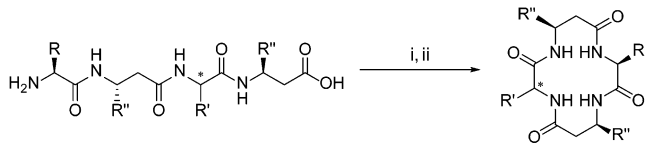

i: 0.2 mM in CH_2Cl_2, PyBOP (0.3 mM), Hünig's base (0.6 mM), 16-72 h

ii: TFA:H_2O:TIPS (95:2.5:2.5) or TFA:H_2O:TIPS:EDT (87.5:2.5:2.5:7.5)

Scheme 1. Cyclization and sidechain deprotection of twelve peptides.

Table 1: Peptides studied and their yields, aqueous solubility, and recovery rate in human cells (HT-29 cells).

Name	Sequence[a,b]	Yield[c] [%]	Aqueous solubility[d]	Recovery[e] [%]
1	$[Cys\text{-}\beta Ala]_2$	39	soluble[f,g]	–
2	Cys-βAla-*D*Cys-βAla	43	soluble[f,g]	–
3	$[Met\text{-}\beta Ala]_2$	82	insoluble	–
4	$[His\text{-}\beta Ala]_2$	65	soluble	106 ± 3
5	$[Asp\text{-}\beta Ala]_2$	41	soluble[f]	120 ± 11
6	Cys-βAla-Met-βAla	37	soluble[f,g]	–
7	Cys-βAla-His-βAla	77	soluble[g]	–
8	Cys-βAla-Asp-βAla	51	soluble[f]	334 ± 42
9	Cys-βAla-Phe-βAla	40	insoluble	–
1a	$[Cys\text{-}\beta Asp]_2$	39	soluble[f]	106 ± 10
8a	SAsp-βAla-Asp-βAla	75	soluble[f]	416 ± 25
8b	$[SAsp\text{-}\beta Ala]_2$	66	soluble[f]	74 ± 17

[a] Three-letter code of AAs. [b] Head-to-tail cyclic peptides. [c] Over cyclization, deprotection and purification. [d] At 120 mM. [e] In HT-29 human cells and with 10 mM CA. [f] With the addition of 2 equiv NaOH or 1 equiv $Ca(OH)_2$. [g] Precipitates in cell media.

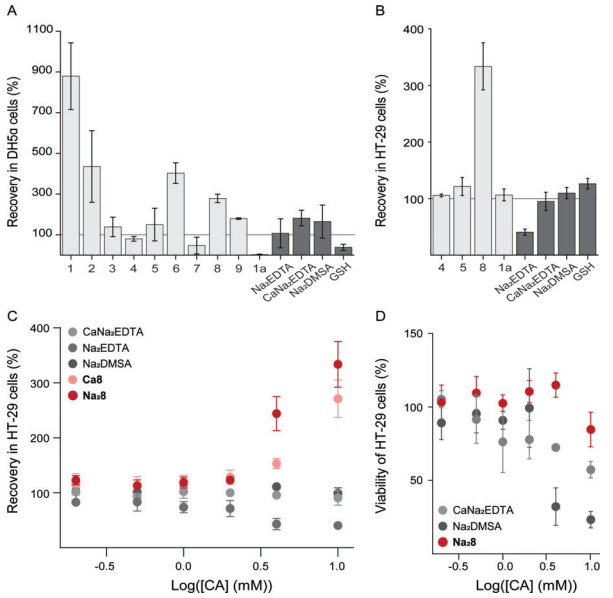

Figure 2. A) Recovery of DH5α cells treated with $Pb(NO_3)_2$ (12 mM) followed by the administration of **1**–**9**, **1a**, and reference compounds (10 equiv; 5 h after the addition of Pb^{2+} ions; values are calculated relative to cells poisoned with Pb^{2+} ions as the negative control), B) recovery of HT-29 cells treated with $Pb(NO_3)_2$ (2 mM) followed by the administration of peptides **4**, **$Na_2$5**, **$Na_2$8**, **$Na_2$1a**, and reference compounds (5 equiv; 1 h after the addition of Pb^{2+} ions; values are calculated relative to cells poisoned with Pb^{2+} ions as the negative control), C) dose-dependent recovery of HT-29 cells treated with $Pb(NO_3)_2$ (2 mM) followed by the administration of **$Na_2$8**, **Ca8**, Na_2EDTA, $CaNa_2$EDTA, and Na_2DMSA (1 h after the addition of Pb^{2+} ions; values are calculated relative to cells poisoned with Pb^{2+} ions as the negative control), D) dose-dependent viability of HT-29 cells treated with **$Na_2$8**, $CaNa_2$EDTA, and Na_2DMSA.[30]

shield potential binding. The sidechain protected peptides were cyclized in solution at an ultrahigh dilution of the peptide in CH_2Cl_2 until full conversion was obtained (Scheme 1, Table 1).[30] HR-ESI-MS and ^1H and ^{13}C NMR indicated exclusively intramolecular cyclization to form the desired tetramers. Noteworthy, replacing one or both βAla with Gly or Ala yielded a mixture of cyclic tetramers and octamers, corroborating the contribution of βAla to the synthesis.

The peptides were then assessed for their ability to detoxify Pb^{2+} ions (Figure 2 A–C; Supporting Information, Figures S1–S6). We developed two assays for a rapid and reliable screening of potential CAs both with bacteria (DH5α cells) and human cell-culture (HT-29 cells).[30,32] Briefly, cells were first exposed to $Pb(NO_3)_2$ slightly below the minimal inhibitory concentration and then treated with various concentrations of the investigated CA, ranging between 0.1–10 equivalents. The viability of the cells was determined by colony counting or the crystal violet assay[33] for bacteria and human cells, respectively.[30] The results were compared to poisoned cells that were not treated with any CA as the negative control and to the non-treated cells as the positive control, indicating their recovery and rescue values, respectively.[30]

Among the nine peptides tested, four exhibited excellent results in detoxifying poisoned *E. coli* compared to the benchmark compounds (Figure 2 A; Supporting Information, Figure S2). All of the peptides contain at least one Cys and an additional residue that can bind Pb^{2+}; Cys, DCys, Met, and Asp for peptides **1**, **2**, **6**, and **8**, respectively. Treatment of bacteria with **1** increased the recovery more than 8-fold compared to the negative control. Substitution of one of the LCys with DCys (peptide **2**) reduced the detoxification ability by half. This shows that the binding moieties should point in the same direction, capturing Pb^{2+} ion in its favored unique hemidirected geometry. Surprisingly, the homo-bifunctionalized peptides **3**–**5** showed insufficient activity, indicating a low metal selectivity or Pb^{2+} affinity. Noteworthy, the linear analogs of peptides **1**–**8** were also tested and showed hardly any detoxification ability (Supporting Information, Figure S3). Thus, the cyclization preorganizes the ligands and endows their metal affinity by improving the coordination properties.

Even though highly active in recovering bacteria, peptides **1**, **2**, and **6** showed low media solubility, reducing their effectiveness as CAs. Attempts to solubilize them, including different pH, formulations with PEG, or co-solvent systems with DMSO, failed. Therefore, we synthesized an analog of **1** where βAla is substituted by βAsp (**1a**). This peptide showed high solubility as a Na or Ca salt, but its detoxification ability in bacteria was unsatisfactory (Figure 2 A; Supporting Information, Figure S2). Its low activity might be related to either a) a competition in coordinating Pb^{2+} between the

carboxylates and the thiolates, which destabilizes the complexation, or b) a decrease in its metal selectivity due to the coordination of alkali or alkaline earth metal ions.

Nevertheless, we tested **1a** and the other soluble peptides for their ability to recover poisoned human cells (Figure 2B; Supporting Information, Figures S4,S5). When necessary, reference compounds and peptides were solubilized as Na salts. Among all compounds, **$Na_2$8** detoxified Pb^{2+} to the greatest extent, with a recovery rate of 334 ± 42%, compared to 110 ± 4% of Na_2DMSA and 95 ± 16% of $CaNa_2$EDTA. **$Na_2$8** also showed the highest rescue rate (viability compared to non-treated cells; Supporting Information, Figure S4) of 90 ± 2%, compared to 49 ± 6% of Na_2DMSA and 42 ± 3% of $CaNa_2$EDTA. This peptide is thus considerably more potent in detoxifying Pb^{2+} than both the benchmark drugs and glutathione (GSH) as a natural reference peptide (Figure 2B,C; Supporting Information, Figures S4,S5).

Next, we tested whether the counter cations affect the activity of **8** (Figure 2C; Supporting Information, Figure S4) since EDTA administration as a CA was changed from Na salt to $CaNa_2$EDTA to decrease the undesired depletion of Ca^{2+} ions.[34] Unlike EDTA, which shows a higher activity and lower toxicity as a Ca salt, **8** is barely affected by the counter cation. This finding indicates that **8** possesses a higher affinity to Pb^{2+} than to Ca^{2+}. The lower activity of **Ca8** in high concentrations is associated with a slightly lower solubility of this salt compared to **$Na_2$8. 8** was also found to have low toxicity (Figure 2D; Supporting Information, Figure S8), especially compared to Na_2DMSA and $CaNa_2$EDTA, as it reduces the viability of only 15 ± 5% of the population.

However, **8** tends to form a precipitate with Pb. We therefore aimed at improving its solubility, both in the *apo* and *holo* forms. We envisioned that thiolation of Asp to form β-mercaptoaspartic acid (SAsp)[35] enables a hybrid between the soluble carboxylate and the binding thiolate. In addition to enhanced solubility, the coordination properties of this amino acid should be improved, as it can form a stable five-membered ring upon a bidentate Pb binding.

Therefore, two analogues of **8** were synthesized: cyc-[SAsp-βAla-Asp-βAla] (**8a**) and cyc-[SAsp-βAla]$_2$ (**8b**; Figure 3A). Their detoxification abilities in human cells were evaluated and compared with that of **$Na_2$8. $Na_2$8a** proved superior to **$Na_2$8**, already at low concentrations, reaching a recovery rate of above 400% and a rescue rate of 114 ± 9% (Table 1, Figure 3B; Supporting Information, Figure S7).[36] Additionally, **$Na_2$8a** itself did not affect the viability of the cells, indicating its safe administration (Supporting Information, Figure S9). **$Na_2$8b**, on the other hand, enabled no detoxification (Table 1, Figure 3B; Supporting Information, Figure S7) and showed high toxicity (Supporting Information, Figure S9). To better understand the differences between these three peptides, we measured the reduced thiol concentration of each of them using the Ellman's test.[30] While **8** and **8a** were barely oxidized, half of **8b** was oxidized shortly after dissolving it in water. In addition to its rapid oxidation and lower solubility, we also hypothesize that **8b** does not effectively bind Pb^{2+}. Quantum mechanics (QM) calculations of **8b** support these findings, revealing an unstable complexation between the peptide and Pb^{2+} ion.[30]

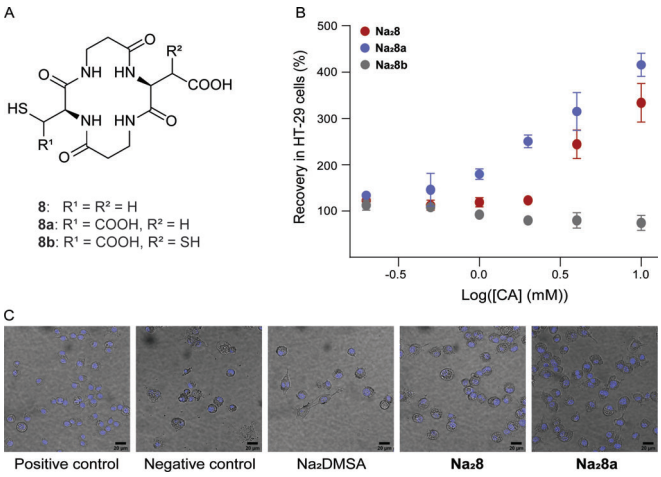

Figure 3. A) Peptides **8**, **8a** and **8b**, B) dose-dependent recovery of HT-29 cells treated with $Pb(NO_3)_2$ (2 mM) followed by the administration of **$Na_2$8**, **$Na_2$8a**, and **$Na_2$8b** (1 h after the addition of Pb^{2+} ions; values are calculated relative to cells poisoned with Pb^{2+} ions as the negative control), C) microscopy images (superimposed bright-field and blue channel) of HT-29 cells treated with $Pb(NO_3)_2$ (2 mM) followed by extensive washing and the administration of Na_2DMSA, **$Na_2$8**, and **$Na_2$8a** (2 mM; 2 h after the addition of Pb^{2+} ions, nuclei stained with Hoechst 33342). Positive control contains non-poisoned cells, whereas the negative control depicts Pb-poisoned cells treated with no CAs.[30]

Microscopy images of Pb-poisoned human cells treated with Na_2DMSA, **$Na_2$8**, or **$Na_2$8a** corroborated the recovery quantifications of these CAs. The cells were compared to an untreated culture (positive control; Figure 3C; Supporting Information, Figure S10) and cells without the administration of a CA (negative control). While Na_2DMSA did not improve the apparent viability of the cells, and most of them showed no vital morphologies, treatment with **$Na_2$8** and **$Na_2$8a** proved effective in preserving cell viability (Figure 3C; Supporting Information, Figure S10). Noteworthy, the medium was replaced 2 h after the addition of Pb^{2+} ions, and the cells were extensively washed prior to the administration of the CAs.[30] These results shed light on the detoxification mechanism of **8** and **8a** and suggest that the peptides can also cross the cell membrane and bind intracellular Pb^{2+} ions.

The complex between **8a** and Pb^{2+} was characterized as a monomeric species, both by HR-ESI-MS (Figure 4A; Supporting Information, Figure S11) and a Job's plot titration following the ligand-to-metal charge-transfer (LMCT) at $\lambda_{max} = 269$ nm (Supporting Information, Figure S12). λ_{max} at this wavelength indicates a binding fashion that combines both the thiolate and the carboxylate ligands.[37]

UV-titration of **8a** with $Pb(NO_3)_2$ revealed a tight binding of peptide **8a** to Pb^{2+} with an observed K_D value of below 1 μM (Figure 4B,C).[38] This binding affinity is remarkable for a monomeric complex with a ligand containing only one thiolate group and with comparison to the affinity of the drugs.[39,40] We then assessed the selectivity of **8a** to Pb^{2+} compared to Ca^{2+} and Zn^{2+} ions in physiologically relevant concentrations by performing UV-monitored back-titrations (Figure 4D–F). These metals were chosen since Pb replaces them in metalloproteins and since EDTA and DMSA bind them non-selectively. Adding even 10 equivalents of $ZnCl_2$ or

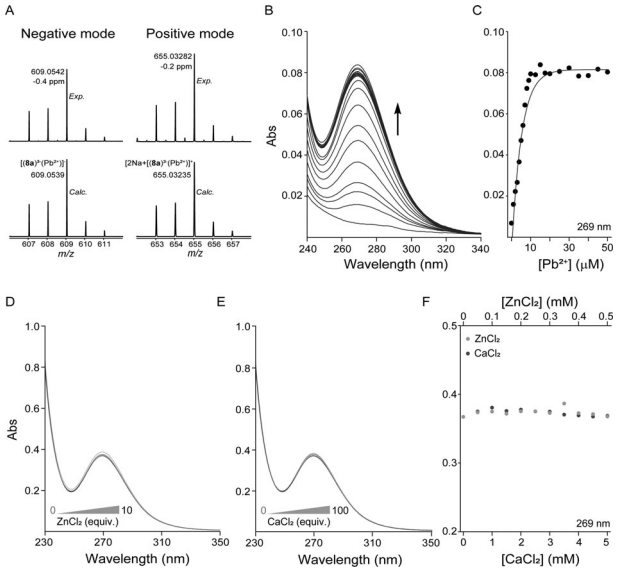

Figure 4. A) HR-ESI-MS spectra of negative and positive modes of **8a**-Pb complex, B) UV titration of **8a** (10 μM) with $Pb(NO_3)_2$ (0–50 μM; 0–5 equiv), C) calculated (line) and experimental (circles) absorbance of **8a**-titration with Pb^{2+} at 269 nm, D) back-titrations of **8a**-Pb (50 μM) with $ZnCl_2$ (0.05–0.5 mM) and E) $CaCl_2$ (0.5-5 mM) where the blue and black spectra are in the absence or presence of 10 or 100 equiv of $ZnCl_2$ or $CaCl_2$, respectively, F) absorbance of the **8a**-Pb complex (50 μM) with 0–10 equivalents of $ZnCl_2$ and 0–100 equivalents of $CaCl_2$ at 269 nm.[30]

100 equivalents of $CaCl_2$ to the **8a**-Pb complex did not affect the spectrum of the latter, indicating high stability of the complex and high metal selectivity of the peptide. Remarkably, the addition of similar equivalents MCl_2 to *apo* **8a** did not change the spectrum of the peptide (Supporting Information, Figure S13), demonstrating a low binding affinity between **8a** and these metals.[30]

We then used QM(DFT-D3)//COSMO-RS calculations to analyze the equilibrium structures of the $[M(\mathbf{8a})\cdot\{H_2O\}_4]^-$ complexes (Figure 5). This was done by employing the composite protocol previously calibrated to obtain stability constants of metal complexes within about 2 kcal mol^{-1} relative accuracy.[30,41] Supported by the UV and MS experiments, the computations clearly show that the most stable complex of **8a** with Pb^{2+} is tridentate, where both carboxylates and the thiolate bind the ion (Figure 5A). Zn^{2+} ion binds similarly, whereas Ca^{2+} is coordinated via the two carboxylates. The predicted complexation Gibbs free energies ($\Delta G_{complexation} = RT\ln K_D$) for the most stable complex of each ion explain the remarkable differences in binding affinities of **8a** to the three ions, corroborating the metal selectivity achieved by this peptide (Figure 5).[30] Specifically, the K_D for Pb^{2+} is predicted to be in the range of 10–100 nM, whereas Ca^{2+} shall bind only in a triple-digit millimolar range, and Zn^{2+} shall reside primarily in solution.

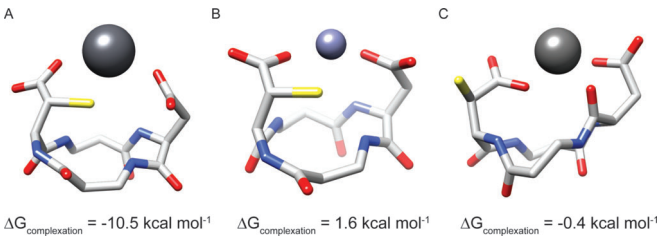

Figure 5. Calculated lowest-energy structures and predicted complexation Gibbs free energies of $[M(\mathbf{8a})\cdot\{H_2O\}_4]^-$ with A) Pb^{2+}, B) Zn^{2+}, and C) Ca^{2+} ions.[30] Hydrogen atoms and four coordinated (or second-sphere) water molecules were omitted for clarity.

On the contrary, the calculated $\Delta G_{complexation}$ for the $[M(\mathbf{8b})\cdot\{H_2O\}_4]^{2-}$ complexes were 22.5, 9.5, and 7.9 kcal mol^{-1}, for $M = Ca^{2+}$, Pb^{2+}, and Zn^{2+}, respectively; perfectly in line with experimental findings that **8b** is incapable of detoxifying Pb^{2+} ions.[30,42]

To further examine the drugability of **8a**, we determined its stability in human blood serum at 37 °C.[43] Thanks to its cyclization and the incorporation of non-proteinogenic amino acids, **8a** showed high resistance during the examined period of 48 h, where no oxidation or degradation products were observed, and the peptide remained intact (Supporting Information, Figure S14).

In conclusion, we successfully obtained a peptide that recovers Pb-poisoned human cells more than 4-fold higher than the state-of-the-art drugs for treating Pb poisoning. Unlike these drugs, the peptide revealed no toxicity, even at high concentrations. Our studies manifest the great potential of peptides and the reported scaffold in particular to become the next-generation CAs against Pb poisoning. Lastly, this sequence may be further leveraged for the development of also selective Pb sensors[44,45] and remediation systems.

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Conflict of interest

T.A.M. and M.S.S. are named inventors on a patent application (EU20211457.5) filed by the University of Zurich on the peptide family described in this work. C.M.M., T.K., M.K., and L.R. declare no competing financial interest.

Keywords: chelation therapy · lead poisoning · metal selectivity · peptides · rational design

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Communications

Lead Poisoning

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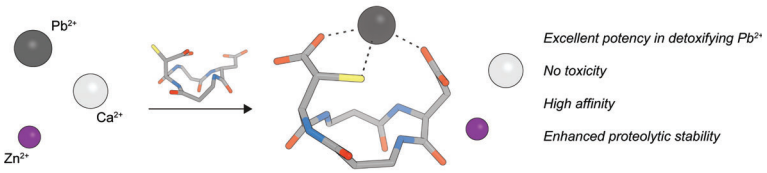
Potent Cyclic Tetrapeptide for Lead Detoxification



Cyclic tetrapeptides were designed and screened for their ability to recover Pb-poisoned bacteria and human cells. Excellent potency was identified with two members, outcompeting the benchmark clinical chelating agents. The leading peptide was further characterized, revealing an outstanding metal affinity and selectivity, indicating its great potential as a next-generation treatment for Pb poisoning.