Increased in vitro Anti-HIV Activity of Caffeinium-Functionalized Polyoxometalates

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Polyoxometalates (POMs), molecular metal oxide anions, are inorganic clusters with promising antiviral activity. Herein we report increased anti-HIV-1 activity of a POM when electrostatically combined with organic counter-cations. To this end, Keggin-type cerium tungstate POMs have been combined with organic methyl-caffeinium (Caf) cations, and their cytotoxicity, antiviral activity and mode of action have been studied. The novel compound, $Caf_4K[\beta_2-CeSiW_{11}O_{39}] \times H_2O$, exhibits sub-nano-molar antiviral activity and inhibits HIV-1 infectivity by acting on an early step of the viral infection cycle. This work demonstrates that combination of POM anions and organic bioactive cations can be a powerful new strategy to increase antiviral activity of these inorganic compounds.

Introduction

One intriguing class of inorganic materials which has attracted significant interest in the field of medicinal chemistry are molecular metal oxides, or polyoxometalates (POMs). POMs are metal-oxo anions based on high-valent transition metals, often Mo or W.[1] Pioneering studies have shown that POMs are promising materials with antibacterial, antiviral and antitumor properties.[2–5] One typical approach to tune POM reactivity is their functionalization by incorporation of hetero-elements such as metal cations. This concept has been successfully employed to design POMs with antiviral properties, and a range of metal-functionalized POMs have been reported as broad-spectrum inhibitors of enveloped DNA and RNA viruses as well as retroviruses such as HIV-1 and HIV-2.[6–10] In one study, it was shown that the sandwich-type cerium-functionalized species $K_{13}[Ce(SiW_{11}O_{39})_2] \times 26H_2O$ is one of the most potent anti-HIV POMs, with an IC_{50} of ~30 nM by binding to the viral envelope protein gp120.[11] Their functionalization with covalently or noncovalently linked organic species is an additional approach to introduce secondary bioactivity into POMs.[12] Arguably the simplest and most versatile strategy is the electrostatic combination of POM anions with organic cations, leading to the formation of organic-inorganic POM salts which combine the properties of both components.[13] This principle has for example been used to combine POM anions with bactericidal tetra-alkylammonium cations to give POM composites with high antibacterial efficiency.[14–16]

Here, we build on these concepts and explore how cerium-functionalized tungstate Keggin POMs can be combined with organo-cations, to improve antiviral activity against HIV-1 infection. As a model system, we selected the caffeinium cation, because the precursor, caffeine, is a natural compound with various promising therapeutic applications and expected to be non-toxic.[17] In addition, earlier studies suggested that caffeine and caffeine-related methylxanthines can affect HIV replication, by acting on the integration step of the virus life cycle.[18,19]

In this project, we report caffeinium-functionalized POM species and provide initial insights into their anti-HIV activity together with mechanistic understanding of the combined mode of action of the cation and anion moieties. This new approach could open the pathway for bringing together organic and inorganic antiviral agents to harness their full synergistic potential in developing next-generation antiviral treatments.

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Results and Discussion

Briefly, the caffeinium-containing POM-salt was synthesized by cation metathesis as follows: the mono-cerium-functionalized lacunary Keggin anion was obtained by reaction of Ce(III) with the monolacunary $K_8[\beta_2\text{-SiW}_{11}O_{39}]\times 13 H_2O$ in water to give $K_5[\beta_2\text{-CeSiW}_{11}O_{39}]\times H_2O$ (hereafter: K_5Ce-POM$\times H_2O$, for synthetic and analytical details see SI). The methyl-caffeinium cation 1,3,7,9-tetramethyxanthinium (hereafter: CAF) was synthesized by methylation of caffeine (for details see SI). The caffeinium-POM compound $Caf_4K[\beta_2\text{-CeSiW}_{11}O_{39}]\times H_2O$ (Caf-POM) was obtained by combining aqueous solutions of CAF and Ce-POM, and selective precipitation of the product. Sample identification and purity confirmation were performed by NMR spectroscopy, CHN elemental analysis, inductively coupled plasma optical emission spectroscopy (ICP-OES) and FT-IR spectroscopy (for details see SI). The stability of the compound under the experimental conditions in aqueous phosphate buffer solution (PBS) at pH 7.4 (10 mM) was analyzed by UV-Vis spectroscopy and showed no significant changes over the course of one week (for details see SI).

The antiviral activity of Caf-POM and Ce-POM (reference compound) was first evaluated against lentiviral pseudotypes carrying either the glycoproteins of HIV-1 (HIV-1 gp120/41), or the vesicular stomatitis virus (VSV-G; for details see SI). For this, virions were treated with the compounds and then used for inoculation of TZM-bl reporter cells. Infection rates were determined 2 days later by a luminescence based assay (see SI) and revealed a concentration-dependent inhibition of both viral pseudotypes (Figure 1). All stocks were prepared in DMEM medium supplemented with 10% fetal calf serum. Both POMs inhibited HIV-1 pseudotype infection, with Caf-POM being ~15-fold more potent (IC_{50}: 0.3 nM) than Ce-POM ($IC_{50} = 4.6$ nM) (Figure 1a). For VSV–G pseudotypes, antiviral efficacy was generally lower, with IC_{50} values of Ce-POM of 13.7 nM and Caf-POM of 111 nM. Thus, Caf-POM exerts a high sub-nanomolar activity against HIV-1.

To verify that the observed antiviral activity is not due to a potential cytopathic/cytotoxic effect on TZM-bl cells, a cell viability assay was performed. None of the compounds reduced cellular metabolic activity when tested at final cell culture concentrations of up to 100 μM, which are orders of magnitude higher than the antiviral activities (Figure 2).
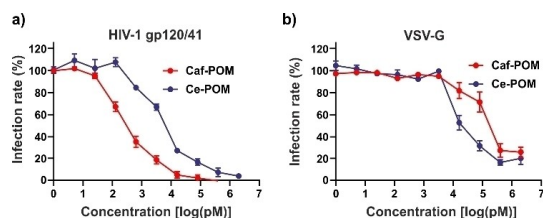

Figure 1. Effect of Caf-POM and Ce-POM on a) HIV-1 gp120/41 and b) VSV-G pseudotype infection. Virions were exposed to serial dilutions of the compounds and used to inoculate TZM-bl cells. 2 days later, infection rates were determined by quantifying ß-galactosidase activities in cellular lysates. Shown are mean values derived from three independent experiments each performed in triplicates ($\pm$ standard deviation).
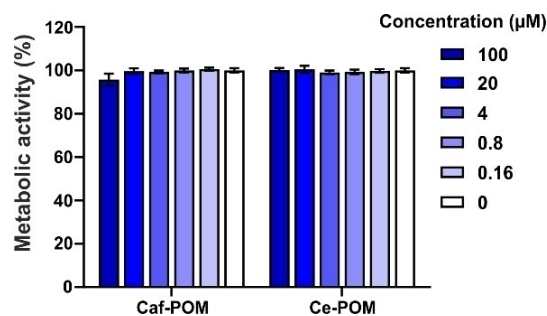

Figure 2. Effect of Caf- and Ce-POMs on the metabolic activity of TZM-bl cells. 10.000 TZM-bl reporter cells were incubated for 48 hours with the indicated concentrations of POMs before cell viability was determined by measuring intracellular ATP levels. Values shown are mean values derived from one experiment performed in triplicates ($\pm$ standard deviation).

Previous studies suggested that POMs inhibit virion attachment to the target cell.[14] To assess whether Caf-POM act by a similar mechanism, HIV-1 pseudoparticles were first exposed to the compound for 15 min and then used to inoculate TZM-bl cells. Alternatively, cells were treated with Caf-POM for 24 hours, washed to remove unbound compound and then infected. Infection rates were determined two days later and confirmed a sub-nanomolar IC_{50} of 0.9 nM of Caf-POM under the conditions of virion treatment (Figure 3). In contrast, Ca-POM lost a considerable amount of its antiviral activity if removed from the cells prior to infection. This finding suggests that the Ca-POM targets the viral particle and/or prevents its interaction with the target cell.

Having demonstrated that the Caf-POM may target an early step in the viral life cycle, we next investigated whether the time the virions are exposed to the POMs affects antiviral activity. HIV-1 pseudoparticles were incubated for 360, 180, 60, 30, 15 or 0 min with both POMs, and then the mixtures were used to infect TZM-bl cells. Infection rates were determined two days later and showed a time-dependent increase in the antiviral activity of the Caf-POM (Figure 4a, 4c) and the Ce-POM
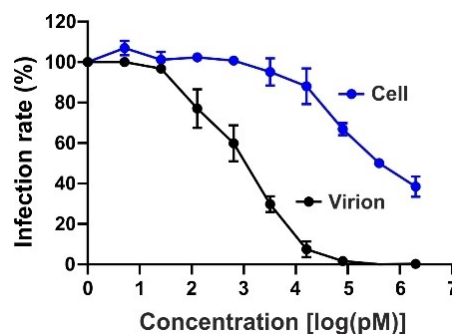

Figure 3. Caf-POM inhibits an early step in the HIV-1 life cycle. Caf-POM was either pre-incubated with TZM-bl reporter cells (blue lines) for one day prior to infection, or mixed with HIV-1 pseudoparticle (black lines) and then added onto TZM-bl cells. Infection rates were determined two day later by quantifying ß-galactosidase activities in cellular lysates. Shown are mean values derived from three independent experiments each performed in triplicates ($\pm$ standard deviation).

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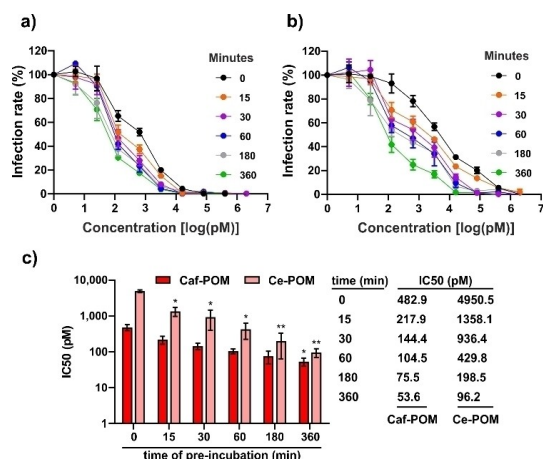European Chemical
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Figure 4. The antiviral activity of Caf- and Ce-POMs increases over time. HIV-1 particles were exposed for indicated times to serial dilutions of Caf-POM (a) and Ce-POM (b). Thereafter, mixtures were used to inoculated on TZM-bl cells and infection rates were determined two days later. Shown are mean values ± standard deviation of two independent experiments performed in triplicates. (c) IC_{50} values of experiments shown in a and b were calculated using Graph Pad Prism. Statistical differences between IC_{50} values was assessed with the unpaired t test. * $p < 0.05$; ** $p < 0.01$; n = 2 ± SEM.

(Figure 4b, 4c). For example, an IC_{50} value of 483 pM was obtained when the Caf-POM was mixed with HIV-1 and immediately added to the cells (0 min) (Figure 4a, 4c). After 1 h of incubation, the IC_{50} decreased to 104 pM, and after 6 hours to only 53 pM (Figure 4a, 4c), corresponding to a 9-fold enhanced antiviral effect. Ce-POM (IC_{50}: 4.950 pM) inhibited infection at the 0 min time point ∼10-fold less effectively as Caf-POM (IC_{50} 483 pM), largely confirming observations shown in Figure 1. However, with increasing incubation times, a comparably more pronounced antiviral effect was observed for the Ce-POM, with an IC_{50} value of only 96 pM at 360 min, corresponding to more than 50-fold enhanced antiviral effect. Collectively, these findings suggest that both POMs target the viral particle in a time dependent manner. The Caf-POM shows faster viral inactivation kinetics as the Ce-POM, but both compounds exert almost similar antiviral activities if incubated with the HIV-1 particles for 6 hours.

To gain more insight into the antiviral mechanism, a liposome leakage assay was performed which uses virion-mimicking lipid vesicles to evaluate membrane-disrupting activity. This is an important criterion, as envelope disruption would irreversibly inactivate HIV-1 virions and could also indicate broad antiviral activity. To this end, vesicles containing self-quenching concentrations of carboxyfluorescein dye were treated with Caf-POM or Ce-POM. Leakage of the fluorescent dye and thus disruption of the vesicle membrane was then assessed by emission spectroscopic analysis of the reaction solution over 60 min. Here, we utilized vesicles consisting of DOPC/SM/Chol (for details see SI, molar ratio 45:25:30 mol-%) to mimic the HIV viral membrane. Interestingly, both POM exerted dose-dependent membrane-disrupting activity. A substantially higher and more rapid effect was observed for Caf-POM as compared to Ce-POM (Figure 5a), highlighting that the
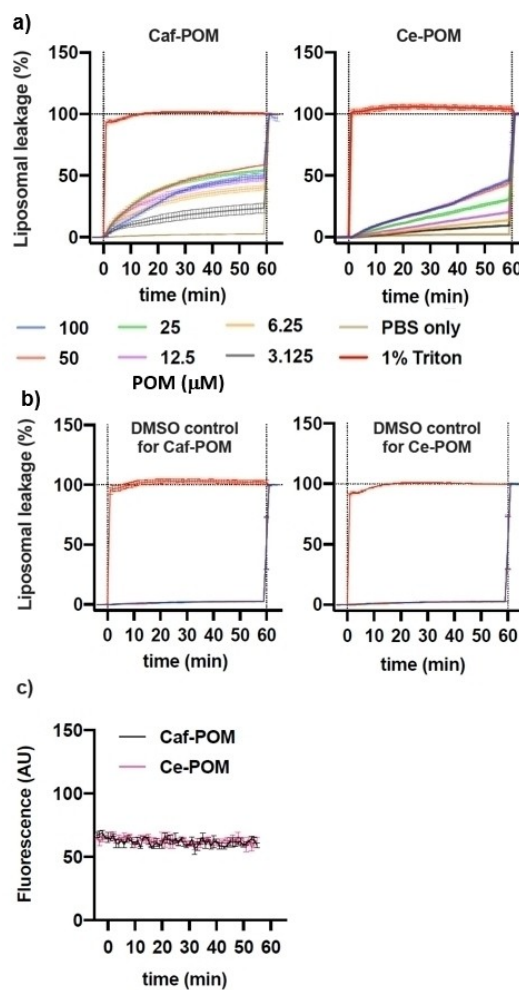

Figure 5. POMs disrupt membranes of virus-like liposomes. Virus-like liposome (VLL) with a membrane composition of DOPC/SM/Chol (45/25/30 mol%) were synthesized with 50 mM carboxyfluorescein cargo and purified by SEC. 2.25×10^9 particles were added to 96 well plates and baseline fluorescence measured for 5 min while incubating at 37 deg C. (a) Indicated concentrations of POMs were then added (first dashed line) and plates incubated for 1 h. Finally, Triton X-100 was added to all wells at a final concentration of 1% (second dashed line) to induce total lysis; values were baseline-subtracted and normalized to the signal obtained for total lysis. (b) DMSO equivalent to the amounts used in POM preparations did not induce leakage. (c) POM in PBS at the highest dose tested (100 μM) did not exhibit autofluorescence or an increase in fluorescence after 1 h incubation. Data shown are means ± SEM form two experiments performed in triplicates.

combined action of cation and anion results in an accelerated membrane-disruptive action. Of note, DMSO only did not induce liposome leakage (Figure 5b) and POMs itself did not exhibit autofluorescence (Figure 5c) demonstrating that the observed changes in fluorescence are caused by released carboxyfluorescein. The disruption of the lipid membrane could be explained by a hydrolytic mechanism based on the Lewis-acidic cerium(III) ion in the POM.[20] However, the concentrations of both POMs required for substantial membrane-disrupting activity within 1 h are in the micromolar range, indicating that (also) other mechanisms are likely involved in antiviral activity observed at picomolar concentrations.

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It is suggested that beyond the electrostatic interaction of the components of Caf-POM, there could be an additional face-to-face stacking (lone pair-π hole interaction) between the purine base and the Ce-POM. It was reported that these interactions occur at the largest faces of the anion.[21] In this structure, this could be the reason for the presence of one K^+ and four Caf^+ to balance the charges as the POM has four largest faces (leaving the large faces nearby the cerium discriminated for steric hindrance effect). POMs can interact with different amino acids of viral proteins, mainly through electrostatic interactions and hydrogen bonds. In addition to this, the caffeinium compound could introduce cooperative effects through the inclusion of a second noncovalent interaction (i.e. π-stacking or cation-π interactions) to form anion-π–π and anion-π-cation triads with the amino acids situated at the protein surface.[22] All these interactions could play an important role in the mechanism of action of Caf-POM against the HIV infection.

Conclusion

In conclusion, we report the design of synergistic polyoxometalate-organocation compounds where caffeinium cations are combined with cerium-tungstate polyoxometalates, leading to novel, combined anti-HIV activity with negligible cytotoxicity. The combination of organocation and POM anion results in enhanced anti-HIV activity as shown by the significantly lower IC_{50} values observed for the caffeinium-containing compound compared with the caffeinium-free reference. In agreement with previous POM-virus studies, the compound is suggested to target an early step in the viral life cycle, probably by a direct interaction with the virus resulting in reduced entry. In addition, we observe that the compound shows modest time-dependent increase of the antiviral activity which we propose could be associated with the gradual destruction of the viral particles. To gain insight into this hypothesis, a liposome leakage assay was performed with virion-mimicking model membranes. The results show some membrane-disrupting activity of both POMs, but a slightly increased activity for Caf-POM. While the effects are much lower than required to fully explain the antiviral activity, it indicates that the POM may interact with viral membranes, possibly leading to interference with viral attachment and, to a lesser extent, membrane disruption and, consequently, inactivation of the virion. In conclusion, Caf-POM represents a highly potent anti-HIV-1 agent and its further investigation as microbicide to block sexual e.g. HIV-1 transmission is warranted.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

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