New potent inhibitors of aquaporins: silver and gold compounds inhibit aquaporins of plant and human origin

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Abstract Silver and gold compounds were tested as potential inhibitors of aquaporins of plant- and human origin. Silver as $AgNO_3$ or silver sulfadiazine inhibited with high potency (EC_{50} 1–10 μM) the water permeability of the peribacteroid membrane from soybean (containing Nodulin 26), the water permeability of plasma membrane from roots (containing plasma membrane integral proteins), and the water permeability of human red cells (containing aquaporin 1). Gold as $HAuCl_4$ was less effective but still inhibited peribacteroid membrane water permeability (EC_{50} = 10 μM). Silver and gold are more potent inhibitors of aquaporins than the presently widely used mercury containing compounds.

Key words: Aquaporin; Silver inhibition; Gold inhibition; Mercury inhibition; Nodulin 26; Plasma membrane integral protein

1. Introduction

Specific, high affinity inhibitors are powerful tools for the study of enzyme systems in their natural environment and are potential candidates for manipulation of enzyme action in an agricultural or therapeutic context. Until now mercury compounds, and mercuric chloride in particular, had the highest known affinity for aquaporin inhibition, and have been used routinely to test for aquaporin activity in plants and animals. These compounds are notoriously unspecific [1] and were often used in high concentration to inhibit aquaporins, if they inhibited at all. It has also been demonstrated for plants that $HgCl_2$ can act as a protonophore [2]. Their high toxicity precludes any commercial application.

Mercury inhibition is thought to occur via covalent modification of cysteine residues within the water pore and in other regions of the protein causing either block or conformational changes leading to inhibition of water transport [3,4]. Other transition elements are also known to interact with sulfhydryl groups but have not been systematically investigated with respect to water permeation through aquaporins. We describe in this paper powerful inhibition of water transport through aquaporins caused by silver and gold compounds. Three broad classes of aquaporins were assessed for their sensitivity to silver and gold by examination of the inhibitory action on the water permeability of native membranes that have been reported to contain high densities of the particular aquaporins.

The peribacteroid membrane (PBM) of soybean nodules contains at high density the Nodulin 26 aquaporin NOD26 [5]. NOD26 represents the class of aquaporins found in plants called the Nodulin like integral proteins of which there are nine in the *Arabidopsis* genome [6]. NOD26 has been found to conduct water as well as glycerol and some small electrolytes [7–10]. Vesicles isolated from the PBM show high water permeability and can be used to test the function of NOD26 in its native membrane [11].

Another group of plant aquaporins are the plasma membrane integral proteins (PIPs) that contain two subgroups with 13 members in *Arabidopsis* [6]. The plasma membrane of storage roots contain PIPs at high density [4,12]. For the plasma membrane of various cell types it has been problematic to isolate fractions which displayed active water channels, but this problem has recently been overcome [13]. We are now able to obtain plasma membrane fractions with active water channels from a variety of plant tissues.

The third aquaporin type tested in this investigation is human aquaporin 1 that densely studs the human red cell membrane [14].

2. Material and methods

Soybean plants (*Glycine max* L. cv. Stephens) inoculated with *Bradyrhizobium japonicum* USDA110 were grown as described in [11]. PBM vesicles were obtained following the procedure of Christiansen and coworkers [15]. Symbiosomes were broken by osmotic shock and by vortexing in a solution containing 10 mM carboxy-fluoresceine to load the resulting PBM vesicles with this dye. The final vesicle pellet was resuspended in a small volume of 130 mM sucrose 5 mM K-Pi buffer pH 7.8 and either used fresh or stored at −80°C. For shrinking experiments dye-loaded vesicles (8–10 μg protein/ml) were resuspended in 130 mM sucrose, 10 mM HEPES–KOH, pH 7, and injected vs. 330 mM sucrose, 10 mM HEPES–KOH, pH 7. Vesicles' shrinking was detected in a stopped flow fluorimeter (Applied Photophysics, Leatherhead, UK) as reduced emission at >515 nm (dye self-quenching) with decreasing vesicle size.

Vesicle size was determined by dynamic laser light scattering in a NICOMP 380 particle sizer (PSS-NICOMP Particle Sizing Systems, Santa Barbara, CA, USA). The instrument was calibrated against latex beads of known diameter distributions. The observed mean vesicle diameter of 220 nm corresponded well to previously reported vesicle size data obtained by electron microscopy [11].

For inhibitor experiments vesicles in 130 mM sucrose, 10 mM HEPES–KOH, pH 7, was mixed with 200 times concentrated inhibitor (4 μl inhibitor/800 μl vesicles) and injected vs. hyperosmotic sucrose containing the same inhibitor concentration. For dose–response experiments, data collection commenced 2.5 min after addition of the

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inhibitor. All inhibitor solutions were made up fresh daily. Silver sulfadiazine (silver 4-amino-*N*-2-pyrimidinylbenzenesulfonamide, Aldrich Chemical Company) was dissolved in dimethyl sulfoxide (DMSO). In these experiments all runs (including the control) contained 0.5% DMSO, which had no significant effect on the observed shrinking rate.

Beet root PM vesicles were obtained following the modified isolation procedure of Gerbeau et al [13] in a phase system adapted to beet root vesicles by the choice of 6.6% (w/w) PEG/Dextran (G. Amodeo, personal communication). Shrinking of PM vesicle was tested as increased light scattering at 500 nm.

Human red cells were obtained from the local blood bank. Cells were washed once in 10 volumes 330 mM sucrose, 10 mM HEPES–KOH, pH 7.4 (5 min, 1000×*g*). Packed red cells were diluted 1 in 560 with the same solution and injected vs. 530 mM sucrose, 10 mM HEPES–KOH, pH 7.4. Inhibitor experiments were conducted as with the plasma membrane vesicles.

Dose–response curves were fitted using Prism (GraphPad) software to the following equation:

$$y = \min + (\max - \min)/(1 + ([\mathrm{Inh}]/\mathrm{EC}_{50})^{n}), \quad (1)$$

where y was either the relative rate constant for shrinking (max = 1) or the osmotic water permeability (P_{os}), [Inh] is the concentration of inhibitor, EC_{50} is the concentration for 50% inhibition, and n the Hill slope.

3. Results

3.1. Time course of inhibition of vesicle shrinking by $AgNO_3$

Fig. 1A shows the time course of inhibition of water flow in PBM vesicles. In a stopped flow fluorimeter PBM vesicles suspended in 130 mM sucrose, 10 mM HEPES–KOH, pH 7 were injected vs. 330 mM sucrose, 10 mM HEPES–KOH, pH 7, to create a 100 mM sucrose gradient, resulting in water flow out of the vesicles and hence, vesicle shrinking. Silver nitrate (5 μM) rapidly reduced the shrinking rate of PBM vesicles. Individual time courses were fitted with a single exponential to obtain a rate constant for shrinking that was used to calculate P_{os} [16]. The P_{os} is plotted against incubation time after addition of 5 μM $AgNO_3$ in Fig. 1B. Within the first minute P_{os} decreased from above 400 μm s^{-1} to 130 μm s^{-1} to reach a final value of 22 μm s^{-1}, which is approaching the background osmotic water permeability of a lipid bilayer. After an initial rapid reduction, the kinetics of which could not be resolved, the subsequent time course of inhibition followed an exponential decay with a half-life of less than 30 s. An exponential decay of activity indicates an irreversible mechanism of inhibition, where the rate of inhibition at any given time is determined by the number of aquaporin molecules that are still active in the membrane. The addition of 1 mM mercaptoethanol failed to restore activity (data not shown). The high initial P_{os} of the PBM that we have measured here compared to our previous report [11] is related to an improved protocol for membrane vesicle extraction, and to an inhibition of the PBM P_{os} by increased osmotic concentration (Niemietz and Tyerman, unpublished). In the experiments reported here we have used a lower initial osmotic concentration (130 mM sucrose) compared to our previous report (330 mM sucrose).

3.2. Dose–response curve for $AgNO_3$ on PBM water permeability

To further characterize $AgNO_3$ inhibition of water flow in the PBM, vesicles were incubated with increasing concentrations of $AgNO_3$ (Fig. 2A). The observed water permeability was plotted against log [μM $AgNO_3$] and fitted with Eq. 1. Under these conditions half-maximal inhibition of water permeability was observed for 2.58 μM $AgNO_3$ with a Hill slope not significantly different to 1.

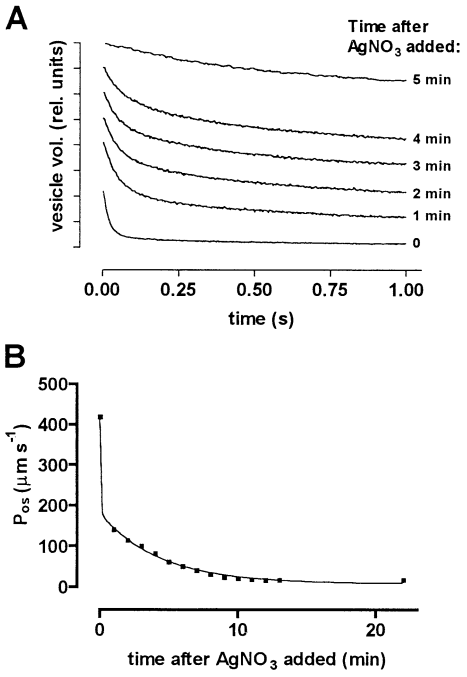

Fig. 1. Time course of $AgNO_3$ (5 μM) inhibition of shrinking rate of PBM vesicles in 130 mM sucrose, 10 mM HEPES–KOH, pH 7, injected vs. 330 mM sucrose, 10 mM HEPES–KOH, pH 7. A: Individual injections performed at the indicated time after exposing PBM vesicles to 5 μM $AgNO_3$. B: Corresponding decrease in P_{os} obtained by fitting single exponential time courses to the individual traces. P_{os} was calculated from the exponential rate constant using a measured vesicle diameter of 220 nm.

3.3. Dose–response curve for silver sulfadiazine on PBM water permeability

To test the possible effect of an organic moiety on the potency of silver inhibition, we incubated PBM vesicles with silver sulfadiazine, a topical antimicrobial agent used in the management of wound infection particular for burns [17]. Fig. 2B shows a dose–response curve similar to that obtained for silver nitrate with an inhibition constant (EC_{50} = 1.47 μM) of the same order of magnitude as for silver nitrate, but with a Hill slope of 2.2.

3.4. Dose–response curve for $HgCl_2$ on PBM water permeability

To compare the potency of silver inhibition to the known effect of $HgCl_2$ on water flux through NOD26, PBM vesicles were incubated with increasing concentrations of $HgCl_2$ as described for $AgNO_3$ (Fig. 2C). The observed inhibition constant EC_{50} for $HgCl_2$ was 38.2 μM and the Hill slope was 1.50. The extent of inhibition (55%) was less complete than with $AgNO_3$ (80%) or silver sulfadiazine (90%). PBM vesicles were about 20 times more sensitive to Ag than to $HgCl_2$.

3.5. Transition elements on PBM water permeability

To further characterize the nature of the observed inhibition of silver ions, PBM vesicles were incubated with numerous other transition elements (Fig. 3). Of the elements tested at 100 μM concentration only silver, mercury and gold significantly reduced water permeability of PBM vesicles.

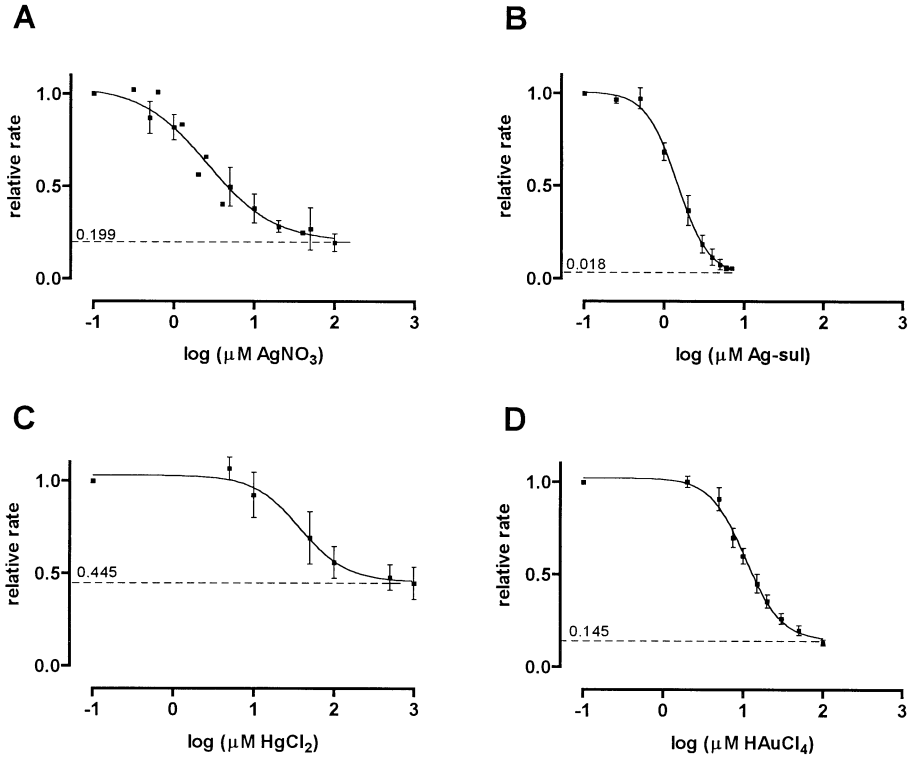

Fig. 2. Dose–response curve for inhibition of water flow in PBM vesicles by transition elements. Every point is an average of at least five injections with the number of individual experiments (different preparation of membrane vesicles) being three to five. The rate constant for shrinking is normalized relative to the rate constant without inhibitor. A: $AgNO_3$, (B) silver sulfadiazine, (C) $HgCl_2$, (D) $HAuCl_4$. Data were plotted on a logarithmic scale and fitted with Eq. 1, fit parameters given in text (Prism software). Horizontal dashed lines with numerical values indicate the minimum relative rate constant as obtained from the fits.

3.6. *Dose–response curve for $HAuCl_4$ inhibition of PBM water permeability*

To quantify the inhibitory potency of gold, PBM vesicles were incubated with increasing concentrations of the gold tetrachloride ($HAuCl_4$). Fig. 2D shows a dose–response curve for $HAuCl_4$ inhibition of PBM shrinking. The observed EC_{50} of 10.8 μM is not as low as the value observed for silver inhibition, but is still three times lower as the EC_{50} for mercury inhibition and the extent of inhibition was 85%. The Hill slope was 1.95.

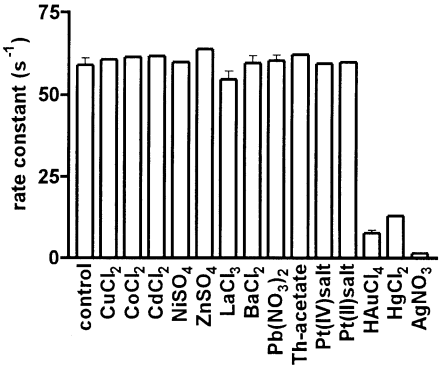

Fig. 3. Effect of numerous transition elements on shrinking rate of PBM vesicles. PBM vesicles in 130 mM sucrose, 10 mM HEPES–KOH, pH 7, were incubated for 5 min with 100 μM of the respective salt and then injected vs. 330 mM sucrose, 10 mM HEPES–KOH, pH 7, also containing the metal salt ($n = 3$ preparations).

3.7. *Effect of $AgNO_3$ on water permeability of plant plasma membrane vesicles*

Fig. 4 shows the effect of increasing concentrations of $AgNO_3$ on water permeability of plasma membrane vesicles derived from beet root (*Beta vulgaris* L.). Silver inhibits with an EC_{50} of 13.2 μM and Hill slope of 2.37. The extent of inhibition was 74%.

3.8. *Effect of silver compounds on water permeability of human red blood cells*

Fig. 5 shows the effect of increasing concentrations of $AgNO_3$ and of silver sulfadiazine on relative rate of shrinking of human red blood cells. Silver nitrate inhibits osmotic shrinking of human red blood cells with an EC_{50} of 3.90 μM and Hill slope of 1.44. The extent of inhibition was 68%. For silver sulfadiazine the EC_{50} was 1.24 μM and Hill slope of 2.60. The extent of inhibition was 75%.

4. Discussion

We have found silver to be the most potent inhibitor of aquaporins so far described. Silver inhibition is rapid and is not reversible with mercaptoethanol. In PBM vesicles the inhibition constant for silver is about 40 times lower than that for mercury. For human red cells the inhibition was about 200× more potent than mercury compounds [18]. Of numerous other transition elements tested, only gold also inhibited water flux in the PBM. Silver inhibition was not restricted to the PBM but was just as effective on water flow from plant plasma membrane vesicles and from human

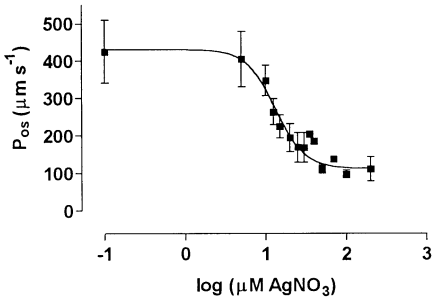

Fig. 4. Dose–response curve for $AgNO_3$ inhibition of shrinking rate of plasma membrane (PM) vesicles from beet root. PM vesicles in 130 mM sucrose, 10 mM HEPES–KOH, pH 8, were incubated with increasing concentrations of $AgNO_3$ for 2.5 min before being injected vs. 330 mM sucrose, 10 mM HEPES–KOH, pH 8 ($n = 5$ preparations).

red blood cells, making it a more general feature of aquaporins.

The mechanism of silver and gold inhibition is most likely due to their ability to interact with sulfhydryl groups of proteins. The thiol moiety of cysteine has a pK_a of around 8.5 that may be lowered to 6 when this moiety is in the neighborhood of other amino acids [19]. The thiolate then easily participates in coordination of transition metal ions. In addition to this the SH group of cysteine has a strongly nucleophilic character [19], which allows its derivatization by nucleophiles such as activated sulfonyl groups as could be the case for silver-sulfadiazine. Another amino acid to possibly interact with silver is histidine [20], but this reaction between silver and the imidazole N of histidine is only supposed to occur at silver concentrations of more than 200 μM.

In the case of aquaporins it is likely that silver reacts with the sulfhydryl group of a cysteine in the vicinity of the conserved NPA motif and thus effectively blocks the constriction region of the channel. However, the non-reversibility by mercaptoethanol may suggest a different mode of inhibition to that caused by mercury. Both mammalian and plant aquaporins [21–26] and the glycerol facilitator from *Escherichia coli* [27] have been crystallized and modelled. Aquaporin 1 from bovine red cells has been described with a resolution down to 2.2 Å [25]. The protein is a homotetramer with each monomer providing an independent pore. An extracellular vestibule narrows down to a channel and widens again on the cytoplasmic side. The constriction region of the channel provides the selectivity filter for water transport. Both cysteine-191 and histidine-182 protrude into the channel at this point. The inner diameter of the channel is 2.8 Å. Even though the three-dimensional structure of NOD26 has not been resolved in such detail, the similarity between the aquaporins that have been structurally resolved so far is so close [25,28,29] that it is likely that NOD26 basically shares this channel architecture.

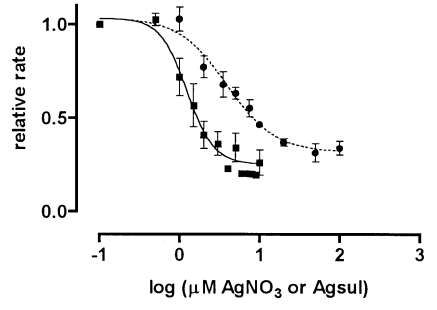

Fig. 5. Dose–response curve for silver inhibition of shrinking of human red blood cells. Red cells in 330 mM sucrose, 10 mM HEPES–KOH, pH, 7.4 were incubated for 2.5 min with increasing concentrations of $AgNO_3$ (dashed line, $n = 3$ preparations) or silver-sulfadiazine (solid line, $n = 5$ preparations) before injected vs. 530 mM sucrose, 10 mM HEPES–KOH, pH 7.4, containing the same inhibitor concentration.

As shown in Fig. 3 only a few elements with an ionic radius similar to Ag^+ inhibit water flow through NOD26. It is striking how close the assigned diameter of the Ag^+ ion (2.5 Å) matches the predicted pore diameter of 2.8 Å. The Hg^{2+} ion is slightly smaller (2.2 Å diameter), whereas the gold ion is slightly larger (2.7 Å diameter) (ionic radii from [30]). All three elements are very reactive towards cysteine residues. This combination of reactivity and size most likely constitutes the basis for the observed inhibition. There was no specific pattern in the Hill slopes for the different inhibitors in each of the membrane systems, although the Hill slope for silver sulfadiazine was greater than that for $AgNO_3$. A Hill slope greater that one may indicate cooperativity in inhibition. The higher Hill slope for silver sulfadiazine may indicate greater accessibility to intramembraneous sites because the molecule is hydrophobic.

The potency of silver inhibition is much higher than that of mercury. In the case of plant plasma membrane aquaporins, silver inhibition was observed when 100 μM $HgCl_2$ failed to reduce water flow (data not shown). This suggests that other aquaporins that in the past have been described as mercury-insensitive might well be inhibited by silver. Silver inhibition is otherwise rare [20,31] making silver a potentially more selective tool to test for presence of aquaporins in experimental systems. The ionic nature of the inhibitors needs to be considered because of interactions with other ions (e.g. Cl^-) and with cation binding sites in plant cell walls. $HAuCl_4$ dissociates to the $AuCl_4^-$ anion and therefore would probably not interact with cation binding sites. Silver sulfadiazine is already used in clinical treatment of burns [17] where the Ag^+ ion is thought to be the active component in inhibiting microbial growth. However, we have found that silver sulfadiazine has a distinctly different pattern of inhibition of aquaporins compared to $AgNO_3$ which freely dissociates, and this may be related to the high hydrophobicity of the silver sulfadiazine molecule.

In conclusion it can be said silver inhibition is both potent and specific, making silver – and possibly gold – not only a new diagnostic tool to test water channel activity, but also opens up avenues to manipulate water channels in agricultural and clinical applications.

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