Differential Effects of Salen and Manganese-Salen Complex (EUK-8) on the Regulation of Cellular Cadmium Uptake and Toxicity

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Cadmium (Cd) stimulates the production of reactive oxygen species (ROS) and causes cell damage. We investigated here the feasibility of using a cell permeable superoxide dismutase/catalase mimetic, EUK-8, to reduce the Cd-induced ROS and cytotoxicity in Chinese hamster ovary cells. EUK-8 reduces the ROS level caused by Cd treatment. EUK-8 also curtails propidium iodide (PI) influx and increases the viability of Cd-treated cells. The efficacy of EUK-8 as a Cd antidote diminishes gradually when added at a later stage of Cd treatment. EUK-8 blocks Cd transport into cells. It is ineffective in accelerating the efflux of metals from the cells. EUK-8 is a Mn-salen complex. Mn decreases the uptake and cytotoxicity of Cd, while salen perturbs the membrane integrity and increases the uptake and cytotoxicity of Cd. Salen is able to bind Cd, and the Cd-salen complex formed does not perturb the integrity of cell membranes and thus the influx of metal is not enhanced. Our results reveal a differential effect of salen and Mn-salen complex on the transport of Cd with subsequent different levels of cell damage. Key Words: EUK-8; superoxide dismutase/catalase mimetic; cadmium; manganese; transport; salen; cell viability.

Cadmium (Cd) is an abundant and widely dispersed toxicant that is continuously accumulated in the environment due to industrial activities. The accumulation of Cd is of great concern because it affects human health through environmental or occupational exposure. Animal and epidemiological studies suggest that Cd is one of the causative agents of pulmonary, prostate, and testicular cancers (Waalkes et al., 1992). Cadmium is thus classified as a human carcinogen by the International Agency for Research on Cancer (IARC, 1993).

The mechanism(s) for Cd toxicity is not well understood. It has been demonstrated that Cd treatment produces ROS that cause cell damage (Zhong et al., 1990). Cd does not produce ROS through Fenton-type reaction (O’Brien and Salacinski, 1998). Instead it may interact with lipids and cause lipid peroxidation (Manca et al., 1994) with consequential pro-

production of ROS. Alternatively, it replaces iron from cellular binding sites to increase the free iron level and leads to iron-induced oxidations (Fenton-type reaction) (Wardeska et al., 1986). Cd also reacts with intrinsic thiol-containing molecules and causes an increase in ROS by reducing the cellular antioxidative capacity (Hussain et al., 1987). These factors may add synergistically to the elevation of cellular ROS. Indeed, an increase in hydrogen peroxide, superoxide anion, and hydroxyl radicals after Cd exposure has been reported in several studies (Hassoun and Stohs, 1996; O’Brien and Salacinski, 1998; Oya et al., 1986). The addition of ROS scavengers or antioxidant enzymes effectively reduces the occurrence of Cd-induced chromosomal aberrations, DNA strand breakages, mutagenicity, and cytotoxicity (Figueiredo-Pereira et al., 2002; Ochi and Ohsawa, 1985; Oya et al., 1986). In addition to ROS, Cd can increase the production of nitric oxide (Hassoun and Stohs, 1996). These findings imply that oxidative and nitrosative stresses induced by Cd treatment play a critical role in DNA damage. Cd also causes irreversible G2/M arrest (Chao and Yang, 2001). However, it is not clear whether the ROS produced is related to cell cycle arrest as it is in other transition metals, such as vanadium (Zhang et al., 2001a) and chromium (Zhang et al., 2001b).

Endogenous antioxidant enzymes, such as SOD and catalase, can neutralize cellular superoxide anions and hydrogen peroxide respectively. However, the administration of antioxidant enzymes for the reduction of cellular ROS is clinically inefficient and has had limited success. Synthetic SOD or catalase mimetics (Baudry et al., 1993) have been demonstrated to be useful SOD/catalase mimetics (Mn-salens), a group of low molecular weight, cell-permeable complexes, have been demonstrated to be useful SOD/catalase mimetics (Baudry et al., 1993). Chemists have studied the synthetic metal-salen complexes for more than half a century. However, experimental and clinical applications of the chiral salen derivatives have attracted attention only since the 1990s (Yoon and Jacobsen, 2003).
Salen ligands bind Mn ions through four atoms. The status of the Mn ion determines whether the salen complex is an SOD or catalase mimic (Sharpe et al., 2002). As an SOD mimic, the Mn(III) in the salen complex is reduced to Mn(II) by superoxide anion and yields oxygen. The Mn(II) is then re-oxidized to Mn(III) by another superoxide anion and produces hydrogen peroxide. To act as a catalase mimic, the Mn-salen is oxidized to an oxomanganese [Mn(V)O2-] complex by hydrogen peroxide to yield water. The oxidized complex is regenerated to the initial Mn-salen by reacting with another molecule of hydrogen peroxide, producing water and oxygen. A recent study has shown that the oxomanganese complex can oxidize nitric oxide (Sharpe et al., 2002). These findings indicate that the Mn-salen is potentially able to reduce damage caused by both oxidative and nitrosative stresses. Indeed, the Mn-salen complexes protect cells from oxidative damage in several animal models including Alzheimer’s disease (Bruce et al., 1996), Parkinson’s disease (Pong et al., 2000), stroke (Baker et al., 1998), motor neuron disease (Jung et al., 2001), multiple sclerosis (Malfroy et al., 1997), and excitotoxic neural injury (Rong et al., 1999).

It is well established that Cd stimulates ROS production. We report here the utilization of EUK-8, one of the Mn-salen, to reduce the cellular damage caused by Cd exposure. We found that EUK-8 effectively reduces ROS production and cell death in the presence of Cd. However, the Mn-salen protects cells from Cd damage mainly by blocking the influx of Cd into cells and consequently reduces ROS production in cells.

**MATERIALS AND METHODS**

**Cell culture and chemicals.** Chinese hamster ovary (CHO K1) cells were cultured as monolayers at 37°C in McCoy’s 5A medium supplemented with 10% heat-inactivated fetal bovine serum, 0.22% sodium bicarbonate, 100 U/ml ampicillin, and 100 μg/ml streptomycin, in 5% CO2/95% air and 100% humidity. Reagents for cell culture were purchased from GIBCO. Cadmium chloride (CdCl2) was obtained from Merck. Salen [N,N’-bis(salicylidene) ethylenediamine] and other reagent grade chemicals were purchased from Sigma unless specified. EUK-8 [manganese N,N’-bis(salicylidene)ethylenediamine chloride] was prepared according the procedures described by Sharpe et al. (2002). This complex showed the same biological responses as that of a commercially available product (Sigma) in this study.

**Determination of ROS.** Cells were treated with 1 or 4 μM Cd in the presence or absence of 30 μM EUK-8 for the indicated times. They were harvested 30 min after the addition of 5 μM H2DCF-DA (Calbiochem). Cellular fluorescence was measured using flow cytometry (FACScalibur, Becton-Dickinson) with excitation and emission wavelengths at 488 and 530 nm, respectively.

**Cell viability assay using PI exclusion.** The integrity of the plasma membrane was assessed by determining the ability of cells to exclude PI. Cells were trypsinized and collected by centrifugation. They were then washed once with PBS and resuspended in PBS containing 20 μM PI. The cells were kept at room temperature in the dark for 15 min. The levels of PI incorporation were determined by flow cytometry (FACScalibur, Becton-Dickinson).

**Clonogenic survival assay.** Two hundred cells were seeded in a 60 mm dish then treated with Cd for 2 h and the metal was removed. Cells were cultured for seven days without disturbance. The cells were then fixed and stained with 1% (w/v) crystal violet dissolved in 30% ethanol. Colonies containing at least 50 cells were counted. The survival fraction was determined by dividing the number of colonies in the treated sample by that of the non-treated sample.

**Determination of metal content.** Cellular metal content was measured by the method developed by Gagne et al. (1990). Briefly, cells were treated with Cd then washed twice with PBS containing 100 μM EDTA (PBS-EDTA) and harvested by trypsinization. The cells were resuspended in PBS-EDTA and cell numbers were determined. They were collected by centrifugation at 2000 rpm for 5 min then resuspended in 1 ml of 10 mM Tris-acetate (pH 7.4), 50 mM sucrose, 1 mM β-mercaptoethanol, 0.002% NaN3, and 8 mM HNO3. After incubating at 65°C for 12 h, the samples were diluted at least four-fold with deionized water and the metal contents determined with an atomic absorption spectrophotometer (GF3000, GBC).

**Electron paramagnetic resonance (EPR) spectroscopy.** The concentration of free Mn was determined by EPR as described previously (Fendorf et al., 1993). EPR spectra were recorded on a Bruker EMX-10 with 100-kHz field modulator, 20 mW microwave power and 100 G scan width. For quantitative determination of free Mn, the EPR cell was filled with a Mn solution of known concentration. The magnetic field was then swept, resulting in a Mn hyperfine sextet structure. The magnetic field was then centered at the down-field peak for the quantitative measurement of free Mn. Spectra were recorded at room temperature and samples were freshly prepared. The EPR signal intensity was determined from the height of the fourth derivative spectral peak. A calibration curve was established by measuring the signal intensity generated from 10, 20, 50, and 100 μM of MnCl2. The free Mn content in the sample was determined by the same procedure and calculated by interpolating the signal intensity into the standard curve.

**Spectroscopic analysis of salen-metal binding.** The experiments were performed on a HITACHI U2000 spectrophotometer. Salen (1 mM) was incubated with 0.05, 0.1, 0.25, 0.5, 1, 5, or 10 mM of metal for 24 h then analyzed by spectrophotometry. Differential spectra were obtained in the range of 300 to 600 nm. Binding of salen with metal produces a difference spectrum that exhibits a maximum at 460 nm (Mn) or 430 nm (Cd). The apparent dissociation constant (Kd) was calculated from the plot of 1/ΔA vs. 1/[S] according to the following equation:

\[
\frac{1}{\Delta A} = \frac{K_d}{\Delta A_{\text{max}}} \times \frac{1}{[S]} + \frac{1}{\Delta A_{\text{max}}}
\]

where ΔA is the absolute value of the difference between maximum (metal-salen) and minimum (salen) absorption. [S] is the initial metal concentration, and ΔA is the absorbance change upon complete formation of the complex (Youngs et al., 2000).

**Statistical analysis.** Means and standard deviations of samples (performed in triplicate) were calculated from the numerical data generated in this study. Significant differences between treatments were determined by Student’s t-test.

**RESULTS**

It is known that Cd may damage cells by stimulating the production of ROS, including hydrogen peroxide and superoxide anions (Hassoun and Stohs, 1996; O’Brien and Salacinski, 1998; Oya et al., 1986). The presence of an antioxidant might facilitate the removal of Cd-induced ROS and reduce cell damage. Since EUK-8 is a superoxide dismutase/catalase mimic, we examined here the effectiveness of this complex in removing Cd-induced ROS. Cells were treated with 1 or 4 μM Cd for 24 h in the presence or absence of 30 μM EUK-8. Cd stimulated ROS production in a dose-dependent manner. Increases of 1.5- and 2-fold in ROS level were detected with
Cd causes lipid peroxidation, which damages the integrity of cell membranes and allows the diffusion of PI into cells. We examined the cell permeability of PI with Cd treatment in the absence or presence of EUK-8. Cells were treated with 1 or 4 μM Cd for 24 h and PI was added after cell harvest. The level of PI in the cell was analyzed by flow cytometry. As shown in Figure 1B, a 3- to 10-fold increase in PI uptake was detected in Cd treated cells. For cells treated with 1 μM Cd, EUK-8 can inhibit this PI influx completely. EUK-8 can still block more than 50% of the PI influx when cells were treated with 4 μM Cd.

We also used a clonogenic survival assay to demonstrate the effect of EUK-8 on reducing the Cd-induced cytotoxicity. Cells were treated with 1, 4, or 8 μM Cd for 2 h in the absence or presence of 30 μM EUK-8. The viability of the cells after the treatment was examined. As shown in Figure 1C, cell viability was reduced to 60 and 5% of that of the untreated controls after the administration of 4 and 8 μM Cd, respectively. In the presence of EUK-8, close to 100% viability was observed with the Cd treated cells. These results demonstrate clearly that EUK-8 effectively prevents Cd toxicity and is potentially a useful Cd detoxifier.

We then investigated the time point of EUK-8 addition for the protection of Cd-exposed cells. EUK-8 was given at various time intervals to cells that had been treated with 4 μM Cd. Cells were harvested 24 h after administration of Cd. The permeability of PI was used as an indicator of cell damage. We observed an 11-fold increase in PI uptake for cells that had been exposed to Cd. EUK-8 blocked 90% of the PI uptake when added within 4 h of Cd treatment (Fig. 2). The efficacy of EUK-8 diminished if the complex was added at a later stage of Cd treatment. Only 20% of PI influx, as compared to that of the cells treated with Cd for 24 h, was inhibited when EUK-8 was given 12 h after Cd exposure. This finding indicates that Cd toxicity cannot be
attenuated when EUK-8 is given after prolonged Cd exposure, implying that Cd may exert its toxicity after moving into the cells. In addition, the ROS level did not increase in CHO K1 cells that had been treated with 4 μM Cd for 2 h (data not shown), even though cell viability was decreased (Fig. 1C). This decrease in cell viability was prevented in the presence of EUK-8. The results suggest that EUK-8 reduces Cd toxicity by inhibiting Cd transport. The decrease in ROS level after administering EUK-8 may then be attributed to the blocking of Cd transport with a consequential avoidance of Cd-induced ROS synthesis in cells.

EUK-8 is a Mn-salen complex. The toxicity of Cd might be reduced by replacing the Mn in EUK-8 and forming a Cd-salen complex. We incubated EUK-8 (10 μM, 100 μM, or 1 mM) with 113Cd (1 mM, 96.1 atom %) and analyzed the 113Cd profile by nuclear magnetic resonance (NMR). We could not detect any change in the Cd-NMR spectrum (data not shown). This result demonstrates that metal replacement did not occur freely or chemically.

Alternatively, EUK-8 might stimulate the efflux of Cd from cells. Cells were treated with 4 μM Cd for 2 h. The Cd was removed and a fresh culture medium with 30 μM EUK-8 was added. The cellular metal content was determined 12 h after medium change. Figure 3B shows clearly that EUK-8 does not increase the efflux or assist the removal of accumulated Cd from cells.

We subsequently investigated whether a specific component of EUK-8 or the integral complex is responsible for the inhibition of Cd transport. We examined the effect of salen, Mn and Mn-salen (EUK-8) on cellular Cd uptake. Cells were pre-treated with 30 μM salen, MnCl2 or EUK-8 for 30 min, followed by the addition of 4 μM Cd to the same culture medium. Cellular Cd content was determined 2 h after the addition of Cd. EUK-8 blocked approximately 50% of the Cd uptake. However, Mn alone was more effective and eliminated 80–85% of the Cd transport. Interestingly, the salen treatment brought a more than 1.5-fold increase of Cd into the cells compared to the control (Fig. 4A). This result suggests that Mn, free or its complex, is the major component in preventing Cd influx.

Since salen increases Cd accumulation, the viability of the cells was expected to decrease after the treatment. The cell survival fraction, as compared to that of the untreated cells, indeed dropped from 50% for Cd-treated cells to 25% for Cd-treated cells with the addition of salen (Fig. 4B). Giving EUK-8 or Mn to Cd-treated cells abrogated the Cd toxicity. In this experiment, salen itself did not cause any cytotoxic effect. This result demonstrates clearly that salen accelerates the influx of Cd and results in an enhancement of Cd toxicity.

Knowing that Mn itself can reduce Cd toxicity, and that EUK-8 is a complex of salen and Mn, we investigated the possibility that residual Mn in the EUK-8 preparation might play a key role in inhibiting Cd uptake. The free Mn in EUK-8 was thus determined using electron paramagnetic resonance (EPR). A linear calibration curve for free Mn was established (Fig. 5) and utilized in quantifying the free Mn in 10 mM EUK-8. We detected only 9 μM of free Mn, which is less than 0.1% of the total Mn in EUK-8 (assuming salen binds a stoichiometric amount of Mn). For a 30 μM EUK-8 solution, we theorized that there were approximately 0.03 μM free Mn. We examined whether Cd toxicity could be reduced with that quantity of Mn by the PI permeability assay. The Cd-induced PI permeability

![Figure 3](https://academic.oup.com/toxsci/article-abstract/85/1/551/1674358/554-YANG-CHIU-AND-LIN)
for cells treated with 1 μM Cd for 24 h could not be attenuated even in the presence of 1 μM Mn (data not shown). These results demonstrate that EUK-8, but not the residual free Mn in the EUK-8 solution, reduces Cd toxicity.

The effect of salen on Cd transport has never been reported. Hence, we investigated the mechanism of the enhanced cellular Cd uptake by salen. Possibly, salen causes cell membrane leakage and increases the diffusion of Cd into cells. The integrity of the cell membrane was thus examined after salen treatment. Cells were treated with 30 μM salen and metal (Cd or Mn) was added 30 min later. PI permeability was determined after the treatment. *Significantly different (p < 0.05) from that of salen-treated samples. (B) Cells were treated with 4 μM Cd, salen, or the Cd/salen mixture (pre-mixed in room temperature for 30 min) for 2 h. PI permeability and cell viability (C) was compared after the treatment. Control: cells without treatment.

FIG. 4. Effects of salen, Mn, and Mn-salen on Cd uptake and cytotoxicity. Cells were pre-treated with 30 μM EUK-8, MnCl₂, or salen for 30 min followed by 4 μM Cd for another 2 h. Cellular Cd content was determined by AAS (A), and cell viability was determined by a clonogenic survival assay (B). Each value represents a mean ± SD of three samples.

FIG. 5. Determination of free Mn level in the EUK-8 by EPR. A calibration curve for EPR analysis was established using 10, 25, 50, and 100 μM MnCl₂ in the assay. Free Mn in 10 mM EUK-8 was determined according to the calibration curve, and indicated by the arrow.

FIG. 6. Effect of salen and metal/salen mixture on the integrity of cell membrane and cell viability. (A) Cells were treated with (solid bar) or without (open bar) 30 μM salen for 30 min before the addition of either 4 μM Cd or Mn. Culturing was continued for another 2 h. PI permeability was determined after the treatment. *Significantly different (p < 0.05) from that of salen-treated samples. (B) Cells were treated with 4 μM Cd, salen, or the Cd/salen mixture (pre-mixed in room temperature for 30 min) for 2 h. PI permeability and cell viability (C) was compared after the treatment. Control: cells without treatment.
result indicates that salen indeed perturbs the integrity of the cell membrane and affects Cd transport.

Other experiments were also conducted to investigate the toxic effect of salen/Cd if they are premixed. Salen and Cd were mixed at equal molarity and incubated at room temperature for 30 min. The mixture (4 μM) was then added to the cell culture and after 2 h PI permeability was examined. As shown in Figure 6B, PI influx of cells treated with the Cd/salen mixture was not different from that of the untreated control cells. The cellular Cd content was also determined after the treatment. There was no difference in Cd accumulation between cells that received the same concentration of Cd-salen mixture and those that received Cd only (data not shown). A clonogenic survival assay also revealed that cells exposed to the Cd/salen mixture had a similar viability as those exposed to Cd only (Fig. 6C).

Since cells treated with the pre-incubated salen/Cd mixture did not have enhanced Cd uptake or cell damage, it may be the case that the salen binds the metal and the complex moves into cells without disturbing the membrane integrity. Therefore, we investigated whether binding occurs between salen and metal. Salen was mixed with increasing concentrations of Cd or Mn, and the spectroscopic changes were monitored. The spectroscopic change occurs immediately after mixing salen and metal, and reaches maximum absorption after 30 min (data not shown). Figure 7A shows an increasing absorption peak at 460 nm wavelength after the mixing. This peak increased in a dose-dependent manner. The dissociation constant between salen and Mn was calculated to be 1.4 mM⁻¹. There was also a spectroscopic change when salen was mixed with Cd. A decrease of absorption at 430 nm was noted for the reaction. This decrease in absorption is again a function of Cd concentration (Fig. 7B). The dissociation constant was calculated to be 12.5 mM⁻¹. The finding indicates that binding between salen and metal does occur. The affinity of Mn towards salen is approximately 10-fold higher than that of Cd binding to salen.

DISCUSSION

As superoxide dismutase/catalase mimetics, Mn-salen complexes are expected to reduce ROS level and protect cells from oxidative stresses. It has been demonstrated that Mn-salens can effectively reduce oxidative damage in animals and are potentially useful for clinical applications (Doctrow et al., 1997). Cd is known to stimulate ROS synthesis and to cause cell damage (Zhong et al., 1990). We therefore explored the possibility of using the Mn-salen complex (EUK-8) to counteract the Cd-induced ROS and cell damage. Cells were treated with 8 μM Cd for 2 h. After removing the metal, cells were cultured for an additional 24 h and an increase in ROS were observed in cells. The ROS can be reduced by adding EUK-8 after Cd removal (data not shown). The results suggest EUK-8 has an antioxidative role in Cd-induced oxidative stress. In addition to its antioxidative activity, EUK-8 is also a potent inhibitor of Cd toxicity in CHO cells. EUK-8 blocks the uptake of Cd into cells. This inhibition is not cell-specific and has been observed in cells derived from lung (A549), liver (Hep G2), and kidney (HEK293) (data not shown). These tissues are targets of Cd toxicity. This finding extends further the possibility of future applications of Mn-salens in attenuating deleterious effects of cytotoxic agents.

Several chemicals have been used to reduce the cytotoxicity of Cd. Most of them are chelators of Cd. To act as antidotes against heavy metal toxicity, the chelators should bind metal effectively and specifically, and should not themselves be toxic to cells. Ideally, the chelator should be able to assist the efflux of Cd from cells. Several chelators have been evaluated for their efficiency in reducing Cd toxicity (Fischer, 1995) and have been found to be inadequate. Thus the application of chelating agents for clinical usage is still under development. For EUK-8, its capacity to reduce Cd toxicity does not result from metal binding; instead, it blocks the cellular uptake of the metal. Although EUK-8 does not enhance the release of metal
from cells, its function provides another possibility for protecting cells from Cd damage.

The exact mechanism(s) for Cd transport is not well understood. Studies have indicated that Cd may pass through cell membranes by diffusion, carrier-mediated transport, or ion channels. Cd is not an essential element for organisms. Therefore, transport of Cd into cells by carrier or channel may rely on routes utilized by other similar elements (Foulkes, 2000). For example, Cd can be transported into cells via the calcium channel because of the similarity in radius (Shibuya and Douglas, 1992).

Due to the lack of a specific entrance mechanism for Cd, competition for transportation occurs when Cd is present with the element that shares the same pathway. Several Cd transport systems have been found in bacterial, yeast, plant, and mammalian cells (Hao et al., 1999; Li et al., 1997; Salt and Wagner, 1993; Shibuya and Douglas, 1992). However, only a few transporters that are associated with Cd uptake have been identified for these systems. In mammalian cells, divalent metal transporter 1 (DMT1) was shown to be involved in the intestinal absorption of Cd (Park et al., 2002). DMT1 regulates iron (Fe) absorption at the apical membrane of enterocytes. The presence of Cd inhibits Fe transport. Knockdown of DMT1 expression in Coca-2 cells resulted in a reduction in both Cd and Fe uptakes (Bannon et al., 2003). These results indicate the importance of DMT1 in Fe and Cd transport. Another transporter, natural resistance associated macrophage protein (Nramp), has also been shown to be capable of transporting Cd (Okubo et al., 2003). Notably, both transporters (DMT1 and Nramp) are also associated with Mn transport, and thus competition for entrance is expected.

There is no apparent chemical similarity between Cd and Mn. However, competition of uptake between Mn and Cd has been reported in several metal transporters and ion channels of different organisms. Recently, a Cd resistant cell line derived from a metallothionein-null mouse cell revealed a suppression of high-affinity Mn transport (Himeno, 2002). Competitive and kinetic analyses showed that low concentrations of Cd and Mn shared the same pathway for cellular transport. Competition of uptake between Mn and Cd was also found in HeLa, PC12, and Coca-2 cells (Himeno, 2002).

Although the presence of these channels or transporters described above in CHO cells remains to be clarified, suppression of Cd uptake by Mn is also observed (Fig. 4A). This evidence demonstrates the mutual inhibition of Cd and Mn for entry into cells. Notably, the inhibition of Cd uptake also occurred in the presence of a Mn-complex (EUK-8). We have speculated that the residual free Mn in the EUK-8 preparation might affect the uptake of Cd. Using EPR to estimate the quantity of free Mn, we found less than 0.1% of free Mn in the EUK-8 preparation (Fig. 5). In our experiments, 30 μM of EUK-8 was added to the culture medium. This quantity of free Mn (0.03 μM) was not capable of reducing the toxic effect caused by 1 μM Cd treatment. This result indicates that EUK-8 is the main component involved in inhibiting Cd uptake by cells. Our results demonstrate that not only free Mn ions but also Mn complexes are able to attenuate Cd toxicity.

It is well known that zinc (Zn) suppresses cellular uptake of Cd. Even though Zn and Cd belong to the same transition metal series, they do not share the same transport system in mammalian cells. However, the presence of Zn reduces the uptake of both Cd and Mn ions (Himeno, 2002). This finding suggests that Zn and Mn employ different mechanisms in inhibiting Cd uptake. Zn possibly interferes with component(s) or effector(s) that may be required for Cd transport and hence reduces the Cd ion uptake. The exact mechanism necessary for EUK-8 to act as a Cd antidote also remains to be clarified. EUK-8 may inhibit Cd uptake via a similar mechanism as Zn. However, we do not exclude the possibility that EUK-8 suppresses Cd uptake by the same mechanism as Mn, although transport of the lipophilic Mn-salen complex more likely occurs through simple diffusion.

Salen ligands are useful starting materials for the development of asymmetric catalysts. The ligands bind metal through four atoms with a structure similar to the porphyrin framework in heme-based oxidative enzymes. The metal-salen complex may possess different catalytic activities depending on the status and the nature of the metal bound (Yoon and Jacobsen, 2003). Mn-salen complexes are potentially useful for clinical applications because of their catalytic abilities to remove ROS produced from oxidative stress (Doctrow et al., 1997). However, chromium-salen complexes stimulate ROS production, which further causes DNA cleavage and apoptosis (Balamurugan et al., 2002; Sugden and Martin, 2002). These findings show the diverse characteristics of salen and its complexes.

Salen ligand also shows different biological effects depending on whether or not it has metal in the structure. Exposing cells to salen causes leakage of cell membranes (Fig. 6A) although no lethal effect has been observed for short-term exposure (Fig. 4B). This leakage is not due to lipid peroxidation because salen itself does not enhance ROS production under the conditions employed (data not shown). Metal ions can diffuse into cells through the leaks. Therefore, cells pretreated with salen followed by Cd administration exhibit 50% more Cd accumulation (Fig. 4A). A different result arises if metal and salen are pre-mixed before application to cells. Salen can bind either Mn or Cd (Fig. 7). We did not observe any difference in cell permeability when either metal-salen complex was administered to the cells (Figs. 1B and 6B). Therefore, metal is most likely brought into cells by the lipophilic carrier (salen) through simple diffusion. Although Cd is chelated by salen before moving into cells, it can still exert cytotoxicity. This phenomenon is quite different from that observed in other Cd chelators which bind Cd and reduce cytotoxicity by preventing the Cd from interacting with cellular molecules. Possibly, Cd is released from the complex in the cell or the Cd-salen complex is cytotoxic itself.
In summary, we found that a Mn-salen complex is an effective antidote to Cd toxicity. This complex blocks the entrance of Cd into cells and thus reduces the deleterious effects of Cd. Notably, the components of the complex show different effects in attenuating Cd toxicity. Mn inhibits Cd uptake and reduces its toxicity whereas salen enhances Cd uptake and increases its toxicity. Our results may provide further insight into the usage of Mn-salen complexes for future clinical applications.

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