

# Evaluation of the Toxic Potency of Selected Cadmium Compounds on A549 and CHO-9 Cells

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*Cytotoxicity of cadmium sulphide, oxide and chloride was tested using A549 and CHO-9 cells. Metabolic activity of cells (MTT test) and cell membrane permeability (NRU test) were used as cytotoxicity endpoints. The results revealed unexpectedly low toxicity of cadmium sulphide as compared to chloride and oxide. This preliminary report does not provide any explanation for this effect, but the result may nevertheless be interesting for future studies of toxicity mechanisms of cadmium compounds. First cadmium compounds caused damage or change in the permeability of cell membranes, then inhibition of metabolic activity of mitochondria. It cannot be ruled out that the cell lysosomes are at first exposed to the effect of cadmium.*

cadmium sulphide    cadmium chloride    cadmium oxide

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## 1. INTRODUCTION

Cadmium is an anthropogenic metal of special importance with respect to incidence of cancer [1]. It is emitted into the atmosphere, mainly in processes of combustion of oils, coal and waste. It is currently used in production of electrodes in nickel-cadmium batteries as cadmium oxide (CdO) (83%) and as a pigment in ceramic products, plastic and glass (8%). It is also a component of anti-corrosion coats (7%), polymer stabilizers (1.2%), nonferrous alloys, photovoltaic devices, etc. (0.8%) [2]. Occupational exposure to cadmium compounds is a problem for workers in mining, metallurgy and chemical industry. The development of nanotechnology entails an increase in synthesis and the use of cadmium sulphide (CdS), mainly as quantum dots [3]. CdS is a well-known semiconductor, it is used in the electronics industry in photocells, photoresistors and light-emitting diodes. It is applied as a component of a photovoltaic (solar) cell. CdS is also used as a pigment in plastics, as an additive in polymer nanocompos-

ites and as a curing agent in tyres. Despite increasingly wide application of CdS, its toxicity has not been investigated extensively. Toxicological studies, both in vivo and in vitro, dealing with toxicity of inorganic cadmium compounds, have mainly focused on evaluating cadmium chloride (CdCl<sub>2</sub>) and CdO. Therefore, those compounds, with a relatively well known toxic effect, have been taken for comparative analysis of toxicity of CdS.

The aim of this study was to evaluate the cytotoxic potency of CdS as compared to CdCl<sub>2</sub> and CdO. The tests were conducted on two cell lines: nonspecialized (fibroblast-like type) cells of the Chinese hamster ovary (CHO-9) and human lung cancer cells (A549). CHO-9 cells are regarded as an appropriate model for investigating the cytotoxic effect of xenobiotics, including metals [4, 5]. A549 cells resemble regular cells because they retain the properties of alveolar epithelial cells such as expression of CYP1A1, CYP1B1 and CYP2B6 [6] and an ability to form DNA adducts [7]. Many researchers regard A549 cells as a suitable model to study the effect of air pollution on

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This study was prepared on the basis of the results of a research task carried out within the scope of the first stage of the National Programme “Improvement of safety and working conditions” partly supported in 2008–2010—within the scope of research and development—by the Ministry of Science and Higher Education. The Central Institute for Labour Protection - National Research Institute was the Programme’s main co-ordinator.

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human respiratory system, including examination of dust and insoluble compounds, because A549 cells possess a strong capability of phagocytosis [7, 8, 9]. We evaluated the cytotoxic effect of cadmium compounds with a test used to assess cell membranes integrity (NRU test) and a test which determines the metabolic activity of mitochondria (MTT-reduction assay). The tests have been standardized and validated in numerous studies [10]. These are currently the most frequently used and recommended methods to assess cytotoxicity [11]. We also determined the oxidative potential in cells exposed to the compounds under study by determining the total concentration of lipid peroxides in cells.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

For cell cultures, the following were used: F-10 (Ham) medium with L-glutamate, Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS) and antibiotic-antimycotic from Gibco (Life Technologies, UK); 0.25% trypsin/EDTA and the 0.4% trypan blue from Sigma Chemical, USA.

For cell viability assays, the following were used: 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), neutral red solution (NRU), Hank's balanced salt solution, Dulbecco's phosphate buffered saline, dimethylsulphoxide (DMSO), Bradford's kit from Sigma; the PerOx (TOS) test kit (Immundiagnostik, Germany).

The cadmium compounds—CdO, cadmium chloride hemi(pentahydrate) ( $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$ ) and CdS—were purchased from Sigma.

### 2.2. Cell Culture and Treatment

The line of Chinese hamster ovary cells CHO-9 was kindly provided by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw, Poland. The line of human lung cancer cells A549 was kindly provided by the Technische Universität in Berlin, Germany.

The CHO-9 cells were cultured as a monolayer in F-10 (Ham) medium, whereas A549 cells in

DMEM, both supplemented with 7% FBS and 1% antibiotic-antimycotic (1 ml/100 ml medium) in sterile tissue culture flasks (Nunc, USA) and maintained at 37 °C under the humidified atmosphere of 95% air and 5%  $\text{CO}_2$  (pH 7.2–7.4). The cells were subcultured twice of week. Before the experiment, cell suspension was prepared and cell viability was assessed with the trypan blue exclusion assay [12]. Cells whose viability was over 90% were used in cytotoxicity assays and total oxidative status assessment.

The cells were exposed to different concentrations of the cadmium compounds. The stock solutions were prepared directly before the cytotoxicity tests by dissolving them in distilled water. Concentrations for the experiment were prepared in the culture medium, appropriate for a specific cell line. Suspensions rather than solutions of substances could only be obtained for insoluble compounds. In such cases, “concentration” is understood to denote the amount of material per unit of volume. Solutions (suspensions) were mixed and sonified to make them homogenous. The following concentration ranges were used for cytotoxicity tests: 1–30  $\mu\text{g/ml}$  for CdO and  $\text{CdCl}_2 \times 2.5 \text{ H}_2\text{O}$  and 20–500  $\mu\text{g/ml}$  for CdS.

### 2.3. Cytotoxicity Assays

Experimental cell cultures were seeded in 96-well microplates at a density of  $8 \times 10^3$  cells/well. After 24 h of incubation, different concentration of cadmium compounds were added to the cells, and incubated for 24 or 72 h at 37 °C. A medium without a test compound was added to the control wells. After 24- or 72-h exposure, viability of cells (reduction in the number of viable cells) was assessed with the neutral red uptake (NRU) and MTT reduction assays, according to INVITOX Protocols No. 64 and No. 17, respectively [13, 14]. The absorbencies were measured at 540/450 nm (for NRU) and 570/620 nm (for MTT) filter, using a Synergy 2 microplate reader (BioTek, USA). Cytotoxicity assays were performed in at least three independent replications. Depending on the compound concentration, viability of exposed cells as compared to control (100%) was calculated and presented as a dose-effect curve. Based on the analysis of a series of

dose–effect curves for each compound the  $IC_{50}$  value (inhibitory concentration, i.e., concentrations producing 50% reduction of number viable cells) was determined using nonlinear regression analysis with 95% confidence interval.

## 2.4. Total Oxidative Status (TOS) Assay

TOS of the cells exposed to the tested compounds was determined by measuring the total concentration of lipid peroxides in cells in accordance with the procedure provided by the manufacturer [15].

Briefly: after 24-h exposure to the test compounds, cells were lysed by freezing at 80 °C (in three thaw–freeze cycles). Then, the cells were thawed, sonicated (30 min) and centrifuged (13000 rpm). The levels of peroxides in the samples were determined by the reaction of horseradish peroxidase with tetramethylbenzidine (TMB) dichloride in the presence of hydrogen peroxide. The reaction with the enzyme yields a soluble, blue-coloured product. The enzymatic reaction is quenched by adding 2 M  $H_2SO_4$ , which leads to the solution changing its colour to yellow. The absorbance of the dye was measured at the wavelength of 450 nm using a Synergy 2 microplates reader (BioTek, USA). Using the measured absorbance values, total levels of peroxides in tested samples ( $\mu\text{mol } H_2O_2$ ) were determined according to the formula provided by the manufacturer, and then converted to milligrams of protein in individual samples. The oxidative potential of the compounds was expressed as percentage of control, which comprised unexposed cells (control = 100%). Protein was determined with Bradford's method [16]. Each experiment was performed in at least two replications.

## 2.5. Statistical Analysis

Conformity of the distribution of results ( $\log IC_{50}$ ) with normal distribution was examined with the Kolmogorov–Smirnov test. The significance of differences between distributions of  $\log IC_{50}$  values was evaluated with Student's *t* test for the  $\log IC_{50}$  values which met the criteria

of normal distribution or with Wilcoxon's test for those which do not. Calculations were performed with R package<sup>1</sup>.

TOS data were analysed with Student's *t* test for comparison between two groups. Differences were considered statistically significant at  $p < .05$ . Statistica<sup>2</sup> version 7.1 was used.

## 3. RESULTS

All the compounds studied were found to affect cell viability in dose dependent manner. Figures 1–2 represent a comparison of the cytotoxicity of tested compounds on the basis of  $IC_{50}$  values. They are shown in the logarithmic scale to picture the differences between the compounds more clearly. The high values of  $IC_{50}$  indicate low toxicity of the compounds ( $IC_{50}$  values are presented in labels above histogram bars).

Comparison of the  $IC_{50}$  values for the cadmium compounds in the 24- and 72-h experiments on both cell lines in most cases revealed statistically significant differences between their cytotoxic effect ( $p < .001$ ).  $CdCl_2$  showed slightly higher toxicity as compared with  $CdO$  (except for the results of MTT test in the 24-h experiment and NRU test in the 72-h experiment, when compounds were equally toxic).

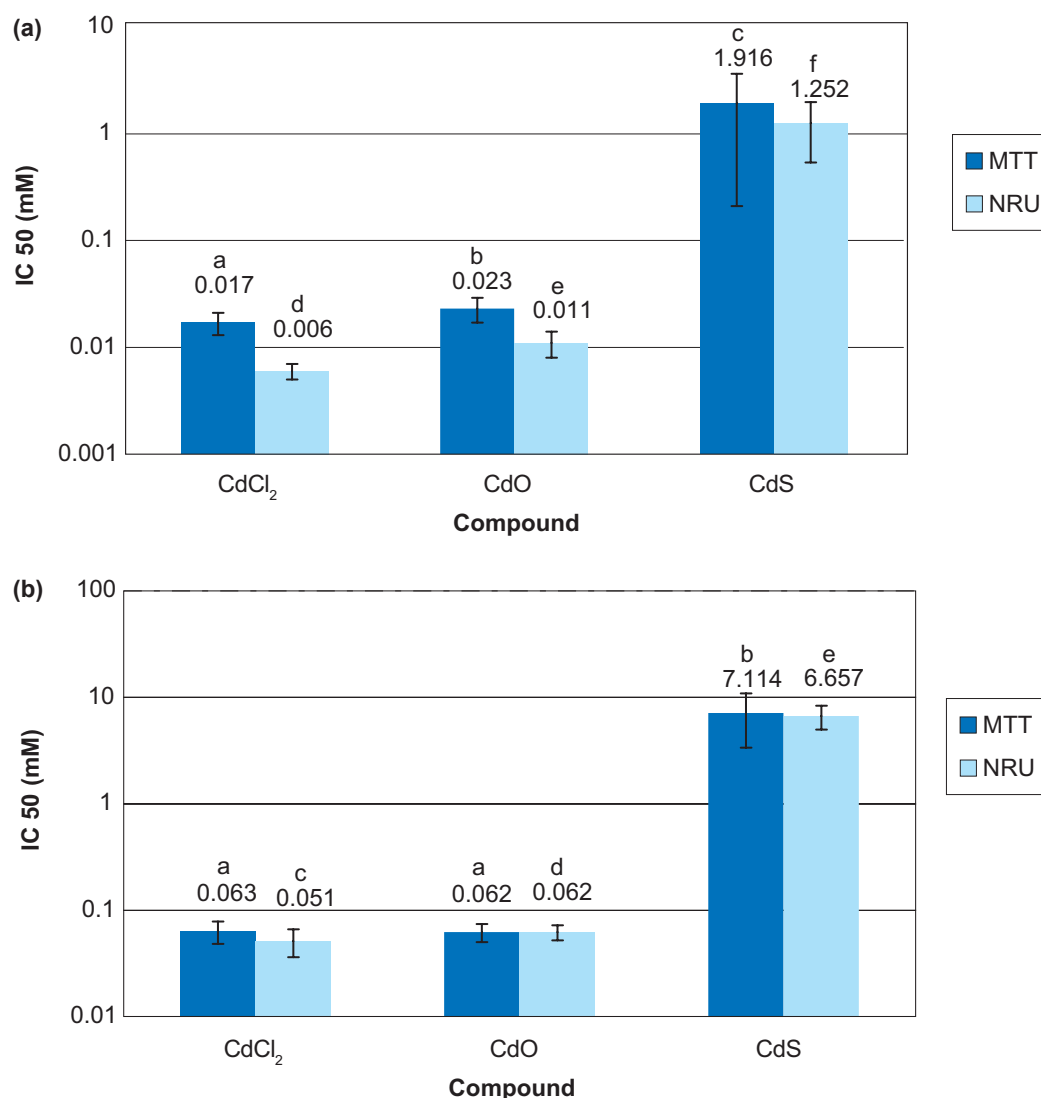
The highest  $IC_{50}$  values were recorded for  $CdS$  in both cells, both tests, both in the 24- and 72-h experiments. The  $IC_{50}$  values determined for  $CdS$  were over 100 times higher compared to  $CdCl_2$  and  $CdO$ .

Our findings indicate that cadmium compounds in the first instance caused cell membrane damage, and then metabolic disorders. The  $IC_{50}$  values calculated from the results of the NRU tests were, in most cases, lower than the values determined with MTT tests on both cell lines, both in the 24- and 72-h experiments.

We also observed differences in the sensitivity of the cells to the tested compounds. The  $IC_{50}$  values determined on the basis of both tests in both types of experiments were lower for CHO-9 cells than that for A549 cells.

<sup>1</sup> <http://www.R-project.org>

<sup>2</sup> <http://www.statsoft.com/Products/STATISTICA/Product-Index>



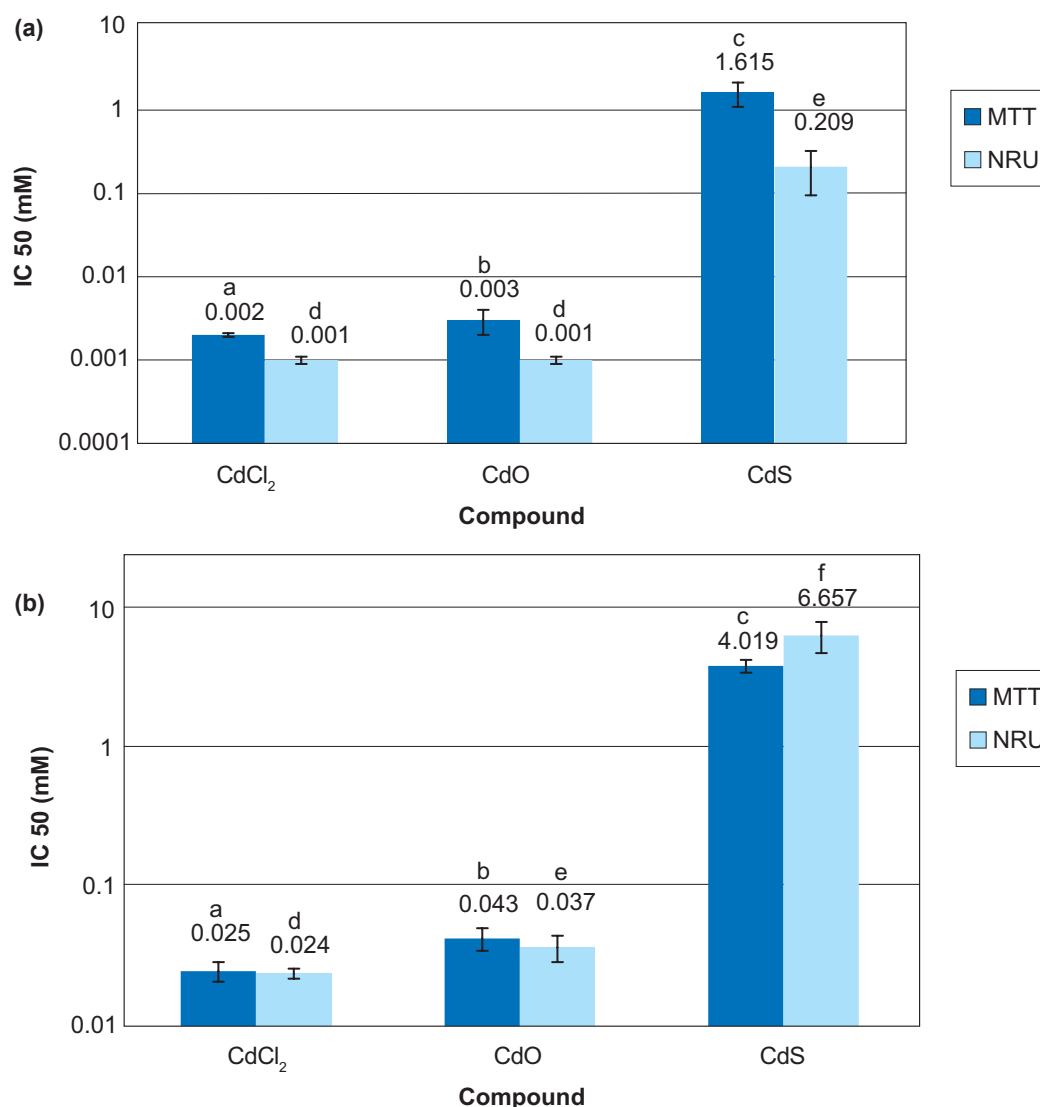
**Figure 1. Cytotoxicity of cadmium chloride (CdCl<sub>2</sub>), oxide (CdO) and sulphide (CdS) on (a) CHO-9 and (b) A549 cells after 24-h exposure.** Notes. Cytotoxicity of cadmium compounds was determined with NRU or MTT assay and are plotted in the order of the concentrations that inhibit cell growth by 50% compared with untreated controls (IC<sub>50</sub> values). Each bar represents  $M \pm SD$  from at least 3 experiments. In each panel, different letters over histograms indicate the significance of differences between distributions of log IC<sub>50</sub> values with  $p < .001$ .

Figure 3 shows a comparison of the total concentration of lipid peroxides (mmol H<sub>2</sub>O<sub>2</sub>/mg of protein) in cells exposed to the tested cadmium compounds and the control (control = 100%). All compounds under study caused an increase in the total concentration of lipid peroxides in cells as compared to the control. The release of lipid peroxides was recorded at high cytotoxic concentrations, higher than the IC<sub>50</sub> concentrations determined in MTT and NRU tests on the respective cell lines. The maximum release of peroxides was recorded at ~500% as compared to the control.

No differences have been shown to exist in the toxic effect between CdO and CdCl<sub>2</sub> in equimolar concentrations, either on A549 or CHO-9 cells. CdS was shown to have the weakest toxic effect.

#### 4. DISCUSSION

For most heavy metals, there have been reports which indicate that the bioavailability of a metal and, in consequence, its toxic effect, is affected by its physicochemical form and the resulting solubility in water [17, 18, 19].

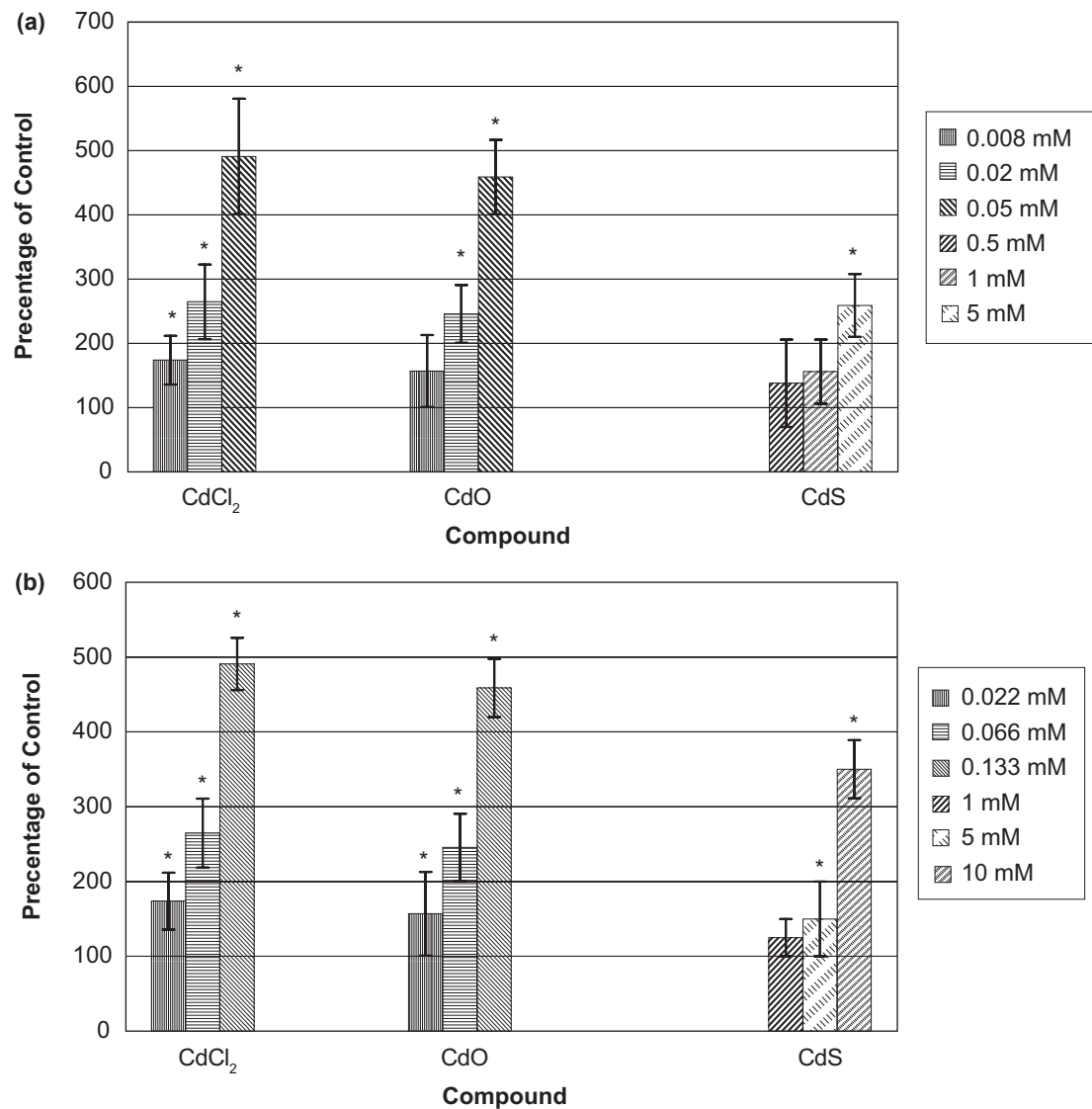


**Figure 2. Cytotoxicity of cadmium chloride (CdCl<sub>2</sub>), oxide (CdO) and sulphide (CdS) on (a) CHO-9 and (b) A549 cells after 72-h exposure.** Notes. Cytotoxicity of cadmium compounds was determined with NRU or MTT assay and are plotted in order of the concentrations that inhibit cell growth by 50% compared with untreated controls (IC<sub>50</sub> values). Each bar represents  $M \pm SD$  from at least 3 experiments. In each panel, different letters over histograms indicate the significance of differences between distributions of log IC<sub>50</sub> values with  $p < .001$ .

The results indicated that solubility in water was not the main factor which affected toxicity of cadmium compounds. CdCl<sub>2</sub> (readily soluble in water) proved to be more toxic than virtually insoluble CdO in all tests performed on both cell lines, both in the 24- and 72-h experiments. The differences in the toxicity of both compounds were low but statistically significant in most cases ( $p < .001$ ). Schwerdtle and Hartwig obtained similar results [20]. In a comparative study of the genotoxic effect of particulate CdO and soluble CdCl<sub>2</sub> on A549 cells, they demonstrated a com-

parable degree of toxicity based on the oxidative damage of DNA.

The toxic effect of cadmium compounds Cd(II) is related to the route of entry of metal ions into the cells. Cd<sup>2+</sup> ions are transported into the cells through the divalent metal transporter DMT-1 membrane ion channel, by mimicking Ca<sup>2+</sup> ions [21]. In this context, the lowest cytotoxicity of CdS in comparison with CdCl<sub>2</sub> and CdO is very interesting. Toxicity of CdCl<sub>2</sub> and CdO in both experiment arrangements was over 100 times higher than that of CdS. We have no explanation



**Figure 3. Effect of cadmium chloride (CdCl<sub>2</sub>), oxide (CdO) and sulphide (CdS) on production of total lipid peroxides in (a) CHO-9 and (b) A549 cells.** *Notes.* Cells were treated with different concentrations of the compounds for 24 h. H<sub>2</sub>O<sub>2</sub> was used to detect total lipid peroxidation. Results were calculated as mmol H<sub>2</sub>O<sub>2</sub>/mg of proteins in the sample and expressed as percentage of control, which comprised unexposed cells (control = 100%). Each bar represents  $M \pm SD$  from at least 2 experiments. \* = significantly different values ( $p < .05$ ) in comparison with control.

for this phenomenon, but it seems interesting for future studies of toxicity mechanisms of cadmium compounds.

The difference in the effect of CdO, CdCl<sub>2</sub> and CdS was also shown in an in vivo experiment. Glasser, Kloppel and Hochrainer evaluated the concentration of cadmium in homogenate of lung tissue of Wistar strain rats, after inhalation exposure to CdCl<sub>2</sub> (at the concentration of 0.1 mg/m<sup>3</sup> Cd), CdO (0.1 mg/m<sup>3</sup> Cd) or CdS (1 mg/m<sup>3</sup> Cd) for 30 days [22]. They found the concentration of cadmium in the tissue of rats exposed to CdO to

be twice as high as in those exposed to CdCl<sub>2</sub>. Concentration of cadmium in tissues of rats exposed to CdS was over 10 times lower as compared to those exposed to CdO. The mechanisms which are responsible for the difference in toxicity between CdO and CdS (both are scarcely soluble) have not been elucidated.

Mitochondria are regarded as the main target structure of cadmium action on the subcellular level. Damaging the function of mitochondria by cadmium compounds has been confirmed in vivo [23] and in vitro studies [24].



The cadmium compounds under study were quicker to damage cell membranes or to change their permeability than to inhibit metabolic activity of cellular mitochondria (in most cases, the values of  $IC_{50}$  determined for cadmium compounds in NRU tests were lower than in MTT tests). Although the NRU test generally evaluates the integrity of all cell membranes, it cannot be ruled out that it also points to the mechanism of action in the case of cadmium, namely to lysosomes. The idea of cadmium damaging lysosomes is being corroborated. Fotakis, Cemeli, Anderson, et al. examined hepatic cancer cells exposed to  $CdCl_2$  and found lysosomes to be primarily vulnerable to the effect of cadmium [25]. Changes in lysosomes ("swelling") took place earlier and at lower concentrations of the compound than damage to mitochondria, DNA or formation of oxygen reactive species. Fotakis et al. link the fact to formation of cadmium complexes with metallothioneine, which accumulate in lysosomes and then are degraded and released to cytosol. Their results of in vitro research correlate well with clinical observations in humans, where an increase in concentration of lysosomal enzymes in blood is an early symptom of kidney damage.

Pro-oxidative action of cadmium compounds is well documented both in vitro and in vivo studies [2]. This study attempts to compare the strength of the toxic effect of cadmium compounds based on the oxidative potential in the exposed cells. In most cases, the order of the compounds with respect to their toxic effect was similar to the results of cytotoxicity studies. However, the tested compounds induced overproduction of peroxides at definitely cytotoxic concentrations, much higher than the  $IC_{50}$  values, both in CHO-9 and in A549 cells. The result may indicate that damage to cell membranes and mitochondria function disorders are more sensitive parameters to be used in assessing early cell damage caused by cadmium than the pro-oxidative effect of the compounds. This has been confirmed by experiments on hepatocytes exposed to  $CdCl_2$ . Reactive species of oxygen were formed in cells at concentrations of the compounds which caused the cell mortality rate to reach 80% [24].  $CdCl_2$  also

induced oxidative damage to DNA in cancer cells at concentrations which were relatively higher than those causing lysosome damage (20  $\mu M$ ), which corresponded to lowering the cell viability by 70% [25].

This study has revealed considerable differences in sensitivity of A549 and CHO-9 cells to cadmium compounds. The values of  $IC_{50}$  found in the tests for all compounds were lower for CHO-9 than for A549 cells. Human lung cancer cells seem to be a less sensitive experimental model for studying the general cytotoxic effect of cadmium compounds. Despite the differences in sensitivity, the results on both cell lines are consistent, which strengthens our conclusions.

## 5. CONCLUSIONS

- Comparative studies of the cytotoxic effect of CdS, CdO and  $CdCl_2$  have demonstrated unexpectedly low toxicity of CdS. This may be interesting for further studies of the toxicity of cadmium compounds.
- Cadmium compounds needed less time to damage cell membranes or to lower their permeability than to inhibit metabolic activity of mitochondria. It cannot be ruled out that lysosomes are the first target of cadmium.
- Damage to cell membranes and mitochondria function disorders are more sensitive parameters to be used in assessing early cell damage caused by cadmium than releasing lipid peroxides.

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