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The influence of ebselen on the toxicity of cisplatin in LLC-PK$_1$ cells

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Abstract—LLC-PK$_1$ cells have been used as an *in vitro* model to study the nephrotoxicity of the antitumor drug cisplatin. A concentration-dependent cytotoxicity of cisplatin, measured as lactate dehydrogenase leakage and amount of protein remaining attached to the culture plate, was observed. At a cisplatin concentration of 0.4 mM cell viability was reduced to 21% after 72 hr. Ebselen, a seleno-organic compound capable of forming selenol intermediates through reaction with thiols, was found to protect LLC-PK$_1$ cells against cisplatin-induced toxicity at low concentrations (5–15 μM). The ebselen-induced protection against cisplatin toxicity in this *in vitro* test system apparently correlates well with a similar protection previously observed *in vivo* in mice and rats.

Cisplatin (*cis*-diamminedichloroplatinum II) is an important cytotoxic drug which is used in the treatment of a variety of human neoplasms [1]. However, severe side-effects, notably toxicity to the kidneys, the gastrointestinal tract, the peripheral nerves and the bone marrow lower the therapeutic index of cisplatin [2]. Nephrotoxicity is one of the most important toxicities of cisplatin in humans. The events responsible for the toxicity of cisplatin occur shortly after administration of the drug [3]. Signs of kidney toxicity, however, occur after several days, mainly in the S_3 segment of the proximal tubule [4, 5]. The precise molecular mechanism of cisplatin-induced nephrotoxicity is unknown. In cells, due to the low chloride concentration, cisplatin is hydrolysed to positively charged monoaquo- and diaquo species [6]. These species are probably the active toxicants. Based on *in vitro* experiments it has been suggested that lipid peroxidation is not a major cause in cisplatin-induced nephrotoxicity [7]. The nephrotoxicity of cisplatin has been attributed to binding of platinum to critical protein sulfhydryl groups [8]. Renal brush border enzymes, however, have been excluded as primary targets of toxicity *in vivo* [9]. Alteration of glutathione (GSH*) levels and depression of macromolecule synthesis in the kidney have also been suggested to play a role in cisplatin-induced nephrotoxicity [6].

Several attempts have been made to reduce cisplatin-induced nephrotoxicity [10]. Induction of chloruresis by hypertonic saline infusions, for example, protects against cisplatin nephrotoxicity because this treatment leads to a reduction in the concentrations of cisplatin and reactive metabolites in the kidney, during the first hours after administration of cisplatin [11]. An important alternative approach is the use of chemoprotectors. Most chemoprotectors contain a nucleophilic moiety [12]. Diethyldithiocarbamate, for example, reduces the nephrotoxicity but not the antitumour activity of cisplatin, probably because this compound can selectively remove platinum from platinum–sulfhydryl complexes but not from platinum–DNA adducts [13]. An important problem in the clinical

* Abbreviations: GSH, glutathione; LDH, lactate dehydrogenase.

application of diethyldithiocarbamate, however, is its intrinsic toxicity [14].

The seleno-organic compound ebselen (2-phenyl-1,2-benzisoselenazol-3(2*H*)-one) does not have such limitations. Ebselen has a low toxicity, probably because of its strongly bound selenium moiety [15]. Ebselen can form a selenol, through a chemical reaction with thiols such as GSH [16, 17]. Selenols are nucleophiles which may be able to form a complex with cisplatin, thereby inactivating the drug.

In vitro test systems would greatly facilitate the search for novel chemoprotectors and their underlying mechanisms of action. Boogaard *et al.* [18] have shown that proximal tubular cells freshly isolated from rat kidney are a suitable *in vitro* model to study the toxicity of a variety of nephrotoxicants, including cisplatin. Another interesting *in vitro* test system is the LLC-PK_1 cell line. This cell line expresses many characteristics of proximal tubular cells and can be maintained as a continuous cell line [19]. The LLC-PK_1 cell line has been used to study the nephrotoxicity of aminoglycosides [20], cysteine-*S*-conjugates [21] and, more recently, cisplatin [22].

The primary aim of this study was to examine the influence of ebselen on the toxicity of cisplatin in the LLC-PK_1 kidney cell line. In order to evaluate this *in vitro* model, we have compared the results obtained in this *in vitro* test system with those from recently published *in vivo* studies in the rat.

Materials and Methods

Chemicals. Ebselen was a gift of Rhone-Poulenc Natterman (Cologne, F.R.G.). Chemicals for preparing the mobile phase of the chromatographic system were of HPLC grade, all other chemicals were of analytical grade.

Synthesis of cisplatin. Cisplatin was synthesized according to the synthetic and test procedures described previously [23, 24].

In vitro *model.* LLC-PK_1 cells were obtained from Flow Laboratories (Zwanenburg, The Netherlands) and maintained by serial passages in 75-cm^2 plastic culture flasks in an atmosphere of 5% CO_2–95% air at 37°. The complete medium consisted of E 199 medium (Flow Laboratories), supplemented with 2 mM L-glutamine and 5% fetal calf serum. When cell growth reached saturation density, subcultures were prepared by treatment with 0.05% trypsin in E 199 medium containing 0.02% EDTA.

All experiments were performed on passages 200–210. The cell culture procedure was essentially as described by Gstraunthaler *et al.* [19]. Briefly, cells were seeded in 24-well culture dishes (2.0 cm^2/well) and allowed to grow to confluence (2.4×10^5 cell/cm^2) over 8 days in serum containing 5% fetal calf serum. The cells were then maintained in serum-free medium for 2 days before drug treatment. Only monolayers in which dome formation occurred were used for the experiments.

Cisplatin was dissolved in a sterile salt solution (114 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.8 mM Na_2HPO_4, 0.2 mM NaH_2PO_4, 16 mM $NaHCO_3$, 5.5 mM glucose) saturated with 5% CO_2–95% air at 37° (pH 7.4). Ebselen was dissolved in 2% dimethylsulfoxide in the sterile salt solution.

Viability assay. Monolayers of LLC-PK_1 were lysed with trypsin and cell viability was assessed by measuring lactate dehydrogenase (LDH) activity, according to the method of Stevens *et al.* [21], and by measuring the amount of protein remaining attached to the culture plate with bovine serum albumin as standard, according to the method of Lowry *et al.* [25].

Cytotoxicity of cisplatin and ebselen in LLC-PK_1 cells. Monolayers of LLC-PK_1 cells were washed twice with physiological saline and then exposed to cisplatin or ebselen for 60 min. After incubation, the monolayers were washed three times with physiological saline to remove residual drug. Subsequently, the cells were grown in medium containing 5% fetal calf serum. Cell viability was assessed at different time points. In control experiments, monolayers were exposed to the vehicle instead of drug.

The influence of ebselen on the cytotoxicity of cisplatin in LLC-PK_1 cells. Monolayers of LLC-PK_1 cells were washed twice with physiological saline and incubated with ebselen for 60 min. Subsequently, monolayers were washed three times with physiological saline and incubated with cisplatin as described above. In control experiments, ebselen was replaced by the vehicle.

Statistics. Student's *t*-test, unpaired, was used to evaluate the significance of differences between experimental groups. The level of significance was set at $P < 0.05$.

Results

Cytotoxicity of cisplatin in LLC-PK_1 cells. The influence of cisplatin at several concentrations on the viability of LLC-PK_1 cells is shown in Fig. 1. Measurements of LDH in the medium and of the amount of protein remaining attached to the culture plate, yielded similar results. Exposure of the quiescent cells to a cisplatin concentration of 0.4 mM for 1 hr, followed by maintenance of the cells in drug-free medium, caused a decrease in cell viability, which was first observed 24 hr after termination of the incubation with cisplatin. Cell viability was further reduced to 21% during the next 48 hr. Under these conditions, a cisplatin concentration of 0.1 mM did not significantly reduce the cell viability, measured up to 72 hr after exposure.

Cytotoxicity of ebselen in LLC-PK_1 cells. Figure 2 shows the influence of various concentrations of ebselen on the viability of the LLC-PK_1 cells. Exposure of the LLC-PK_1 cells to ebselen, 5–15 μM, for 1 hr did not alter their viability as measured by the leakage of intracellular LDH in the medium and by the amount of protein remaining attached to the culture plate, up to 72 hr after exposure. Cell viability was reduced to 32%, 72 hr after exposure to 50 μM ebselen for 1 hr.

The influence of ebselen on the cytotoxicity in LLC-PK_1 cells. The results presented in Fig. 3 demonstrate that pre-incubation of the LLC-PK_1 cells with ebselen, 5–15 μM for 1 hr, protected the cells from a decrease in viability induced by exposure of the cells to 0.2 or 0.4 mM cisplatin. The protective effect of ebselen was concentration dependent: the highest protection was obtained at an ebselen concentration of 15 μM. Pre-incubation of ebselen could not protect the cells against the decrease in viability caused by exposure to a cisplatin concentrations of 0.8 mM for 1 hr (Fig. 3). The protective effect of ebselen as function of the time after exposure to cisplatin is plotted in Fig. 4. As shown, the viability of the cells, measured after exposure to 0.4 mM cisplatin for 1 hr, was during the whole period (up to 72 hr after exposure) significantly higher in cells which were pre-exposed to 10 μM ebselen for 1 hr.

Discussion

The aim of this study was to investigate the influence of ebselen on the toxicity of cisplatin in LLC-PK_1 cells and to study the correlation between the results of this test system with those of *in vivo* experiments.

LLC-PK_1 cell model. LLC-PK_1 cells are porcine kidney cells which express many characteristics of proximal tubule epithelia [19]. The proximal tubular area is the major site of cisplatin nephrotoxicity [5] and the LLC-PK_1 cell line has been used to study the nephrotoxicity of cisplatin [22]. In this cell line several viability assays, such as Tryphan blue exclusion, LDH leakage and the amount of protein remaining attached to the culture plate, can be used with similar results [22].

As demonstrated in Fig. 1, cisplatin caused a concentration-dependent cytotoxicity in LLC-PK_1 cells. This cisplatin-induced cytotoxicity developed relatively

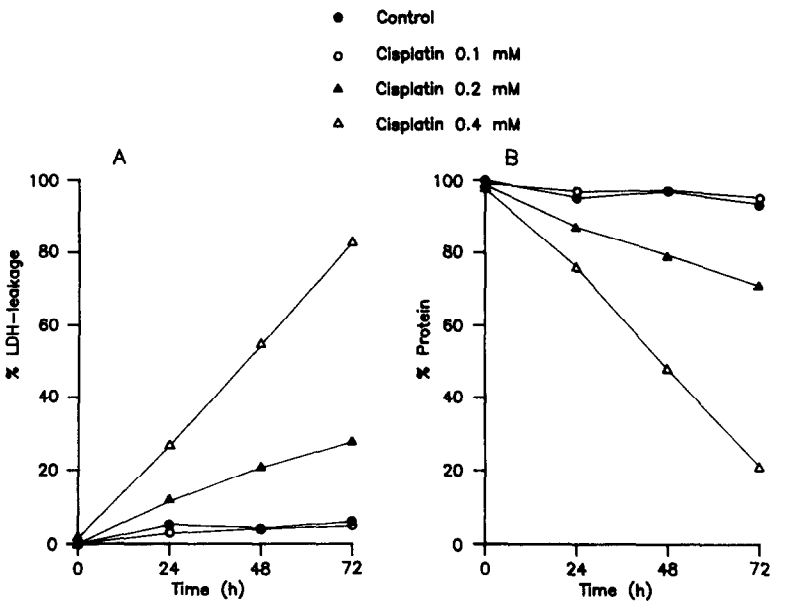

Fig. 1. Cytotoxicity of cisplatin in LLC-PK$_1$ cells. Quiescent cells were exposed to cisplatin for 1 hr, washed and then incubated in fresh medium until assayed for viability by measuring LDH leakage (A) and the amount of protein remaining attached to the culture plate (B). Data represent one typical example out of three independent experiments. SD < 6%.

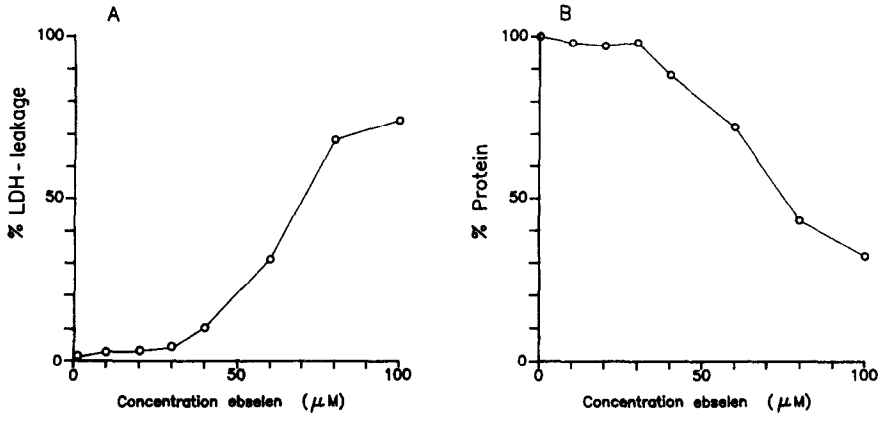

Fig. 2. Cytotoxicity of ebselen in LLC-PK$_1$ cells. Quiescent cells were exposed to cisplatin for 1 hr, washed and then incubated in fresh medium until assayed (t = 72 hr) for viability by measuring LDH leakage (A) and the amount of protein remaining attached to the culture plate (B). Data represent one typical example out of three independent experiments. SD < 6%.

slowly: 24 hr after exposure to 0.4 mM cisplatin, cell viability was much higher than after 72 hr. This delay in cytotoxicity is comparable with the delay in onset of the nephrotoxicity induced by cisplatin *in vivo* [26]. In this study the cells were maintained in quiescent state, before and after exposure to cisplatin. DNA synthesis is of limited importance for cell survival in quiescent cells: therefore, cytotoxicity of cisplatin in quiescent LLC-PK$_1$ cells does not represent the antitumour effects of cisplatin, which correlate with inhibition of DNA synthesis [27]. The antitumour effects of cisplatin are mediated through the formation of platinum–DNA adducts [28]. Montine and Borch [22] have shown that cisplatin-induced toxicity in quiescent LLC-PK$_1$ cells is related to inhibition of post transcriptional processes, especially protein synthesis. Ebselen, at concentrations of up to 15 μM for an exposure time of 1 hr, was not toxic to quiescent LLC-PK$_1$ cells (Fig. 2). Therefore, quiescent LLC-PK$_1$ cells seem to be a reliable *in vitro* model to predict whether ebselen might be able to protect against cisplatin-induced nephrotoxicity *in vivo*.

The cytotoxicity of cisplatin could be prevented by ebselen. Pre-exposure of the cells for 1 hr to ebselen concentrations as low as 5 μM (Fig. 3) provided protection

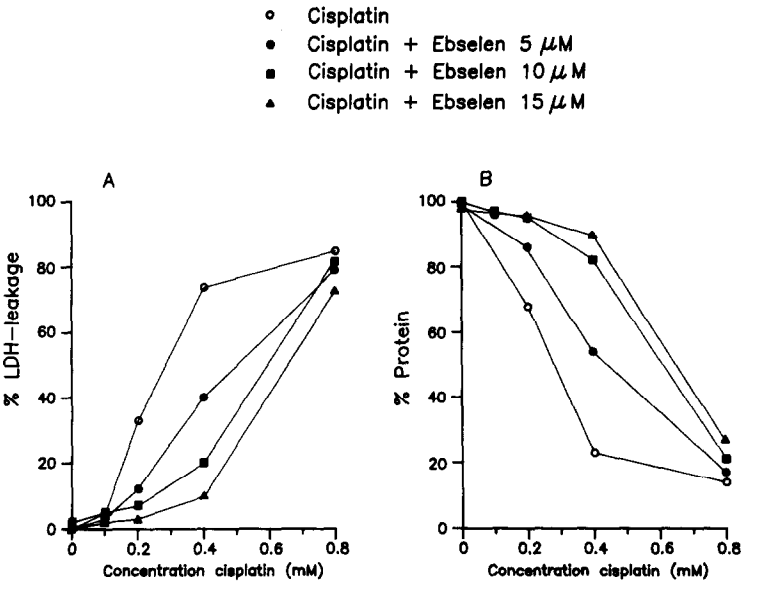

Fig. 3. The influence of ebselen on the cytotoxicity of cisplatin in LLC-PK$_1$ cells. Quiescent cells were exposed to ebselen for 1 hr, washed, exposed to cisplatin for 1 hr, washed and then incubated in fresh medium until assayed (t = 72 hr) for viability by measuring LDH leakage (A) and the amount of protein remaining attached to the culture plate (B). Data represent one typical example out of three independent experiments. SD < 6%.

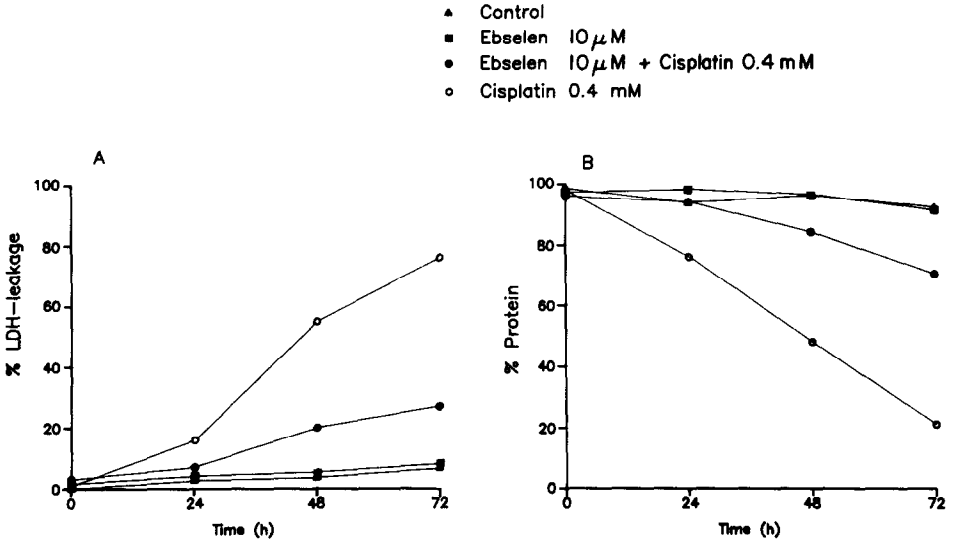

Fig. 4. Time course of the protective effect of ebselen on the cytotoxicity of cisplatin in LLC-PK$_1$ cells. Quiescent cells were exposed to 10 μM ebselen for 1 hr, washed, exposed to 0.4 mM cisplatin for 1 hr, washed and then incubated in fresh medium until assayed for viability by measuring LDH leakage (A) and the amount of protein remaining attached to the culture plate (B). Data represent one typical example out of three independent experiments. SD < 6%.

against the cytotoxic effects induced by exposure of the cells for 1 hr to cisplatin concentrations of up to 0.4 mM. Several other agents have been tested in this model. Mesna, thioureum, thiosulfate and diethyldithiocarbamate also protected against cisplatin-induced cytotoxicity in quiescent LLC-PK$_1$ cells [22].

In vitro–in vivo *correlation*. The results obtained *in vitro* in the LLC-PK$_1$ cell model correlate well with our *in vivo*

experiments, published earlier [29]. Ebselen prevented cisplatin-induced nephrotoxicity in BALB/c mice, when administered 1 hr before a single dose of cisplatin. Montine and Borch [22] have demonstrated that the protective effects of diethyldithiocarbamate, mesna, sodium thiosulfate and thioureum against cisplatin cytotoxicity in quiescent LLC-PK_1 cells also correlate well with the protective effects of these agents against cisplatin-induced nephrotoxicity in rodent models. Interestingly, Hori *et al.* [30] have shown that the rank-order of cytotoxicity of aminoglycosides in LLC-PK_1 cells also correlated well with the rank-order of *in vivo* nephrotoxicity. Apparently quiescent LLC-PK_1 cells are a suitable *in vitro* model to test potential chemoprotectors against nephrotoxicity induced by cisplatin; whether this model is also suitable to test chemoprotectors against nephrotoxicity induced by other agents such as aminoglycoside remains to be established.

Mechanism of protection by ebselen. The precise mechanism of cisplatin-induced nephrotoxicity is unknown. Inactivation of thiol-containing enzymes in the kidney may play an important role in the nephrotoxicity of cisplatin [31]. Nucleophilic agents, which are capable of alkylating cisplatin and its hydrolysis products, can protect rodents against cisplatin-induced nephrotoxicity. Diethyldithiocarbamate [13, 31] and *S*-2-(3-aminopropylamino) ethylphosphorothioic acid (WR-2721) [32] are examples of this type of chemoprotector against cisplatin-induced nephrotoxicity.

Selenium compounds are also a very efficient class of chemoprotectors against cisplatin-induced nephrotoxicity. Sodium selenite [33–35] and selenomethionine [36] have been shown to protect against cisplatin-induced nephrotoxicity. In thiol-rich tissues, such as the kidneys, both compounds are metabolized to selenols (R-SeH) such as methyl selenol and glutathionyl selenol. Selenols are nucleophilic agents which are able to inactivate cisplatin, most probably by forming a platinum-selenium bond via a S_N2 nucleophilic displacement reaction with a chlorine atom as leaving group [37]. Ebselen can form selenol intermediates by reacting with GSH or other thiols [16, 17]. Ebselen-induced formation of selenols will occur primarily in thiol-rich tissues, such as the kidneys. These selenols may be responsible for the protective effects of ebselen against both the cytotoxicity of cisplatin in quiescent LLC-PK_1 cells and the cisplatin-induced nephrotoxicity in rodents. Further studies to establish the clinical value of ebselen as a chemoprotector against cisplatin-induced nephrotoxicity would be worthwhile.

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Soybean trypsin inhibitor and β-amylase induce alveolar macrophages to release nitrogen oxides

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Abstract—Rat alveolar macrophages incubated with soybean trypsin inhibitor and β-amylase produced nitrite in a dose- and time-dependent manner. This production depends on the presence of L-arginine (L-arg) in the culture medium. The precursor of this nitrite was demonstrated as being nitric oxide by bleaching ferredoxin at 410 nm when added to the culture medium. N^G-Monomethyl-L-arginine and the tetrahydrobiopterin biosynthesis inhibitor 2,4-diamino-6-hydroxypyrimidine inhibited the release of nitrite in a dose-dependent manner. Dexamethasone was able to modulate this release. These data indicate that alveolar macrophages are capable of secreting L-arg-derived nitrogen oxides when stimulated with certain alimentary proteins.

Immunostimulated macrophages, either murine peritoneal macrophages [1, 2] or rat Kuppfer cells, mice peritoneal, wound and bone marrow macrophages [3–5] synthesize nitrite (NO_2^-) and nitrate (NO_3^-) from L-arginine (L-arg*) by oxidation of the intermediate nitric oxide (NO) [4]. The true biological role of nitrogen oxides produced by

* Abbreviations: STI, soybean trypsin inhibitor; L-arg, L-arginine; rIFN-γ, recombinant interferon-γ; LPS, lipopolysaccharide; DAHP, 2,4-diamino-6-hydropyrimidine; Fd, ferredoxin; L-NMMA, N^G-monomethyl-L-arginine; DMEM, Dulbecco's modified Eagle's medium.