

## EBSELEN ANALOGUES REDUCE 2-CHLOROETHYL ETHYL SULPHIDE TOXICITY IN A-431 CELLS

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Vesicants are potent blistering agents. The prototype vesicant is sulphur mustard gas, first used in World War I, which still has no effective antidote. We used a mustard gas surrogate 2-chloroethyl ethyl sulphide (CEES) to study the ability of resveratrol (RES) and pterostilbene (PTS), two well-established stilbene antioxidants, ebselen (EB-1), an organoselenium compound, and three EB-1 analogues (EB-2, EB-3, and EB-4) to reduce CEES toxicity in human epidermoid carcinoma cells (A-431). Following a 24-hour incubation of a toxic concentration of CEES (1000  $\mu\text{mol L}^{-1}$ ), we used the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] test to analyse cell viability. Different concentrations of test antioxidants alone (15  $\mu\text{mol L}^{-1}$ , 30  $\mu\text{mol L}^{-1}$  or 60  $\mu\text{mol L}^{-1}$ ) did not decrease cell viability. Treatment with CEES and test antioxidants for 24 h showed that only EB-1 and its analogues EB-2, EB-3, and EB-4 but not the stilbene compounds could rescue the cells from death. EB-1 and EB-4 were the most effective at reducing CEES cytotoxicity and did so in a concentration-dependent manner, while EB-2 and EB-3 demonstrated the least protective effect. In summary, the data described herein indicate that organoselenium antioxidants, especially EB-4, may prove useful as countermeasures to blistering agents.

**KEY WORDS:** *CEES, cell viability, EB-1, EB-2, EB-3, EB-4, human epidermoid carcinoma cells, MTT test, organoselenium, pterostilbene, resveratrol, vesicant countermeasure*

During the First World War, a sulphur mustard compound known as bis(2-chloroethyl)sulfide and commonly referred to as *mustard gas* was developed into a weapon and has since been used in a number of world conflicts. Mustard gas and its analogues have a vesicant or blistering capacity when applied to skin (1, 2). The resulting blisters are slow to heal and pose the risk of secondary infection. Mustard gas also exhibits a wide array of toxicities. From a chemical point of view, vesicants are relatively simple to synthesize and exposure to weapons-grade mustards on the battlefield or in a civilian setting could overload available emergency services (3) and become difficult to manage, since no specific antidote has been

developed to control mustard toxicity. Thus, an effective countermeasure to mustard gas toxicity would be beneficial to the public health.

In addition to causing DNA alkylation, mustards also readily bind to cellular thiols; particularly the free thiol group of the cysteine residue within the cellular antioxidant glutathione (GSH). As GSH is neutralised by mustards, the cell becomes increasingly more sensitive to oxidative stress (4). Oxidative stress can lead to cell death when the production of reactive oxygen (ROS) and/or reactive nitrogen oxide species (RNOS) exceeds the capacity of cellular antioxidant defence mechanisms (5, 6). In recent years, the idea of using antioxidants to decrease the toxicity of

vesicants has gained momentum through results obtained from *in vivo* (7, 8) and *in vitro* studies (9-12). While these antioxidants may serve as effective countermeasures to mustard toxicity in various experimental models, none of them have evolved into an effective therapy to date.

This report describes the results of a study in which several compounds with established or suspected antioxidant activities were tested for ability to reduce the toxicity of a sulphur mustard surrogate *in vitro*. These compounds, from this point forward designated as the test compounds, were resveratrol (RES), pterostilbene (PTS), ebselen (EB-1), and three EB-1 analogues (EB-2, EB-3 and EB-4) which, although not as extensively studied as the parent compound, exhibit structural features common to organoselenium compounds with antioxidant activity (13, 14). Since working with mustard gas poses a significant risk to the investigator and is not commercially available, we used an analogue of mustard gas instead, that is, 2-chloroethyl ethyl sulphide (CEES). This compound is commonly referred to as *half mustard* due to the presence of a single chloroethyl group and forms only monofunctional adducts with DNA. We selected human epidermoid carcinoma cells (A-431) to study the ability of the test compounds to reduce CEES toxicity. This cell line has been used in various *in vitro* dermatological studies, as it provides an affordable alternative to the use of primary skin cultures (9, 12, 15-17).

## MATERIALS AND METHODS

### Cell line

A-431 human epidermoid carcinoma cells (ATCC #CRL-1555) (18) were purchased from the American Type Culture Collection (Manassas, VA, USA).

### Treatment compounds

Unless otherwise indicated, all treatment compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA), as follows: half-mustard (CEES, cat # 242640), resveratrol (RES, 3,4',5'-trihydroxy-trans-stilbene, cat # R5010) and pterostilbene (PTS, 3,5-dimethoxy-4'-hydroxy-trans-stilbene, cat # P1499). Ebselen (EB-1; 2-phenyl-1,2-benziselenazol-3(2H)-one, cat # 70530) and ebselen oxide (EB-3, cat #10012298) were purchased from the Cayman

Chemical Company (Ann Arbor, Michigan, USA). Ebselen analogues (EB-2 and EB-4) were synthesized as described previously (19). The chemical structures of CEES and the compounds tested are shown in Figure 1.

Compounds	Chemical Structure
CEES	
RES	
PTS	
EB-1	
EB-2	
EB-3	
EB-4	

**Figure 1** Structure of CEES and of the tested antioxidant compounds

### Chemicals and other reagents

Unless otherwise indicated, all chemicals used in the study were purchased from Sigma-Aldrich (St. Louis, MO, USA) and included dimethylsulphoxide (DMSO, cat # D8418), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, cat # M2128), Triton-X100 (cat # T8787), BSA (cat # A7906), phosphate buffered saline (PBS, cat # P3813), tris-hydroxymethylaminomethane (TRIS, cat # 154563), phenol solution (cat # P4557), chloroform: isoamyl alcohol (cat # C0549), EDTA (cat # E0399), agarose (cat # A9539), glacial acetic acid (cat # 242853), and glycerol (cat # G5516). Elutriation buffer (cat # 19086) was purchased from Qiagen, Inc. (Valencia, CA, USA). Trypan blue dye (cat # BDH8721-0) was obtained from VWR International (West Chester, PA, USA). Potassium acetate (cat # 529543), ethidium bromide (cat # EM- 4410), NaCl (cat # EM- 7710), and boric acid (cat # EM-203667) were purchased from EMD Chemicals (Gibbstown, NJ, USA).

### *Cell culturing medium*

Cells were grown in tissue culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) containing phenol red, L-glutamine, glucose  $4.5 \text{ mg L}^{-1}$ , and 10 % heat-inactivated foetal bovine serum (FBS) and were supplemented with  $50 \text{ } \mu\text{g mL}^{-1}$  of gentamicin (cat # 15750-060; Invitrogen, Carlsbad, CA, USA). Complete DMEM was obtained by mixing 50 mL of FBS and 550  $\mu\text{L}$  of gentamicin. A-431 skin cells were briefly trypsinised prior to passaging (Invitrogen Corporation, Carlsbad, CA, USA, cat # 10564). Cells were treated in DMEM containing L-glutamine and glucose, but not phenol red and FBS (clear DMEM) (Invitrogen Corporation, Carlsbad, CA, USA; cat # 25300-054).

### *Assays to determine cellular viability*

We mixed 347  $\mu\text{L}$  of CEES with 9.6 mL of ethanol to yield a  $300 \text{ mmol L}^{-1}$  stock solution and stored it at  $4 \text{ }^\circ\text{C}$  until needed. RES (14.8 mg) was dissolved in DMSO (648  $\mu\text{L}$ ) to make a  $100 \text{ mmol L}^{-1}$  stock solution. PTS (208.8 mg) was dissolved in 8125  $\mu\text{L}$  of DMSO and EB-1 (10 mg) was dissolved in 364  $\mu\text{L}$  of DMSO to yield  $100 \text{ } \mu\text{mol L}^{-1}$  solutions. Next, 4.7 mg of EB-2 was dissolved in DMSO (167  $\mu\text{L}$ ) and gently heated. In addition, 2.9 mg of EB-3 and 6.9 mg EB-4 were dissolved in DMSO (172.4  $\mu\text{L}$  and 250  $\mu\text{L}$ , respectively) to make a  $100 \text{ mmol L}^{-1}$  stock solution of each. Finally, all the test compounds ( $100 \text{ mmol L}^{-1}$ ) were dissolved in an appropriate amount of DMSO to make a final stock solution of  $60 \text{ mmol L}^{-1}$  and were stored at  $-20 \text{ }^\circ\text{C}$  until treatment.

MTT reagent was prepared by mixing 0.04 g of thiazolyl blue tetrazolium bromide powder with 8 mL of FBS-free, phenol-free DMEM to yield a final concentration of  $5 \text{ mg mL}^{-1}$ . MTT solubilisation reagent was prepared by mixing 270 mL of isopropyl alcohol, 30 mL Triton-X, and 30 mL of  $1 \text{ mol L}^{-1}$  HCl in a glass amber bottle and was stored at room temperature.

A-431 skin cells were seeded into 24-well plates ( $1 \times 10^5$  cells per well) and treated the next day. Culture medium containing FBS was removed from each well and replaced with 0.5 mL of FBS- and phenol red-free DMEM containing either 0 (control),  $15 \text{ } \mu\text{mol L}^{-1}$ ,  $30 \text{ } \mu\text{mol L}^{-1}$ , or  $60 \text{ } \mu\text{mol L}^{-1}$  of each test compound. Cells were then incubated in parallel with or without CEES ( $1000 \text{ } \mu\text{mol L}^{-1}$ ). After 24 h, the MTT viability assay was conducted. All treatments were performed in triplicate

wells for each test compound, and all experiments were performed a minimum of three times each.

Control wells were supplemented with 0.1 % of DMSO. This concentration of vehicle was neither toxic nor inhibitory in the MTT assay (data not shown).

The MTT assay measures the conversion of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) into a coloured product in living cells. To determine the viability of cells, the MTT assay was performed as described by Mosmann (20), with the incubation time of the MTT reagent being modified as described by Shah et al. (21). To 500  $\mu\text{L}$  of culture medium we added 50  $\mu\text{L}$  of MTT and let it incubate at  $37 \text{ }^\circ\text{C}$  for 1 h. Next we added 550  $\mu\text{L}$  of MTT solubilisation reagent, mixed the wells, and let it incubate for another 10 min, still at  $37 \text{ }^\circ\text{C}$ . The optical density of this purple formazan product was then measured spectrophotometrically in a glass cuvette at 570 nm. The MTT reagent did not react with any of the test compounds at the concentrations used in the absence of cells (data not shown), which validates its use for cell viability studies.

To determine whether or not EB-1 could reduce CEES toxicity in a viability assay not related to the MTT assay, A-431 cells were plated in 6-well plates ( $1 \times 10^6$  cells per well). The following day, culture medium was removed from triplicate wells and replaced with FBS and phenol red-free DMEM containing either the DMSO vehicle (0.1 %) or CEES ( $1000 \text{ } \mu\text{mol L}^{-1}$ ) in the presence or absence of EB-1 ( $15 \text{ } \mu\text{mol L}^{-1}$ ,  $30 \text{ } \mu\text{mol L}^{-1}$  or  $60 \text{ } \mu\text{mol L}^{-1}$ ). After 24 h, culture medium was removed from all wells and the wells were washed once with PBS. Next, 500  $\mu\text{L}$  of trypsin was added to each well and incubated for 10 min at  $37 \text{ }^\circ\text{C}$ . Cells were collected in a microfuge tube and spun in a table top centrifuge at  $1957 \times g$  for 5 min. The supernatant was removed and 500  $\mu\text{L}$  of FBS and phenol red-free DMEM was used to re-suspend the cell pellet. Next, 50  $\mu\text{L}$  of the re-suspended sample was added to an equal volume of trypan blue in a new microfuge tube. Finally, 10  $\mu\text{L}$  of this combined solution was applied to a haemocytometer, which was inspected with a standard light microscope at 20x magnification. Cells without the dye were counted as viable and the number of viable cells per treatment was evaluated.

### *CEES-induced DNA fragmentation*

A-431 skin cells were grown to 90 % confluency in complete DMEM in 6-well plates ( $1 \times 10^6$  cells per

well). The next day, the culture medium was removed and separate wells were incubated with 2 mL of FBS- and phenol-red free DMEM containing different concentrations of CEES (0, 250, 500, 1000, 1500, and 2000)  $\mu\text{mol L}^{-1}$ . These plates were then incubated at 37° C for 16 h to 18 h. DNA fragments were then isolated and analysed according to the method reported by our lab previously (12).

#### Log P determination for EB-1 and its analogues

Log *P*, or the logarithmic partition coefficient of 1-octanol/water, is a critical tool in the determination of drug membrane permeability. This value is used to estimate properties such as absorption, metabolism, and bioavailability (22). It is worth noting that all log *P* values for the organoselenium compounds reported in the Results section were obtained using the Molinspiration Property Calculation Service (www.molinspiration.com) and were not experimentally determined.

#### Statistical analysis

All experiments were carried out in triplicate, with the results being reported as the mean  $\pm$  standard error of the mean from at least three representative experiments. Statistical comparisons were made using either Student's *t*-test (for comparison between two groups) or one-way ANOVA with Neumann-Keuls post hoc test (for comparison across multiple groups) using GraphPad Prism 4.0® software (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered to be significant at  $p < 0.05$ .

## RESULTS

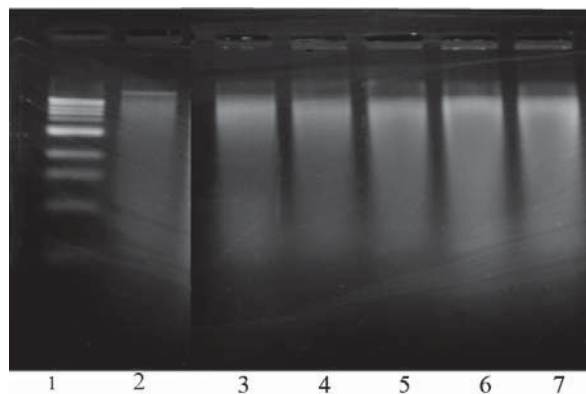
#### CEES cytotoxicity in A-431 cells

At a CEES concentration of 1000  $\mu\text{mol L}^{-1}$ , the MTT assay found ~7 % of the cells viable (Table 1), whereas the trypan blue dye exclusion method found ~14 % of the cells viable (Table 2). Based on this observation, and to ensure a robust toxicity, MTT viability studies with CEES were performed at a cell density of  $1 \times 10^5$  cells per well and a concentration of 1000  $\mu\text{mol L}^{-1}$ .

#### CEES-induced DNA fragmentation

DNA fragments from control and CEES-treated cells [(250, 500, 1000, 1500 or 2000)  $\mu\text{mol L}^{-1}$ ] were isolated and extracted using phenol/chloroform,

treated with RNase, and analysed using agarose gel electrophoresis. CEES caused a concentration-dependent increase in DNA fragmentation, with the highest fragmentation patterns occurring at 1500  $\mu\text{mol L}^{-1}$  and 2000  $\mu\text{mol L}^{-1}$  CEES concentrations (Figure 2).



**Figure 2** DNA fragmentation in A-431 skin cells resulting from treatment with CEES. Lane 1, DNA 1 kb marker; lane 2, control; lane 3, 250  $\mu\text{mol L}^{-1}$  of CEES; lane 4, 500  $\mu\text{mol L}^{-1}$  of CEES; lane 5, 1000  $\mu\text{mol L}^{-1}$  of CEES; lane 6, 1500  $\mu\text{mol L}^{-1}$  of CEES; lane 7, 2000  $\mu\text{mol L}^{-1}$  of CEES

#### Test compounds to counteract the toxicity of CEES

RES is a naturally occurring stilbene and antioxidant and is polyphenolic in nature (Figure 1). When administered alone, RES was not toxic to the skin cells at any of the test concentrations at 24 h (Table 1). When administered in combination with CEES, RES did not rescue the cells from death (Table 1). Similar data were obtained for PTS (Table 1).

In contrast, EB-1 exhibited a concentration-dependent increase in survival reaching about 67 % viability at 60  $\mu\text{mol L}^{-1}$  (Table 1). Only the lowest concentration showed no significant increase in viability. In addition, EB-1 alone exhibited no toxicity to the skin cells at 24 h; moreover, at its highest concentration EB-1 significantly increased cell viability ( $p < 0.001$ ) compared to vehicle-treated cells (Table 1).

EB-2 significantly increased MTT activity at 60  $\mu\text{mol L}^{-1}$  (~10 %) in the absence of CEES co-treatment. In addition, only the 60  $\mu\text{mol L}^{-1}$  concentration of EB-2 provided significant ( $p < 0.001$ ) protection against CEES (Table 1).

EB-3 alone significantly increased A-431 cell viability across the range of test concentrations, but in terms of protection against CEES, only the highest concentration was significantly effective (Table 1).

**Table 1** Effects of selected test compounds on the cytotoxicity of CEES in A-431 skin cells, as determined by the MTT viability assay

Treatment <sup>a</sup>	Viability / % <sup>b</sup>	
	Untreated <sup>c</sup>	CEES-treated <sup>d</sup>
<b>Control</b>	<b>100</b>	<b>6.73±1.06</b>
<u>RES</u>		
RES (15 µmol L <sup>-1</sup> )	102.00±2.01	7.56±0.38
RES (30 µmol L <sup>-1</sup> )	96.60±0.99	7.16±0.20
RES (60 µmol L <sup>-1</sup> )	100.60±3.45	8.27±0.05
<u>PTS</u>		
PTS (15 µmol L <sup>-1</sup> )	98.75±3.72	3.64±0.52
PTS (30 µmol L <sup>-1</sup> )	96.30±3.10	3.64±0.32
PTS (60 µmol L <sup>-1</sup> )	92.87±5.72	3.95±0.19
<u>EB-1</u>		
EB-1 (15 µmol L <sup>-1</sup> )	96.38±1.24	17.42±0.89
EB-1 (30 µmol L <sup>-1</sup> )	89.01±0.94	22.63±1.71 <sup>^</sup>
EB-1 (60 µmol L <sup>-1</sup> )	110.70±4.40 <sup>***</sup>	66.90±2.33 <sup>^^^</sup>
<u>EB-2</u>		
EB-2 (15 µmol L <sup>-1</sup> )	101.20±0.91	3.50±1.03
EB-2 (30 µmol L <sup>-1</sup> )	105.10±0.67	3.90±0.00
EB-2 (60 µmol L <sup>-1</sup> )	109.60±1.16 <sup>***</sup>	11.93±0.47 <sup>^^^</sup>
<u>EB-3</u>		
EB-3 (15 µmol L <sup>-1</sup> )	113.80±1.26 <sup>*</sup>	10.43±0.47
EB-3 (30 µmol L <sup>-1</sup> )	140.90±3.00 <sup>***</sup>	10.62±0.74
EB-3 (60 µmol L <sup>-1</sup> )	149.60±6.06 <sup>***</sup>	20.17±0.80 <sup>^^^</sup>
<u>EB-4</u>		
EB-4 (15 µmol L <sup>-1</sup> )	118.00±3.24 <sup>**</sup>	15.17±0.33 <sup>^</sup>
EB-4 (30 µmol L <sup>-1</sup> )	113.70±0.67 <sup>**</sup>	36.06±2.73 <sup>^^</sup>
EB-4 (60 µmol L <sup>-1</sup> )	123.00±0.77 <sup>***</sup>	82.31±3.59 <sup>^^^</sup>

<sup>a</sup> All cells were cultured for 24 h in the absence or presence of CEES

<sup>b</sup> Values represent the mean ± standard error of the mean from three independent experiments

<sup>c</sup> Significantly different from untreated control cells (vehicle) at <sup>\*</sup>p<0.05, <sup>\*\*</sup>p<0.01 or <sup>\*\*\*</sup>p<0.001 by Student's t-test and ANOVA

<sup>d</sup> Significantly different from CEES-treated control cells at <sup>^</sup>p<0.05 or <sup>^^^</sup>p<0.001 by Student's t-test and ANOVA

EB-4 in turn showed an 18 % to 23 % increase in cell viability when given alone, and in combination with CEES it showed significant protection at all three concentrations (Table 1).

In summary, all of the EB analogues showed some degree of action against CEES cytotoxicity in A-431 skin cells, but EB-4 showed the highest cytoprotective potential.

The trypan blue dye exclusion method confirmed EB-1 efficiency against CEES toxicity without significantly increasing cell viability when given alone (Table 2). The data obtained with the trypan blue dye exclusion assay showed higher standard errors than

data obtained with the MTT assay, which may be attributed to the extensive sample handling required for the trypan assay and the difficulty to distinguish which cells are truly excluding the dye, as some cells appear light blue under the microscope and confound scoring.

Log *P* values were 2.92 for EB-1, 2.10 for EB-2, 2.88 for EB-3, and 2.44 for EB-4. We established no relationship between lipophilicity, as indicated by log *P* values, and protection against CEES; however, a more extensive analysis of lipophilicity and countermeasure effectiveness is warranted before any conclusions are drawn.

**Table 2** Effect of EB-1 on the cytotoxicity of CEES in A-431 skin cells, as determined by the trypan blue dye exclusion assay

Treatment <sup>a</sup>	Viability / % <sup>b</sup>	
	Untreated	CEES-treated <sup>c</sup>
EB-1		
Control	100	13.46±5.52
EB-1 (15 µmol L <sup>-1</sup> )	117.30±8.99	5.89±2.55
EB-1 (30 µmol L <sup>-1</sup> )	71.90±8.19	45.55±7.03*
EB-1 (60 µmol L <sup>-1</sup> )	76.96±13.14	39.35±11.51*

<sup>a</sup> All cells were cultured for 24 h in the absence or presence of CEES

<sup>b</sup> Values represent the mean ± standard error of the mean from three independent experiments

<sup>c</sup> Significantly different from CEES- treated control cells at \* $p < 0.05$  by Student's *t*-test and ANOVA

## DISCUSSION

It has been nearly one hundred years since mustards were first used as chemical warfare agents, yet an effective medical countermeasure is still sought after (23, 24). The cellular mechanisms involved in mustard toxicity are well established and include oxidative stress, DNA damage, and the overactivation of PARP-1 (5, 25, 26). The goal of the present research was to determine if the selected test compounds could reduce the toxicity of a sulphur mustard surrogate *in vitro*. The toxicant used in our studies, CEES, is a monofunctional alkylating agent with vesicant activity *in vivo*. CEES has been utilised by other laboratories to mimic the effects induced by mustard gas (27, 28). The test compounds we used included established antioxidants (RES, PTS and EB-1) and three less studied organoselenium agents (EB-2, EB-3, and EB-4) bearing structural similarity to EB-1.

A-431 human epidermoid carcinoma cells are an inexpensive and easy alternative to using primary skin cell cultures (9, 12, 15, 16). An important limitation with this cell line is that it is tumour-derived and expresses a mutant p53 protein (29, 30). It is therefore possible that these cells respond differently to mustards than normal skin cells would, and this possibility should not be overlooked. In addition, A-431 cells exposed to mustards *in vitro* are unable to mimic the response of normal skin tissue to mustard exposure, which becomes injured and forms fluid-filled blisters. In spite of these limitations, the A-431 cells make an important screening tool for potential countermeasures to mustards that can be tested *in vivo*.

We observed that RES and PTS exhibited no propensity to protect A-431 skin cells from mustard toxicity. RES has been found in at least one *in vivo* study to protect the lungs from mustard injury (8). This discrepancy may be due to the cell type tested

here or to not having used a high enough concentration of the stilbene. The latter however is unlikely, as Radkar et al. (17) found that RES exhibited toxicity in the same cell line with an LC<sub>50</sub> of ~ 60 µmol L<sup>-1</sup>. PTS was also unable to reduce or prevent mustard toxicity in A-431 cells, in contrast to Pereko et al. (31), who found it effective in reducing extracellular reactive oxygen species.

We obtained the best anti-mustard effects with organoseleniums. These compounds contain at least one carbon-selenium bond and are not to be confused with inorganic selenium, which is a trace element in the human diet and has been studied extensively against cancer, immunity and infectious/inflammatory diseases (19, 32). Whereas the prototype organoselenium compound EB-1 has already been reported to protect A-431 skin cells from CEES toxicity (12), the ability of the related compounds EB-2, EB-3, and EB-4 to do so has not been studied so far to the best of our knowledge. The reason why we turned to organoselenium analogues as potential countermeasures to CEES toxicity lies in certain limitations of EB-1. For one, EB-1 binds strongly to plasma proteins when administered intravenously and can not reach the cell in the albumin-bound state. It takes thiol-containing reducing agents to free EB-1 to bind to membrane-associated proteins (33). Moreover, the anti-inflammatory activity of EB-1 is not likely to be involved in the protection against mustard toxicity observed in this study because like other *in vitro* models, A-431 skin cells do not possess a vasculature, connective tissue, or immune cells. As EB-1 highly reacts with protein thiols, its *in vitro* inhibitory activity may be related to nonspecific protein interactions (34). Lastly, EB-1 may inhibit the oxidative burst mechanism used by neutrophils and macrophages during an inflammatory response. The idea that EB-1 can exert an immunosuppressive effect *in vivo* has been explored

previously (35). A similar adverse effect on the immune system is known to be induced by mustard agents. Thus, the combined effect of mustard and EB-1 on immunity may delay healing in the afflicted patient.

In the present study, all three EB-1 analogues reduced CEES toxicity in A-431 cells, with EB-4 exhibiting the highest degree of protection. Masumoto and Sies (36) suggest that EB-3 may be converted to EB-1 in the presence of intracellular glutathione, but our data do not strongly support this hypothesis, as we would have observed a higher degree of cytoprotection by EB-3 if it had been converted to EB-1. Further investigation is required to determine whether EB-3 can serve as an EB-1 prodrug or if it exerts unique anti-mustard activity.

It is worth noting that we established no relationship between anti-mustard action of the organoselenium compounds tested here and hydrophobicity, as measured by Log *P*. In an attempt to take advantage of the antioxidant potential of EB-1 while minimizing its pharmacological shortcomings, numerous analogues of EB-1, including those studied in this work, have been synthesised, with various structural substitutions (19, 37, 38). Alterations to hydrophilicity have been introduced to increase water solubility and bioavailability of EB-1, which may be even more suitable for *in vivo* applications (39). Hopefully these new EB-1 analogues will be more efficient in an animal model, while maintaining the potency equivalent to or greater than EB-1.

It is wise to exercise caution when interpreting the data obtained using selenium compounds in MTT assays. A recent study by Olm et al. (40) indicated that selenite toxicity in cancer cells depended on extracellular reduction by thiols such as cysteine. In our study, the test compounds did not reduce MTT reagent in a cell free system at concentrations up to and including 60  $\mu\text{mol L}^{-1}$  (data not shown). Even so, these compounds may affect extracellular thiol levels. If they do, our *in vitro* system could reduce MTT reagent, produce false positive results, and give the appearance of protection. This may to some extent explain why cells treated with the organoselenium test compounds alone all exhibited higher viability at the highest concentrations than vehicle-treated control cells. Future studies should inquire whether the increased viability observed in the absence of CEES for EB-1 and its analogues may be due to an increase in extracellular thiols.

One reason why MTT experiments were carried out in a culture medium lacking FBS was to minimise any interference from FBS components such as albumin (41). Even though we did not assess the effects of EB-1 on extracellular redox, Schroterova et al. (42) found that MTT assays provide a reliable measure of cell viability in colon cancer cells exposed to diverse selenium-containing compounds at concentrations up to 256  $\mu\text{mol L}^{-1}$ .

We also ran the trypan blue dye exclusion to determine if a non-mitochondrial based assay can measure cytoprotection of organoselenium compounds such as EB-1 against CEES. Although it confirmed that EB-1 protected against CEES, viability of cells treated with EB-1 alone did not increase compared to vehicle-treated controls. The reason is unclear but may be related to low level thiol secretion from organoselenium-treated skin cells. Nonetheless, both viability assays showed that EB-1 protected A-431 skin cells from CEES toxicity.

Although our present study did not determine the mechanism of protection by the organoselenium compounds against CEES toxicity, Kim et al. (43) have suggested that EB-1 activates nuclear transcription via Nrf2. This in turn increases the expression of many phase II enzymes such as heme oxygenase (HO-1), NAD(P)H:quinone oxidoreductase, and gamma-glutamylcysteine synthetase (gamma-GCS), which act against oxidants. Knocking down Nrf2 in the A-431 cells with siRNA would likely abolish the protective effects of EB-1 and its analogues, and future studies may be looking in that direction.

EB-1 is also known to possess a glutathione peroxidase-like activity (44), and this mechanism of protection cannot be ruled out for EB-1 and its analogues.

In summary, EB-1 and its analogues show a potential against mustard toxicity. Previous studies demonstrated the effectiveness of EB-1 *in vitro* (9, 10, 12), this study is the first to propose novel organoselenium compounds (EB-2, EB-3, and EB-4) as agents against mustard toxicity. EB-4 showed the greatest degree of protection against CEES-induced toxicity of all the compounds in this class. Our future studies will investigate whether organoselenium analogues of EB-1 are more efficient in an animal model in comparison with other pharmaceuticals such as topical steroids. We will also look into the mechanisms of protection that these organoselenium compounds provide against CEES toxicity.

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### Conflicts of interest

The authors report no conflicts of interest.

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**Sažetak****ANALOZI EPSELENA SMANJUJU TOKSIČNOST 2-KLOROETIL ETILNOG SULFIDA U A-431-STANICAMA**

Plikavci izazivaju izražene mjehuriće na koži. Najpoznatiji je svakako sumporni iperit, koji se prvi put uporabio u 1. svjetskom ratu i do današnjega dana nema djelotvornog protuotrova. U ispitivanju smo rabili zamjenu za iperit, 2-kloroetil etilni sulfid (CEES) da bismo testirali sposobnost resveratrola (RES) i pterostilbena (PTS), dvaju poznatih stilbenskih antioksidansa, organoselenijeva spoja epselena (EB-1) te njegovih triju analoga (EB-2, EB-3 i EB-4) da smanji toksičnost CEES-a u humanih stanica epidermoidnog karcinoma (A-431). Vijabilnost stanica testirali smo s pomoću 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazol bromida (MTT) nakon 24-satne inkubacije s toksičnom koncentracijom CEES-a ( $1000 \mu\text{mol L}^{-1}$ ). Antioksidansi davani sami u različitim koncentracijama ( $15 \mu\text{mol L}^{-1}$ ,  $30 \mu\text{mol L}^{-1}$ , odnosno  $60 \mu\text{mol L}^{-1}$ ) nisu smanjili vijabilnost stanica. Dvadesetčetverosatna primjena CEES-a i testiranih antioksidansa pokazala je da samo EB-1 i njegovi analozi EB-2, EB-3 i EB-4 mogu spriječiti smrt stanica, ali ne i stilbenski spojevi. EB-1 i EB-4 pokazali su se najdjelotvornijima u ublažavanju toksičnosti CEES-a u skladu s koncentracijom, dok su se EB-2 i EB-3 pokazali najmanje djelotvornima. Ovdje prikazani podaci upućuju na to da organoselenijevi antioksidansi, a napose EB-4, mogu biti korisni protuotrovi plikavcima.

**KLJUČNE RIJEČI:** CEES, EB-1, EB-2, EB-3, EB-4, humane stanice epidermoidnog karcinoma, MTT-test, organoselenij, plikavci, protuotrov, pterostilben, resveratrol, vijabilnost stanica

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