The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Letter

Combined exposure to environmental electrophiles enhances cytotoxicity and consumption of persulfide

Masahiro Akiyama[1], Takamitsu Unoki[2] and Yoshito Kumagai[1]

[1]Environmental Biology Laboratory, Faculty of Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan
[2]Department of Basic Medical Sciences, National Institute for Minamata Disease, Minamata, Kumamoto 867-0008, Japan

(Received April 21, 2020; Accepted April 23, 2020)

ABSTRACT — Environmental electrophiles readily interact with reactive sulfur species (RSS), resulting in decline of cellular RSS levels accompanied by formation of their sulfur adducts. In the present study, we examined the effects of combined environmental electrophiles on consumption of persulfide and on cytotoxicity in HepG2 cells. A convenient assay with SSP4, a fluorometric probe for detection of per/polysulfides, indicated that each environmental electrophile caused compound-dependent consumption of persulfide. Consumption of persulfide by combined exposure to methylmercury (MeHg) and cadmium (Cd) was greater than that of the single exposure. Consistent with this finding, combined exposure to environmental electrophiles (MeHg, Cd, and 1,4-naphthoquinone) exacerbated concentration-dependent cellular toxicity in HepG2 cells compare to single exposure to each compound. As humans are exposed to environmental electrophiles daily, more attention should be paid to the study of combined exposure to environmental electrophiles, which can modulate cellular levels of RSS and disrupt redox homeostasis.

Key words: Environmental electrophiles, Reactive sulfur species, Combined exposure, Exposome

INTRODUCTION

The exposome is defined as the totality of environmental factors that an organism is exposed to from its living environment, lifestyle, and diet (Rappaport and Smith, 2010; Wild, 2005). Environmental electrophiles are considered priority components of the exposome because they exist in multiple forms with high reactivity and ubiquitous distribution (Bohme *et al*., 2016). Examples of environmental electrophiles include naphthoquinones (NQs), formed during the combustion of gasoline and present in fine particulate matter (Cho *et al*., 2004); crotonaldehyde and 1,4-benzoquinone, present in tobacco smoke (Hecht, 1999); methylmercury (MeHg), which accumulates in edible fish such as tuna through biocondensation (Hanna *et al*., 2015); cadmium (Cd), found in rice (Li *et al*., 2017), and acrylamide, formed in foods during heating (Gokmen and Palazoglu, 2008). After entering the body, these chemicals covalently bind to nucleophilic substituents such as protein cysteine residues or DNA nitrogen atoms, creating protein and DNA adducts, respectively, which are associated with enzyme dysfunction or structural alterations and carcinoma (LoPachin and Gavin, 2016; Jan *et al*., 2015; Kanda *et al*., 2014; Saeed *et al*., 2007; Rogers *et al*., 1997).

Some environmental electrophiles, however, are readily trapped by reactive sulfur species (RSS) with high nucleophilicity, such as persulfides and polysulfides (e.g. H_2S_2, H_2S_4), resulting in the formation of their sulfur adducts (Kumagai *et al*., 2019; Shinkai and

Correspondence: Yoshito Kumagai (E-mail: yk-em-tu@md.tsukuba.ac.jp)

Kumagai, 2019). We previously reported that MeHg, Cd, and 1,4-NQ react with RSS to form bismethylmercury sulfide [$(MeHg)_2S$] (Yoshida *et al*., 2011), cadmium sulfide (CdS) (Akiyama *et al*., 2017), 1,4-NQ-SH, 1,4-NQ-S-1,4-NQ, and 1,4-NQ-S-1,4-NQ-OH (Abiko *et al*., 2017a, 2017b). In addition, $(MeHg)_2S$ has been identified as a metabolite of MeHg in the liver of mice (Yoshida *et al*., 2011). $(MeHg)_2S$, CdS, and 1,4-NQ-S-1,4-NQ-OH appear to be less electrophilic and therefore less toxic than their parent electrophiles (Yoshida *et al*., 2011; Akiyama *et al*., 2017; Abiko *et al*., 2017b), indicating that RSS reduce the toxicity of electrophiles by forming inactive metabolites. Consistent with this notion, deletion of the RSS-producing enzyme cystathionine gamma-lyase in mice has been shown to increase sensitivity to Cd-induced hepatotoxicity and MeHg-induced toxicity (Akiyama *et al*., 2017, 2019). However, it should be noted that formation of the sulfur adducts in this manner is accompanied by consumption of intracellular RSS, which are involved in cellular redox homeostasis (Ihara *et al*., 2017). For example, we found that exposing cells to MeHg elevated intracellular levels of the endogenous electrophilic molecule 8-nitro-cGMP, accompanied by depletion of RSS and electrophile-induced activation of redox signaling and consequent cell damage (Ihara *et al*., 2017). Because humans are exposed to a variety of environmental electrophiles daily, we postulated that combined exposure to electrophiles with differing structures would enhance consumption of RSS, owing to formation of the sulfur adducts in comparison with RSS consumption following exposure to a single environmental electrophile. In the present study, we examined the effects of single and multiple exposures to electrophiles on consumption of RSS and on cytotoxicity.

MATERIALS AND METHODS

Chemicals

Acrylamide and $CdCl_2$ were purchased from Wako Pure Chemical Industries (Osaka, Japan). Crotonaldehyde and MeHg were obtained from Nacalai Tesque (Kyoto, Japan). 1,2-NQ, 1,4-NQ, and 1,4-benzoquinone were obtained from Tokyo Chemical Industries (Tokyo, Japan). Sulfane Sulfur Probe 4 (SSP4) and sodium disulfide (Na_2S_2) were purchased from Dojindo (Kumamoto, Japan). β-(4-Hydroxyphenyl)ethyl iodoacetamide (HPE-IAM) was obtained from Molecular Biosciences (Boulder, CO, USA). All other reagents and chemicals were of the highest grades available.

Fluorescent probe detection for RSS

Fluorescent probe detection can measure the reactivity of environmental electrophiles with RSS under multiple conditions simultaneously and conveniently by combining SSP4 (a fluorescent probe for RSS detection), Na_2S_2 (an RSS compound), and a multi-well plate. Each environmental electrophile was reacted with 40 μM Na_2S_2 in 20 mM HEPES buffer (pH 7.5) solution at 37°C for 15 min, followed by reaction with 20 μM SSP4 at 37°C for 15 min. After the reaction, the fluorescence intensity was measured at 515 nm (excitation: 482 nm) using a fluorescence spectrophotometer. The residual Na_2S_2 content was normalized by setting the fluorescence intensity when water was added instead of electrophiles as 100% and the upper value.

Liquid chromatography multiple-reaction monitoring mass spectrometry (LC-MRM-MS) analysis of persulfide

Levels of Na_2S_2 were measured as previously described (Akiyama *et al*., 2019). The sample was incubated with 5 mM HPE-IAM at 37°C for 30 min to yield HPE-AM adducts of sulfur nucleophiles including persulfide. Aliquots containing HPE-AM adducts were diluted four-fold with 0.1% formic acid containing known quantities of isotope-labeled internal standards, which were then analyzed by LC-MRM-MS/MS for persulfide determination.

Cell culture

HepG2 cells were obtained from RIKEN Cell Bank (Ibaraki, Japan). The cells were cultured in minimum essential medium α (Wako) containing 10% fetal bovine serum, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), and 2 mM GlutaMAX (Gibco, Carlsbad, CA, USA) in an incubator supplemented with 5% CO_2 at 37°C. Cells were pre-incubated in serum-free medium for 12 hr before treatment with compounds.

Cell viability

The 3-(4,5-dimethylthiazol-2-yl)-2,5-triphenyl tetrazolium bromide (MTT) assay was used to estimate cell viability as described previously (Denizot and Lang, 1986). Briefly, HepG2 cells in 96-well plates were exposed to MeHg with or without cadmium chloride and 1,4-NQ. After 24 hr, cells were treated with 5 mg/mL MTT for 4 hr at 37°C. After removing the medium, dimethyl sulfoxide (100 μL/well) was added to dissolve the formazan precipitate. Absorbance at 540 nm was read with an iMark microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

Statistical significance was assessed using ANOVA with post-hoc correction for multiple comparisons. All statistical analyses were performed using GraphPad Prism (San Diego, CA, USA). $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

We first measured the reactivity of the environmental electrophiles MeHg, Cd, 1,2-NQ, 1,4-NQ, 1,4-benzoquinone, crotonaldehyde, lead (Pb), and acrylamide with RSS separately. In the study, Na_2S_2 was used as a model for RSS because H_2S_2 was reported to be endogenously produced (Akaike *et al*., 2017). We reacted Na_2S_2 with each environmental electrophile and determined its consumption rate in relation to the reaction concentration of the electrophile using fluorescent probes. Incubation of the Na_2S_2 with MeHg, Cd, 1,2-NQ, and 1,4-NQ consumed more than 80% of Na_2S_2 at electrophile concentrations of 20-40 μM (Fig. 1). Incubation with 1,4-benzoquinone and crotonaldehyde consumed approximately 80% of the Na_2S_2 at an electrophile concentration of 80 μM, and little consumption was detected at 40 μM. Pb and acrylamide did not consume any Na_2S_2 at the measured concentrations under these conditions. The findings suggest that there is a structure specificity for interaction of electrophiles with RSS.

Next, we examined the effect of combined exposure to these environmental electrophiles on consumption of RSS and cytotoxicity. Combined exposure to MeHg and Cd resulted in a higher Na_2S_2 consumption rate than single exposures (Fig. 2A), a finding that was supported by quantitative measurements of Na_2S_2 by LC-MRM-MS (Fig. 2B). These findings suggest that combined exposure to environmental electrophiles may enhance the consumption of RSS, including persulfide, resulting in cytotoxicity even at concentrations that would not cause toxicity in single exposures. Consistent with this notion, combined exposure to MeHg and Cd exacerbated concentration-dependent cellular toxicity in HepG2 cells over single exposures (Fig. 3A). Furthermore, combined exposure to MeHg, Cd, and 1,4-NQ resulted in a further increase in cytotoxicity in HepG2 cells over combined exposure

Relative intensity (% of control)

100	90	80	70	60	50	40	30	20	10	0

MeHg	Concentration	0 uM	2.5 μM	5 μM	10 μM	20 μM	40 μM
	Na2S2 (%)	100	95	89	67	32	10
Cd	Concentration	0 μM	2.5 μM	5 μM	10 μM	20 μM	40 μM
	Na2S2 (%)	100	95	90	70	4	4
1,4-NQ	Concentration	0 μM	2.5 μM	5 μM	10 μM	20 μM	40 μM
	Na2S2 (%)	100	100	64	42	27	14
1,2-NQ	Concentration	0 μM	2.5 μM	5 μM	10 μM	20 μM	40 μM
	Na2S2 (%)	100	100	77	32	7	2
1,4-BQ	Concentration	0 μM	5 μM	10 μM	20 μM	40 μM	80 μM
	Na2S2 (%)	100	100	100	100	100	20
Crotonaldehyde	Concentration	0 μM	5 μM	10 μM	20 μM	40 μM	80 μM
	Na2S2 (%)	100	100	100	100	100	19
Pb	Concentration	0 μM	2 μM	20 μM	200 μM	2 mM	20 mM
	Na2S2 (%)	100	100	100	95	96	95
Acrylamide	Concentration	0 μM	40 μM	400 μM	4 mM	40 mM	400 mM
	Na2S2 (%)	100	100	100	100	100	100

Fig. 1. Consumption of persulfide by environmental electrophiles. Na_2S_2 (20 μM) was reacted with eight compounds at five concentrations for 15 min, and residual Na_2S_2 was then measured using a fluorescent probe to detect reactive sulfur species. Numbers indicate percentage changes in Na_2S_2 levels from control levels. MeHg, methylmercury; NQ, naphthoquinone; BQ, benzoquinone.

A

Relative intensity (% of control)

100	90	80	70	60	50	40	30	20	10	0

Combined exposure concentration		MeHg					
		0 μM	2.5 μM	5 μM	10 μM	20 μM	40 μM
Cd	0 μM	100	93	89	67	32	10
	2.5 μM	95	94	87	53	32	5
	5 μM	90	94	85	59	33	2
	10 μM	90	92	87	52	5	1
	12 μM	80	89	68	29	2	0
	16 μM	76	77	56	25	1	0

B

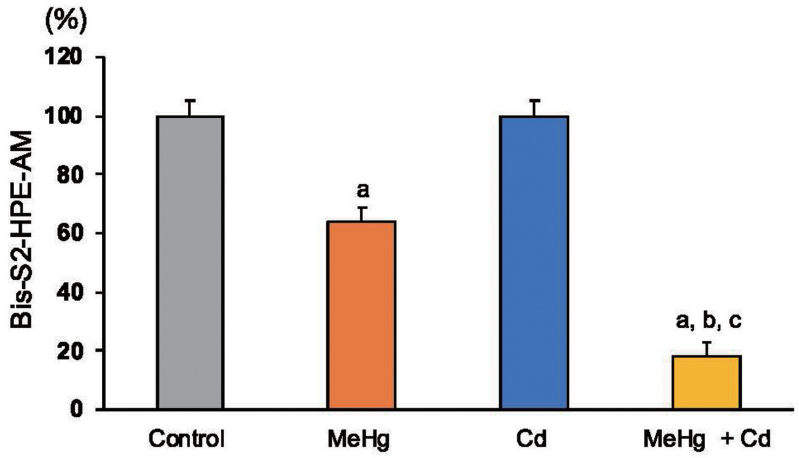

Fig. 2. Combined exposure to environmental electrophiles enhances consumption of persulfide. (A) Na_2S_2 (20 μM) was reacted with a combination of methylmercury (MeHg) and Cd at various concentrations for 15 min, and residual Na_2S_2 was then measured using a fluorescent probe to detect reactive sulfur species. Numbers indicate percentage changes in Na_2S_2 levels from control levels. (B) Na_2S_2 (40 μM) was reacted with MeHg (20 μM) and/or Cd (10 μM) for 15 min, and Na_2S_2 was then measured as bis-S_2-HPE-AM by LC-ESI-MS/MS following labeling with β-(4-hydroxyphenyl)ethyl iodoacetamide. Data are shown as mean (SD) of three independent experiments. [a]$p < 0.01$ compared with control; [b]$p < 0.01$ compared with MeHg; [c]$p < 0.01$ compared with Cd. Statistical significance was assessed by one-way ANOVA followed by Tukey's post-hoc test.

to MeHg and Cd or MeHg and 1,4-NQ (Fig. 3B). These findings indicate that cytotoxicity is exacerbated in proportion to the number of environmental electrophiles in the exposure. In other words, increasing the number of electrophiles in the exposure lowers the threshold of cytotoxicity.

Toxicological and pharmacological studies typically investigate the effects of exposure to a single substance, a premise that not mimic real-world situations or take into account the exposome, a measure of all environmental factors over time and their effects on health (Wild, 2005). Full characterization of the exposome remains a challenge owing to an enormous number of substances and the difficulty of evaluating their single and combined biological effects (Siroux *et al*., 2016; Niedzwiecki *et al*., 2019). Our findings in the present study suggest that combined exposure to environmental electrophiles reduces cellular RSS levels, possibly exacerbating cellular sensitivity to these compounds. Changes in *in vivo* levels of RSS may therefore be an indicator of the biological effects of environmental pollution.

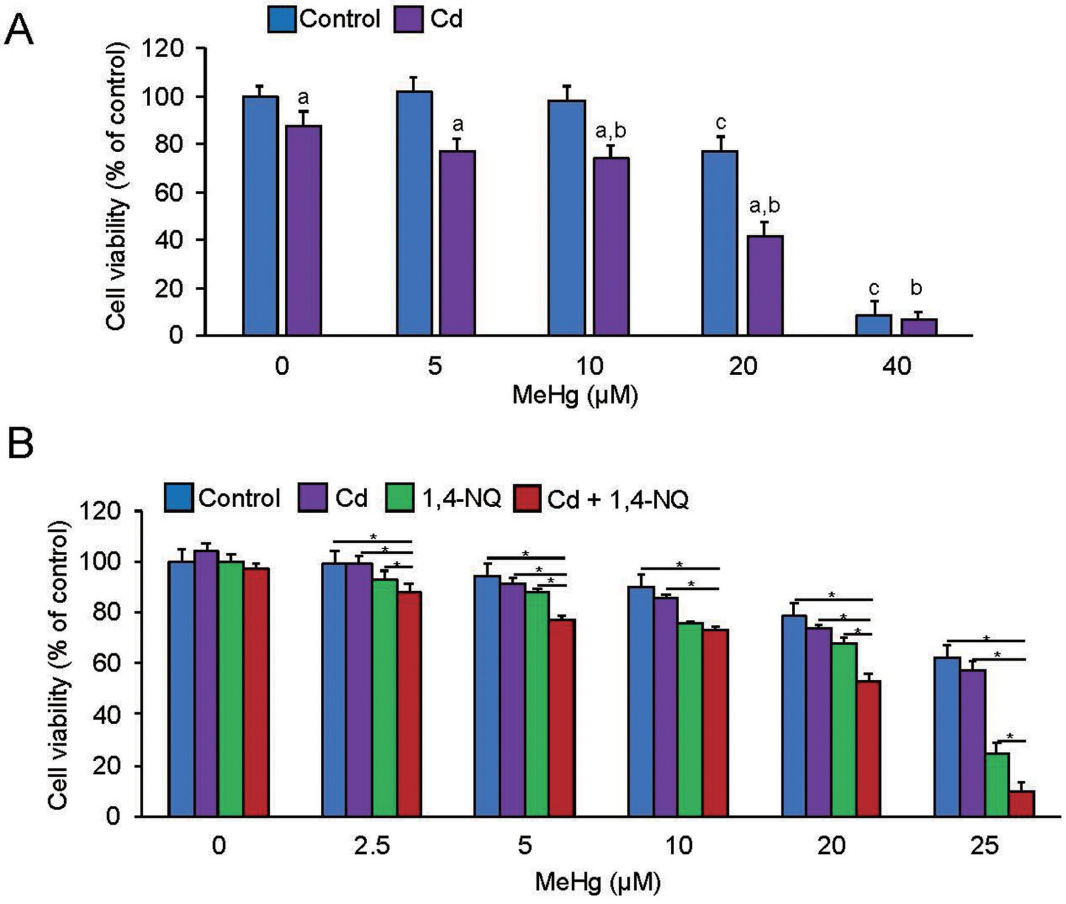

Fig. 3. Combined exposure to environmental electrophiles exacerbates concentration-dependent cytotoxicity. (A) HepG2 cells were exposed to methylmercury (MeHg) at four concentrations with or without 1 μM Cd for 24 hr, and were analyzed for cytotoxicity using the MTT assay. Each value is the mean ± SEM of six independent experiments. $^{a}p < 0.01$ compared with control; $^{b}p < 0.01$ compared with 0 μM MeHg plus Cd; $^{c}p < 0.01$ compared with 0 μM MeHg alone. Statistical significance was assessed by two-way ANOVA followed by Tukey's post-hoc test. (B) HepG2 cells were exposed to MeHg at five concentrations with or without Cd (0.8 μM) or 1,4-naphthoquinone (NQ, 15 μM) or both Cd (0.8 μM) and 1,4-NQ (15 μM) for 24 hr and assessed for cytotoxicity. Each value is the mean ± SEM of four independent experiments.*$p < 0.01$ compared with combined treatment with MeHg, Cd, and 1,4-NQ. Statistical significance was assessed by two-way ANOVA followed by Tukey's post-hoc test.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid (#18H05293 to Y.K.) for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We thank Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Abiko, Y., Sha, L., Shinkai, Y., Unoki, T., Luong, N.C., Tsuchiya, Y., Watanabe, Y., Hirose, R., Akaike, T. and Kumagai, Y. (2017a): 1,4-Naphthoquinone activates the HSP90/HSF1 pathway through the S-arylation of HSP90 in A431 cells: negative regulation of the redox signal transduction pathway by persulfides/polysulfides. Free Radic. Biol. Med., **104**, 118-128.

Abiko, Y., Shinkai, Y., Unoki, T., Hirose, R., Uehara, T. and Kumagai, Y. (2017b): Polysulfide Na2S4 regulates the activation of PTEN/Akt/CREB signaling and cytotoxicity mediated by 1,4-naphthoquinone through formation of sulfur adducts. Sci. Rep., **7**, 4814.

Akaike, T., Ida, T., Wei, F.Y., Nishida, M., Kumagai, Y., Alam,

M.M., Ihara, H., Sawa, T., Matsunaga, T., Kasamatsu, S., Nishimura, A., Morita, M., Tomizawa, K., Nishimura, A., Watanabe, S., Inaba, K., Shima, H., Tanuma, N., Jung, M., Fujii, S., Watanabe, Y., Ohmuraya, M., Nagy, P., Feelisch, M., Fukuto, J.M. and Motohashi, H. (2017): Cysteinyl-tRNA synthetase governs cysteine polysulfidation and mitochondrial bioenergetics. Nat. Commun., **8**, 1177.

Akiyama, M., Shinkai, Y., Unoki, T., Shim, I., Ishii, I. and Kumagai, Y. (2017): The Capture of Cadmium by Reactive Polysulfides Attenuates Cadmium-Induced Adaptive Responses and Hepatotoxicity. Chem. Res. Toxicol., **30**, 2209-2217.

Akiyama, M., Unoki, T., Shinkai, Y., Ishii, I., Ida, T., Akaike, T., Yamamoto, M. and Kumagai, Y. (2019): Environmental Electrophile-Mediated Toxicity in Mice Lacking Nrf2, CSE, or Both. Environ. Health Perspect., **127**, 67002.

Bohme, A., Laqua, A. and Schuurmann, G. (2016): Chemoavailability of Organic Electrophiles: Impact of Hydrophobicity and Reactivity on Their Aquatic Excess Toxicity. Chem. Res. Toxicol., **29**, 952-962.

Cho, A.K., Di Stefano, E., You, Y., Rodriguez, C.E., Schmitz, D.A., Kumagai, Y., Miguel, A.H., Eiguren-Fernandez, A., Kobayashi, T., Avol, E. and Froines, J.R. (2004): Determination of four quinones in diesel exhaust particles, SRM 1649a, an atmospheric PM2.5. Aerosol Sci. Technol., **38**, 68-81.

Denizot, F. and Lang, R. (1986): Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods, **89**, 271-277.

Gokmen, V. and Palazoglu, T.K. (2008): Acrylamide Formation in Foods during Thermal Processing with a Focus on Frying. Food Bioprocess Technol., **1**, 35-42.

Hanna, D.E., Solomon, C.T., Poste, A.E., Buck, D.G. and Chapman, L.J. (2015): A review of mercury concentrations in freshwater fishes of Africa: patterns and predictors. Environ. Toxicol. Chem., **34**, 215-223.

Hecht, S.S. (1999): Tobacco smoke carcinogens and lung cancer. J. Natl. Cancer Inst., **91**, 1194-1210.

Ihara, H., Kasamatsu, S., Kitamura, A., Nishimura, A., Tsutsuki, H., Ida, T., Ishizaki, K., Toyama, T., Yoshida, E., Hamid, H.A., Jung, M., Matsunaga, T., Fujii, S., Sawa, T. Nishida, M., Kumagai, Y. and Akaike, T. (2017): Exposure to Electrophiles Impairs Reactive Persulfide-Dependent Redox Signaling in Neuronal Cells. Chem. Res. Toxicol., **30**, 1673-1684.

Jan, A.T., Azam, M., Siddiqui, K., Ali, A., Choi, I. and Haq, Q.M. (2015): Heavy Metals and Human Health: Mechanistic Insight into Toxicity and Counter Defense System of Antioxidants. Int. J. Mol. Sci., **16**, 29592-29630.

Kanda, H., Shinkai, Y. and Kumagai, Y. (2014): S-mercuration of cellular proteins by methylmercury and its toxicological implications. J. Toxicol. Sci., **39**, 687-700.

Kumagai, Y., Akiyama, M. and Unoki, T. (2019): Adaptive Responses to Electrophilic Stress and Reactive Sulfur Species as their Regulator Molecules. Toxicol. Res., **35**, 303-310.

Li, H., Luo, N., Li, Y.W., Cai, Q.Y., Li, H.Y., Mo, C.H. and Wong, M.H. (2017): Cadmium in rice: transport mechanisms, influencing factors, and minimizing measures. Environ. Pollut., **224**, 622-630.

LoPachin, R.M. and Gavin, T. (2016): Reactions of electrophiles with nucleophilic thiolate sites: relevance to pathophysiological mechanisms and remediation. Free Radic. Res., **50**, 195-205.

Niedzwiecki, M.M., Walker, D.I., Vermeulen, R., Chadeau-Hyam, M., Jones, D.P. and Miller, G.W. (2019): The Exposome: molecules to Populations. Annu. Rev. Pharmacol. Toxicol., **59**, 107-127.

Rappaport, S.M. and Smith, M.T. (2010): Epidemiology. Environment and disease risks. Science, **330**, 460-461.

Rogers, L.K., Moorthy, B. and Smith, C.V. (1997): Acetaminophen binds to mouse hepatic and renal DNA at human therapeutic doses. Chem. Res. Toxicol., **10**, 470-476.

Saeed, M., Higginbotham, S., Rogan, E. and Cavalieri, E. (2007): Formation of depurinating N3adenine and N7guanine adducts after reaction of 1,2-naphthoquinone or enzyme-activated 1,2-dihydroxynaphthalene with DNA. Implications for the mechanism of tumor initiation by naphthalene. Chem. Biol. Interact., **165**, 175-188.

Shinkai, Y. and Kumagai, Y. (2019): Sulfane Sulfur in Toxicology: A Novel Defense System Against Electrophilic Stress. Toxicol. Sci., **170**, 3-9.

Siroux, V., Agier, L. and Slama, R. (2016): The exposome concept: a challenge and a potential driver for environmental health research. Eur. Respir. Rev., **25**, 124-129.

Wild, C.P. (2005): Complementing the genome with an "exposome": the outstanding challenge of environmental exposure measurement in molecular epidemiology. Cancer Epidemiol. Biomarkers Prev., **14**, 1847-1850.

Yoshida, E., Toyama, T., Shinkai, Y., Sawa, T., Akaike, T. and Kumagai, Y. (2011): Detoxification of methylmercury by hydrogen sulfide-producing enzyme in Mammalian cells. Chem. Res. Toxicol., **24**, 1633-1635.