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Joint Toxic Effects of the Type-2 Alkene Electrophiles

Lihai Zhang, Brian C. Geohagen, Terrence Gavin, and Richard M. LoPachin

Department of Anesthesiology (L.Z., B.C.G., and R.M.L.), Montefiore Medical Center, Albert Einstein College of Medicine, Bronx, NY and Department of Chemistry (T.G.) Iona College, New Rochelle, NY

Abstract

Human populations are exposed to complex environmental mixtures of acrolein, methylvinyl ketone (MVK) and other type-2 alkenes. Many members of this chemical class are electrophiles that possess a common molecular mechanism of toxicity; i.e., protein inactivation via formation of stable cysteine adducts. Therefore, acute or chronic exposure to type-2 alkene mixtures could represent a health risk due to additive or synergistic interactions among component chemicals. Despite this risk, there is little experimental information regarding the joint effects of type-2 alkenes. In the present study we used sum of toxic units ($TU_{sum} = \sum TU_i$) to assess the relative toxicity of different type-2 alkene mixtures. These studies involved well characterized environmental type-2 alkene toxicants and included amide (acrylamide; ACR), ketone (methyl vinyl ketone; MVK), aldehyde (2-ethylacrolein; EA) and ester (methyl acrylate; MA) derivatives. *In chemico* analyses revealed that both binary and ternary mixtures could deplete thiol groups according to an additive joint effect at equitoxic and non-equitoxic ratios; i.e., $TU_{sum} = 1.0 \pm 0.20$. In contrast, analyses of joint effects in SNB19 cell cultures indicated that different permutations of type-2 alkene mixtures produced mostly synergistic joint effects with respect to cell lethality; i.e., $TU_{sum} < 0.80$. A mixture of ACR and MA was shown to produce joint toxicity in a rat model. This mixture accelerated the onset and development of neurotoxicity relative to the effects of the individual toxicants. Synergistic effects in biological models might occur when different cellular proteomes are targeted, whereas additive effects develop when the mixtures encompasses a similar proteome.

Keywords

toxicant mixture; acrylamide; additive; synergistic; concentration addition; environmental toxicology

Corresponding author: Richard M. LoPachin, Ph.D. Department of Anesthesiology Montefiore Medical School 111 E. 210th St. Bronx, NY 10467, Fax: (718) 515-4903, Office: (718) 920-5054, lopachin@einstein.yu.edu.

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Conflict of Interests

The authors declare no conflicts of interests.

1. Introduction

Type-2 alkenes such as acrolein, acrylamide (ACR) and methyl vinyl ketone (MVK) have extensive industrial applications and, consequently, human occupational exposure is significant. In addition, these chemicals are recognized as environmental pollutants and dietary contaminants; e.g., acrolein from the combustion of petrochemical fuels and ACR in foods prepared at high temperatures. Type-2 alkenes are also prevalent components of cigarette smoke; e.g., acrolein, ACR and acrylonitrile. Acute or chronic exposure to these type-2 alkenes has been linked to major organ system toxicity and to possible carcinogenicity in humans and laboratory animals (Friedman, 2003; LoPachin et al., 2008a; Parzefall, 2008; Stedman, 1968; Stevens et al., 2008). In addition to environmental intoxication, acrolein, 4-hydroxy-2-nonenal (HNE) and certain other type-2 alkene derivatives are highly toxic by-products of membrane lipid peroxidation associated with cellular oxidative stress. Growing evidence indicates that these endogenous type-2 alkenes play a pathogenic role in diseases that have oxidative stress as a common molecular etiology; e.g., atherosclerosis, Alzheimer's disease, (Butterfield et al., 2010; Dejarnett et al., 2014; LoPachin et al., 2008b, 2009a). The type-2 alkenes are, therefore, an important class of chemicals with respect to environmentally acquired toxicity and endogenous disease pathogenesis.

Type-2 alkene derivatives are characterized by a conjugated α,β -unsaturated structure formed when an electron-withdrawing group (e.g., a carbonyl) is linked to an alkene (see Table 1). The electrons in these conjugated systems are polarizable and thus, such compounds are defined as "soft" electrophiles by the hard and soft, acids and bases theory (HSAB; Pearson, 1990; reviewed in LoPachin et al., 2012). According to this theory, soft electrophiles of the type-2 alkene chemical class will cause toxicity via a common molecular mechanism involving the formation of covalent adducts with soft nucleophiles, which in biological systems are functionally critical thiolate sulfhydryl groups on protein cysteine residues (LoPachin and Gavin, 2012; LoPachin and Gavin, 2014). Substantial experimental evidence now supports this proposed mutual mechanism (e.g., see Barber et al., 2004, 2007; LoPachin et al., 2007a,b, 2009b; Martyniuk et al., 2011, 2013; reviewed in LoPachin and Gavin, 2012, 2014).

As a consequence of both natural and anthropogenic production, type-2 alkenes have a significant environmental presence (e.g., see Bisesi, 1994; Faroon et al., 2008; LoPachin and Gavin, 2012; O'Brien et al., 2005). Human populations are, therefore, exposed to complex mixtures of type-2 alkenes, where the chemical compositions of these mixtures depend upon geographical location, occupation and personnel habits. Although individual environmental type-2 alkene pollutants likely exist at very low concentrations, exposures to mixtures of these chemicals might nonetheless represent a significant human health risk. Specifically, mixtures of type-2 alkenes could exhibit additive or even synergistic toxic effects due to the corresponding common molecular mechanism of action (Altenburger et al., 2003; Kortenkamp, 2007). Therefore, as an initial investigation of type-2 alkene mixture toxicity, we determined the relative abilities of amide (ACR), ketone (MVK), aldehyde (2-ethylacrolein) and ester (methyl acrylate) derivatives to interact. These chemicals exhibit a range of electrophilic reactivity that is quantitatively indicated by the ω values shown in

Table 1. In the absence of steric effects, these values are directly related to the toxic potencies of the electrophiles (reviewed in LoPachin and Gavin, 2012; reviewed in LoPachin and Gavin, 2014).

The sum of toxic units ($TU_{\text{sum}} = \sum TU_i$; see equations 1 and 2) has been widely used to describe the mixture toxicological effects at both equitoxic and non-equitoxic ratios (Cedergreen et al, 2008; Tian et al., 2013a,b). Therefore, our studies employed this method to characterize the potential joint toxicity of the type-2 alkenes. Our experimental design was based on a tiered approach that began with a simplified *in chemico* model to determine interactions at the acellular chemical level. In the next series of studies we defined mixture effects in a simplified biological model - cultured SNB19 glioblastoma cells. Our analyses suggested that the different type-2 alkenes could interact synergistically to produce joint toxic effects. Finally, we determined mixture effects in a mammalian model of subchronic intoxication. This system represents the penultimate model of biological complexity and provided toxicologically relevant evidence for the interactions of environmental chemical toxicants.

2. Materials and Methods

2.1 Chemicals and materials

All reagents were high-performance liquid chromatography grade or better and water was double distilled and deionized. Dimethyl sulfoxide (DMSO), propionaldehyde (PA), acrylamide (ACR), methyl acrylate (MA), 2-ethylacrolein (EA), methyl vinyl ketone (MVK), N-acetyl-L-cysteine (NAC), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma/Aldrich Chemical Company (Bellefonte, PA). Fetal bovine serum (FBS) and Roswell Park Memorial Institute (RPMI) medium and PrestoBlue® Cell Viability Reagent were purchased from Invitrogen (Grand Island, NY).

2.2 Calculations of HSAB Quantum Mechanical Parameters

To calculate Hard and Soft, Aacids and Bases (HSAB) parameters for the electrophiles involved in this study, energy values for the Highest Occupied Molecular Orbital (E_{HOMO}) and the Lowest Unoccupied Molecular Orbital (E_{LUMO}) were obtained using Spartan '14 (version 1.1.8) software (Wavefunction Inc., Irvine CA). For each chemical structure, ground state equilibrium geometries were calculated with Density Functional B3LYP 6-31G* in water starting from 6-31G* geometries. Global (whole molecule) hardness (η) was calculated as $\eta = (E_{\text{LUMO}} - E_{\text{HOMO}})/2$ and the index of electrophilicity (ω) was calculated as $\omega = \mu^2/2\eta$, where μ is chemical potential of the electrophile and $\mu = (E_{\text{LUMO}} + E_{\text{HOMO}})/2$.

2.3 Determination of individual toxicant EC₅₀ values: in chemico analyses

A simplified *in chemico* model (i.e., chemical reactions conducted in a buffer) was used to evaluate the joint effects of type-2 alkenes. This large class of electrophiles has a common mechanism of action involving the formation of adducts with protein cysteine residues (see Introduction). Therefore, we measured N-acetylcysteine (NAC) loss as an index of sulfhydryl adduct formation. NAC (100 μM) was incubated at 24°C in 96-well plates (gentle mixing) with graded concentrations of electrophiles (0.01 – 2000 mM) for 1 hour in Krebs-

HEPES buffer (200 μ L, pH 7.4, DMSO (20%). Following incubation, DTNB (final concentration of 480 μ M) was added to each well, including the NAC standard controls and plates, and incubated for an additional 5 min. Absorbance was read at 412 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). Results are expressed as mean % of control NAC $\pm$ SD ($n = 3-4$) calculated from a NAC standard curve with (5 – 100 μ M) by the following equation: $[\text{SH}]/100 \mu\text{M} \times 100$. EC₅₀ values and 95% confidence intervals for individual toxicants were determined by fitting the concentration-response data to a sigmoidal dose response (variable slope) equation by GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

2.4 Determination of individual toxicant EC₅₀ values: cell culture systems

To investigate joint chemical effects in a biological system, we characterized electrophile interactions in SNB19 glioblastoma cells using viability as an index of cell toxicity. Cells were treated with trypsin, resuspended and seeded (at 20000 cells/ml) in 96 well plates with 5% Roswell Park Memorial Institute (RPMI) solution containing 10% fetal bovine serum (50 ml), 100 U penicillin (5 ml), 0.1 mg/ml streptomycin. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% O₂. After 72 hrs, plated cells were incubated for 48 hrs with graded concentrations of electrophiles (0.01 – 1000 mM), and, at the conclusion of this incubation period, cell viability was determined using PrestoBlue® Cell Viability Reagent following manufacturer's instructions. Results were expressed as mean % of control viability $\pm$ SD ($n = 3-4$). EC₅₀ values and 95% confidence intervals for individual toxicants were determined by fitting the data to a sigmoidal dose response (variable slope) equation by GraphPad Prism 5.04 software (GraphPad Software, Inc., La Jolla, CA). Thus, the preceding studies in cell culture and *in chemico* systems determined the respective EC₅₀ values for individual toxicants. In the next series, we determined TU_{sum} of mixtures by varying toxicant ratios and experimentally deriving the EC_{50-mix} for the mixture of toxicant A and B.

2.5 Determination of mixture EC₅₀ values (EC_{50-mix}) and calculation of TU_{sum}

To evaluate joint toxicant effects in the *in chemico* and cell culture studies, the sum of toxic units at median toxicity (TU_{sum}) was determined. Among the variety of algorithms used to assess the interactions of toxicants, TU_{sum} is a reliable and hence widely used metric (Tian et al., 2013a; Parvez et al., 2009). TU_{sum} can be defined mathematically by the equation (1)

$$\text{TU}_{\text{sum}} = \frac{C_{50-A}}{\text{EC}_{50-A}} + \frac{C_{50-B}}{\text{EC}_{50-B}} \quad (1)$$

where C_{50-A} and C_{50-B} are the respective concentrations of toxicants A and B in a mixture at median toxicity. EC_{50-A} and EC_{50-B} are the EC₅₀ values for the individual toxicants A and B. TU_{sum} can be calculated using the experimentally derived EC₅₀ values for binary mixtures according to equation (2), where k₁ represents selected equitoxic (k₁ = 1) and non-equitoxic (k₁ $\neq$ 1) ratios of toxicants A and B in a mixture (e.g., Table 2; see also Altenburger et al., 2003; Tian et al., 2013a,b).

$$TU_{\text{sum}} = EC_{50-\text{mix}} \times (1+k_1) / (EC_{50-A} + k_1 \times EC_{50-B}) \quad (2)$$

Similarly, the TU_{sum} for ternary mixtures can be calculated using equation (3) where K_1 is a fixed ratio between toxicant A and toxicant B.

$$TU_{\text{sum}} = EC_{50-\text{mix}} \times (1+k_1+k_2) / (EC_{50-A} + k_1 \times EC_{50-B} + k_2 \times EC_{50-C}) \quad (3)$$

Correspondingly, k_2 is the ratio of toxicant A and toxicant C and the EC_{50-C} is the median effective inhibition concentration of chemical C. When $k_1 = k_2 = 1$, the mixture is at equitoxic ratio, whereas $k_1 \neq 1$ or $k_2 \neq 1$, the mixture is at non-equitoxic ratio (Cedergreen et al., 2012).

EC_{50} values and 95% confidence intervals for mixtures were determined by fitting the respective data to a sigmoidal concentration-response (variable slope) equation by GraphPad Prism 5.04 Software (GraphPad Software, Inc., La Jolla, CA). Mean log EC_{50} values ($\pm$ log variance) for the individual and mixture data were analyzed statistically using one-way analysis of variance (ANOVA) and a post-hoc Bonferroni's Multiple Comparison test (GraphPad Prism 5.04 Software, Inc.).

TU_{sum} reflects how toxicants in a binary or tertiary mixture interact. Additive interactions are described when $TU_{\text{sum}} = 1.00 \pm 0.20$, whereas for $TU_{\text{sum}} < 0.80$ synergistic effects are indicated (Tian et al. 2013a).

2.6 Binary toxicant interactions in an animal model

All aspects of this study were in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the Montefiore Medical Center animal care committee. Adult male rats (Sprague–Dawley, 250–275 g; Charles River, NY) were used and were housed individually in polycarbonate boxes. Drinking water and Purina Rodent Laboratory Chow (Purina Mills, Inc., St. Louis, MO) were available ad libitum. The animal room was maintained at approximately 22 °C and 50% humidity with a 12 h light/dark cycle. Rats (6–8 experimental per group) were randomly assigned to experimental groups that received either ACR at 18 mg/kg (0.253 mmol/kg) per day or MA at 40 mg/kg (0.465 mmol/kg) per day in drinking water. An additional experimental group received a mixture of the ACR and MA daily dose-rates in drinking water. The dose-rates used in this study were determined by preliminary range-finding studies (see also LoPachin et al., 2002). The control group received drinking water only. Daily water consumption was determined for all experimental and control groups and toxicant concentrations were adjusted correspondingly (see LoPachin et al., 2002 for additional details).

The onset and development of neurotoxicity induced by ACR and MA, individually and in combination, was measured using gait scoring and changes in body weight determined 2–3 times per week. Previous studies showed that, relative to hindlimb splay, forelimb and hindlimb grip strength and hindlimb extensor thrust, these parameters were highly sensitive

indices of neurotoxicity (LoPachin et al., 2002). Gait scores were analyzed separately using Friedman's repeated measure ANOVA on ranks. A trained, blinded observer who was not involved in animal care or toxicant administration performed the gait analyses. To assess ambulatory behavior in open field, rats were placed in a clear Plexiglas box (90 × 90 cm) and observed for 3 min. Observations were converted to numerical values ranging from 1 to 4. The assigned scores were: 1 = normal gait; 2 = a slightly abnormal gait (slight ataxia, hopping gait and foot splay); 3 = moderately abnormal gait (obvious ataxia and foot splay with limb abduction during ambulation) and 4 = severely abnormal gait (inability to support body weight and foot splay). The average ($\pm$ variance) per diem gain in body weight for animals in the different experimental groups was calculated and analyzed statistically using ANOVA and a post-hoc Bonferroni's Multiple Comparison test (GraphPad Prism 5.0 Software, Inc.).

3. Results

3.1 EC₅₀ values for individual toxicants and corresponding mixtures: in chemico analyses

Figure 1 shows the parallel concentration-dependent effects of individual toxicants on NAC sulfhydryl content. Results indicate the following rank order of individual EC₅₀ values (Table 1); i.e., MVK > EA > MA > ACR. However, this ranking does not correspond to the rank order of electrophilic values (ω ; Table 1); i.e., EA electrophilicity is greater than that of MVK, yet the corresponding EC₅₀ value is less than predicted. This lack of EA correspondence reflects the influence of steric hindrance on the adduct reaction. Specifically, type-2 alkenes have two sites of electrophilic reactivity; i.e., the carbonyl (C₁ carbon atom) and the alkenyl (C₃ carbon atom). Because ethyl acrolein (EA) is an aldehyde, it is less hindered than methylvinyl ketone (MVK) at the carbonyl site, but more hindered at C₃ due to the presence of the ethyl group at the 2 position. Prior studies by our group (Martyniuk et al., 2011) indicated that adduct formation between type-2 alkenes and nucleophilic thiolates (including protein thiolates) takes place at the conjugate site (C₃). Consequently, hindrance by the ethyl group of EA slows the adduct reaction relative to that of MVK which is completely unhindered at C₃. In biological conditions, sulfhydryl thiolates do not form stable adducts at the carbonyl site (C₁) on type-2 alkenes. Instead, the soft nucleophilic thiolate will react more favorably at the C₃ site of a soft electrophile rather than at the harder C₁ carbonyl site (for additional discussion see LoPachin et al., 2009).

Subsequent *in chemico* studies of various toxicant mixtures determined the respective EC_{50-mix} at equitoxic and non-equitoxic values of k_1 (see example in Fig. 2A). Results (Table 2) were used to calculate TU_{sum} values as per equation (2). Statistical analyses indicated that the EC_{50-mix} values for the binary mixtures were significantly different from the EC₅₀ values for individual toxicants. For all binary mixture permutations determined, the respective TU_{sum} values ranged between 0.80 and 1.09 indicating an additive chemical interaction. Analysis of an *in chemico* ternary mixture composed of MVK, MA and ACR (Table 3; Fig 3A) also revealed TU_{sum} values between 0.83 and 1.04 (Fig. 3B) indicating additive interactions among mixture components.

3.2 EC₅₀ values for individual toxicants and corresponding mixtures: cell culture analyses

Figure 4 shows the concentration-dependent effects of individual toxicants on SNB19 cell survival. To determine the interactive potential of electrophiles that are not type-2 alkenes, we evaluated the ability of propionaldehyde (PA) to interact with MVK and ACR. The rank order of the individual EC₅₀ values (MVK>EA>MA>ACR>PA) reflected a combination of electrophilic strengths and steric hindrance in the same fashion as was demonstrated by the *in chemico* experiments (see Subsection 3.1). The respective EC₅₀-mix concentrations were determined at equitoxic and non-equitoxic values of k_1 (see example in Fig. 5A) and were used to calculate TU_{sum} values as per equation (2). Statistical analyses indicated EC₅₀-mix values for the binary mixtures were significantly different from the EC₅₀ values for individual toxicants. The respective TU_{sum} values mostly indicated synergistic chemical interactions (TU_{sum} < 0.80). Exceptions were MA:ACR ($k_1 = 0.05$, TU_{sum} = 0.98) and MVK:ACR ($k_1 = 0.05$, TU_{sum} = 0.94).

3.3 Binary Interactions in an Animal Model

In this study, after 4 days of exposure to a mixture of MA (40 mg/kg/d) and ACR (18 mg/kg/d) in drinking water, rats in this group exhibited a significantly elevated mean gait score (arrow; Fig. 6A). The increase in this parameter was correlated to a significant reduction in weight gain (arrow, Fig. 6B). In contrast, rats in the respective single toxicant treatment groups did not exhibit significant changes in either mean gait scoring or weight gain at the 4 day time point (Figs. 6A and 6B). After 10 days of daily exposure, rats in the MA alone group exhibited a statistically significant increase in mean gait score (arrow, Fig. 6A) that was associated with a small decline in mean body weight (arrow, Fig. 6B). Also at this time point, ACR treated rats did not exhibit changes in mean gait score (Fig. 6A), but did show a significant decline in body weight gain (arrow, Fig. 6B). Neurotoxicity continued to progress in the mixture group (ACR + MA) as evidenced by the changes in mean gait score and weight gain following 10 days of exposure (Fig. 6). After 17 days of intoxication with ACR alone, rats exhibited significant increases in mean gait score (arrow, Fig. 6A) associated with decreases in weight gain (Fig. 6B). Neurotoxicity continued to develop for rats in the MA alone and mixture groups (Fig. 6). After an additional 8 days of intoxication (25 days total; dashed vertical line of Fig. 6), rats in the mixture group were incapable to reaching food and water and were removed from the study. In contrast, rats in the individual toxicant groups remained in the study for an additional 10 days. Experimental groups can also be compared on the basis of respective rates of weight gain. Thus, relative to control rates (6.4 ± 1.0 gm/day), mixture exposed animals exhibited a significantly reduced mean rate (2.4 ± 0.8 gm/day) of daily weight gain. The rate of weight gain in the combination group was also significantly slower when compared to either ACR or MA alone; i.e., 4.0 ± 0.8 gm/day and 5.0 ± 1.2 ; respectively. These data indicate that exposure to the MA/ACR mixture produced statistically significant neurotoxicity within 4 days, whereas exposure to either toxicant alone produced significant changes in measured parameters over a 10–17 day period.

4. Discussion

4.1 In chemico analyses of type-2 alkene interactions

In the present study we determined the joint effects of type-2 alkenes in a simplified *in chemico* system and in a biologically complex cell culture model. Results from both models indicated that mixtures of these soft electrophilic toxicants can produce joint toxic effects. The *in chemico* studies involved an initial analysis of individual EC₅₀ values for toxicant-induced sulfhydryl loss (Table 1). As noted in the Results, the rank order of type-2 alkenes for depletion of NAC sulfhydryl groups was, when steric hindrance was taken into account, consistent with respective electrophilicity values and hence, potencies (Table 1). *In chemico* determinations of mixture EC₅₀ values (EC_{50-mix}) and subsequent TU_{sum} calculations showed that different binary combinations of the selected type-2 alkenes produced additive joint effects at equitoxic and non-equitoxic ratios (k_1); i.e., $TU_{sum} = 1.0 \pm 0.20$ (Fig. 2B; Table 2). Our findings indicated that ternary mixtures also yielded additive joint effects (Fig. 3B; Table 3). As evident in Fig. 2A, the EC₅₀ value for the mixture (EC_{50-mix}) increased relative to the EC_{50-MA}, but decreased when compared to the EC_{50-ACR}. This phenomenon has been noted previously (Altenburger et al., 2003; Chen and Huang, 1996), and indicates that the composite effect of a mixture of chemicals that have a common mechanism but that differ with respect to activity (in this case electrophilicity) is, effectively, a serial dilution of the more active component. The additive effect found in the *in chemico* system is not surprising since all components examined are relatively soft electrophiles that undergo Michael addition reactions with a soft nucleophilic sulfhydryl target.

4.2 Analyses of type-2 alkene joint effects in a cell culture model

In contrast to the *in chemico* findings, analyses of type-2 alkenes in the cell culture model revealed that synergism was the predominant joint effect; i.e., $TU_{sum} < 0.80$. EC₅₀ values for the effects of individual toxicants on cell survival were initially determined (Fig. 4; Table 4) and, like the *in chemico* studies, the corresponding rank order of chemicals (MVK > EA > MA > ACR > PA) was provisionally consistent with the respective electrophilicity values (see Section 3.1). Subsequent determinations of mixture EC₅₀ values (EC_{50-mix}; Fig. 5A; Table 5) and TU_{sum} calculations (Fig. 5B) for the selected chemicals showed that different binary permutations produced synergistic joint effects except at very low values of k_1 which showed additive effects. Although the underlying reason for this ratio-dependent conversion is not known, it has been noted in previous mixture studies (e.g., see Lin et al., 2005; Tian et al., 2013a).

The molecular basis of the type-2 alkene synergistic joint effects might be related to differential targeting of proteomes in the SNB19 cells. Specifically, there is now substantial evidence indicating that aldehyde, ketone, ester and amide derivatives produce toxicity by inactivating protein components of electrophile responsive proteomes (Higdon et al, 2012; LoPachin and Barber, 2006; LoPachin and Gavin, 2014). The extent of proteome inhibition and resulting toxic outcomes are influenced by physicochemical characteristics that determine subcellular distribution and target protein accessibility; e.g., electrophilicity, metabolism, solubility and steric hindrance (LoPachin et al. 2012). Therefore, in combination, a weak soft electrophile such as ACR might have a correspondingly limited

effect on a proteome composed of cysteine-directed proteins, whereas a stronger electrophile mixture component (e.g., MVK) might impair a significantly larger proteome that includes the ACR-specific proteome (see Barber et al., 2004, 2007). Adduct formation among different proteomes is strongly indicated in the case of mixtures containing PA, since this aldehyde is not capable of Michael addition. Consequently, it does not form stable adducts with sulfhydryl groups but can undergo imine (Schiff base) formation with lysine residues.

4.3 Type-2 alkene interactions in an animal model

We have shown that a mixture of ACR and MA can produce joint toxicity in a cell culture model (Table 5). As the next step in biological complexity, we determined whether this chemical mixture could produce joint toxicity in a rat model. Although the type of interaction (additive or synergistic) was not derived, results nonetheless show that the interaction of individual type-2 alkenes (ACR and MA) in a mixture can accelerate the onset and development of neurotoxicity relative to the effects of the individual toxicants (Fig. 6). These animal data provide initial evidence that the preceding cell culture studies might represent a predictive model for identifying the potential joint effects of type-2 alkenes and other environmental chemical toxicants. We have previously proposed that exposure to relatively weak electrophiles such as ACR and MA (Table 1) produces selective neurotoxicity. This selectivity stems from the ability of these toxicants to avoid “adduct buffering” when administered systemically and to access the CNS where they form adducts with nerve terminal proteins that turnover slowly. The progressive accumulation of adduct-inactivated proteins results in cumulative neurotoxicity (reviewed extensively in LoPachin and Gavin, 2012; reviewed extensively in LoPachin and Gavin, 2014). Because ACR and MA are relatively weak electrophiles, it is possible that these toxicants affect a similar nerve terminal proteome (Barber et al., 2004, 2007) and thereby produce an additive joint effect. Data from the cellular studies (Table 5) support this supposition, since only borderline synergism was indicated for an equitoxic mixture of these two chemicals ($TU_{\text{sum}} = 0.75$ when $k_1 = 1$). This result is unlike that for mixtures of ACR with the more highly electrophilic MVK ($TU_{\text{sum}} = 0.46$) or EA ($TU_{\text{sum}} = 0.32$) where the synergistic effects were more pronounced.

4.4 Conclusions

Type-2 alkenes are prominent dietary, environmental and occupational toxicants (e.g., ACR, crotonaldehyde, methylvinyl ketone). Additionally, there is substantial evidence that members of this class are generated endogenously as a consequence of lipid peroxidation associated with many pathogenic processes (Butterfield et al., 2010; Dejarnett et al., 2014; LoPachin et al., 2008b; Luo et al., 2007). The data presented in this paper suggest that environmental exposure to a type-2 alkene mixture might represent a human health risk even though the individual constituents exist at very low concentrations. Furthermore, the potential joint effects resulting from the interaction of environmental and endogenous type-2 alkenes might accelerate the onset and development of disease processes. With respect to mechanisms of joint effects, it is noteworthy that soft electrophilic members of the type-2 alkenes have a common mechanism of toxicity involving the formation of Michael adducts with soft nucleophilic thiolate groups on functionally critical cysteine residues of enzymes and other proteins. Accordingly, we have proposed that the joint effects of type-2 alkene

mixtures that are composed of constituents with diverse electrophilic reactivities are mediated by the selective targeting of different electrophile responsive cellular proteomes. Although it is clear that human populations are exposed to chemical mixtures that likely include the type-2 alkenes and other toxic constituents, most research and governmental legislation is based on the toxicity of individual chemicals (Altenburger et al., 2003; Kortenkamp, 2007). This chemical-by-chemical approach does not recognize “real world exposure” conditions and can therefore underestimate toxic risk. Our data support the argument that the toxic potential of an environmental chemical should be considered within the context of possible mixture interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MVK	methylvinyl ketone
EA	2-ethylacrolein
MA	methyl acrylate
ACR	acrylamide
HNE	4-Hydroxy-2-nonenal
NAC	N-acetylcysteine
GSH	glutathione
HSAB	Hard and Soft, Acids and Bases
E_{LUMO}	Lowest Unoccupied Molecular Orbital energy
E_{HOMO}	Highest Occupied Molecular Orbital energy
PBS	phosphate buffered saline
DMSO	dimethyl sulfoxide
PA	propionaldehyde
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
FBS	fetal bovine serum
RPMI	Roswell Park Memorial Institute
EC₅₀	concentration producing 50% of total effect

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- Human populations are exposed to complex environmental mixtures of type-2 alkenes
- Members of this chemical class are electrophiles with a common toxic mechanism.
- Our findings indicate that type-2 alkenes can interact to cause joint toxicity
- Exposure to type-2 alkene mixtures could be a health risk due to additive/synergistic interactions

Figure 1. Concentration-response curves for depletion of NAC sulfhydryl groups by individual type-2 alkenes. These curves were used to calculate the respective EC_{50-toxicant} values and 95% confidence intervals for individual toxicants.

Figure 2.

(A) Concentration-response curves for a binary mixture of MA and ACR at equitoxic and non-equitoxic ratios (k_1). These curves were used to generate EC_{50-mix} and 95% confidence intervals. (B) The EC_{50-mix} and $EC_{50-toxicant}$ (Fig. 1) values were used in equation (2) to calculate corresponding TU_{sum} values presented in the toxic ratio-effect curve.

Figure 3.

(A) Concentration-response curves for a ternary mixture of MVK, ACR and MA at equitoxic and non-equitoxic ratios (k_1). These curves were used to generate EC_{50-mix} and 95% confidence intervals. (B) The EC_{50-mix} and $EC_{50-toxicant}$ (Fig. 1) values were used in equation (3) to calculate corresponding TU_{sum} values presented in the toxic ratio-effect curve.

Figure 4. Concentration-response curves for relative cell lethality caused by individual type-2 alkenes. These curves were used to calculate the respective EC_{50-toxicant} values and 95% confidence intervals for individual toxicants.

Figure 5.

(A) Concentration-response curves for a binary mixture of MVK and ACR at equitoxic and non-equitoxic ratios (k_1). These curves were used to generate $EC_{50}\text{-mix}$ and 95% confidence intervals. (B) The $EC_{50}\text{-mix}$ and $EC_{50}\text{-toxicant}$ (Fig. 4) values were used in equation (2) to calculate corresponding TU_{sum} values presented in the toxic ratio-effect curve.

Figure 6.

(A) Effects of oral ACR and/or MA on mean ($\pm$ SD) gait score in rats ($n=6-8$ per experimental). Animals were administered ACR (18 mg/kg/d) or MA (40 mg/kg/d) in drinking water alone or in combination. Age-matched control rats received drinking water only. When administered alone, ACR or MA caused cumulative toxicity as assessed by the temporal onset of gait abnormalities. However, the combination of ACR and MA produced significantly earlier increases in gait scores that rapidly approached recumbancy. (B) Effects of oral ACR and/or MA on mean ($\pm$ SD) rate of body weight gain. When administered alone, ACR or MA caused substantial slowing of weight gain. However, the combination of ACR and MA produced an initial truncation of weight gain (up to day 7), which was followed by a significantly slower rate of weight gain. The stipled vertical lines in 6A and 6B indicate the day (25) when the ACR/MA-treated rats were removed from the study. In both 6A and 6B, arrows indicate first experimental day at which mean data were statistically different from corresponding control. Succeeding datum points are statistically significant.

Table 1*In Chemico* Study: Concentration-Response and HSAB Parameters for Individual Toxicants

Toxicant	Structure	Electrophilicity (ω ; eV)	EC ₅₀ (mM)	Solubility (Mol/L)
methyl vinyl ketone (MVK)		3.38	0.108 (0.105–0.112)	5.0
2-ethylacrolein (EA)		3.62	2.21 (1.97–2.47)	0.1
methyl acrylate (MA)		3.22	12.9 (12.1–13.6)	0.6
acrylamide (ACR)		2.62	419 (404–436)	9.0
propionaldehyde		2.32	nd	3.5

Electrophilicity values were calculated for the *s-cis* conformation as shown in each case. EC₅₀-toxicant values for individual type-2 alkenes were calculated based on concentration-response curves in Figure 1 (values in parentheses are 95% confidence intervals).

Table 2

In Chemico Study: Determination of EC_{50-mix} and TU_{sum} for Binary Mixtures

Binary mixtures	k ₁	EC _{50-mix} (mM)	95% confidence intervals (mM)	TU _{sum}	Joint effects
MA : ACR (see Fig. 2A, 2B)	0.06	31	28 to 35	0.89	Additive
	1.1	224	213 to 234	0.99	
	5.9	331	313 to 350	0.92	
MVK : ACR	0.21	61	57 to 66	0.85	Additive
	1.4	226	218 to 234	0.92	
	7.2	314	299 to 326	0.85	
MVK : MA	0.06	0.81	0.76 to 0.85	0.95	Additive
	1.3	6.0	5.8 to 6.2	0.83	
	6.1	9.7	8.7 to 10.7	0.87	
EA : ACR	1.0	230	215 to 246	1.09	Additive
MVK : EA	1.2	1.04	0.97 to 1.10	0.89	Additive
EA : MA	1.0	6.0	5.7 to 6.4	0.80	Additive

Equitoxic and non-equitoxic ratios (k₁) of binary type-2 alkene mixtures were used to generate concentration-response curves (e.g., see Figure 2A) from which EC_{50-mix} and 95% confidence intervals were calculated. The EC_{50-mix} and EC_{50-toxicant} values were used in equation (2) to calculate corresponding TU_{sum} values (Figure 2B).

Table 3

In *Chemico* Study: Determination of EC_{50-mix} and TU_{sum} for Ternary Mixtures

Ternary mixtures	k ₁	k ₂	EC _{50-mix}	TU _{sum}	Joint effects
MVK : MA : ACR (see Fig. 3A)	4.5	5	4.3	0.99	Additive
	0.01	5	9.5	0.83	
	1	1	141	0.98	
	0.1	0.1	171	0.84	
	5	0.01	361	1.04	

Equitoxic and non-equitoxic ratios (k₁, k₂) of ternary type-2 alkene mixtures were used to generate concentration-response curves (e.g., see Figure 3A) from which EC_{50-mix} and 95% confidence intervals were calculated. The EC_{50-mix} (Table 3) and EC_{50-toxicant} values (Table 2) were used in equation (3) to calculate corresponding TU_{sum} values (Figure 3B).

Table 4

SNB19 Cell Culture: Concentration-Response Parameters for Individual Toxicants

Toxicant	Electrophilicity (ω , eV)	EC ₅₀ (mM)	95% confidence intervals (mM)
MVK	3.38	0.043	0.041 to 0.046
EA	3.62	0.11	0.097 to 0.120
MA	3.22	0.53	0.473 to 0.583
ACR	2.62	1.2	1.13 to 1.34
PA	2.32	13	10.2 to 16.6

EC₅₀-toxicant values for individual type-2 alkenes and corresponding 95% confidence intervals were calculated based on concentration-response curves in Figure 4.

Table 5

Cell Culture Study: Determination of EC_{50-mix} and TU_{sum} for Binary Mixtures

Binary mixtures	k ₁	EC _{50-mix} (mM)	95% confidence intervals (mM)	TU _{sum}	Joint effects
MA : ACR	0.05	0.550	0.512 to 0.605	0.98	Synergistic/Additive
	1	0.661	0.609 to 0.717	0.75	
	10	0.931	0.845 to 1.029	0.73	
MVK : ACR (see Fig. 5A, 5B)	0.05	0.093	0.089 to 0.099	0.94	Synergistic/Additive
	1	0.292	0.258 to 0.331	0.46	
	10	0.695	0.660 to 0.731	0.62	
EA: ACR	1	0.214	0.192 to 0.239	0.32	Synergistic
ACR : PA	1	2.73	2.40 to 3.11	0.38	Synergistic
MVK : PA	1	3.37	2.99 to 3.80	0.52	Synergistic

Equitoxic and non-equitoxic ratios (k₁) of binary type-2 alkene mixtures were used to generate concentration-response curves (e.g., see Figure 5A) from which EC_{50-mix} and 95% confidence intervals were calculated. The EC_{50-mix} and EC_{50-toxicant} values were used in equation (2) to calculate corresponding TU_{sum} values (Figure 5B).