Neurotoxic Mechanisms of Electrophilic Type-2 Alkenes: Soft–Soft Interactions Described by Quantum Mechanical Parameters

Richard M. LoPachin,*1 Terrence Gavin,† Brian C. Geohagen,* and Soma Das*

*Department of Anesthesiology, Albert Einstein College of Medicine, Montefiore Medical Center, 111 E. 210th St, Bronx, New York 10467; and †Department of Chemistry, Iona College, New Rochelle, New York 10804

Received March 7, 2007; accepted May 7, 2007

Conjugated Type-2 alkenes, such as acrylamide (ACR), are soft electrophiles that produce neurotoxicity by forming adducts with soft nucleophilic sulfhydryl groups on proteins. Soft–soft interactions are governed by frontier molecular orbital characteristics and can be defined by quantum mechanical parameters such as softness (σ) and chemical potential (μ). The neurotoxic potency of ACR is likely related to the rate of adduct formation, which is reflected in values of σ. Correspondingly, differences in μ, the ability of a nucleophile to transfer electrons to an electrophile, could determine protein targets of these chemicals. Here, σ and μ were calculated for a series of structurally similar Type-2 alkenes and their potential sulfhydryl targets. Results show that N-ethylmaleimide, acrolein and methylvinyl ketone were softer electrophiles than methyl acrylate or ACR. Softness (σ) was closely correlated to corresponding second-order rate constants (k2) for electrophile reactions with sulfhydryl groups on N-acetyl-L-cysteine (NAC). The rank order of softness was also directly related to neurotoxic potency as determined by impairment of synaptosomal function and sulfhydryl loss. Calculations of μ showed that the thiolate state of several cysteine analogs was the preferred nucleophilic target of alkenes electrophiles. In addition, μ was directly related to the thiolate rate constant (k) for the reaction of the Type-2 alkenes with the cysteine compounds. Finally, in accordance with respective μ values, we found that NAC, but not N-acetyl-L-lysine, protected synaptosomes from toxicity. These findings suggest that the neurotoxicity of ACR and its conjugated alkene analogs is related to electrophilic softness and that the thiolate state of cysteine residues is the corresponding adduct target.

Key Words: distal axonopathy; acrylamide; acrolein; Type-2 alkenes; adduct formation; nerve terminal; neurotoxicity.

Exposure of humans and laboratory animals to acrylamide (ACR) produces neurotoxicity characterized by initial inhibition of neurotransmission followed by nerve terminal degeneration (Lehnig et al., 2002a,b; 2003; LoPachin et al., 2004, 2006, 2007; see also LoPachin et al., 2002, 2003). ACR is an α,β-unsaturated carbonyl derivative (Fig. 1A) and is, therefore, classified as a conjugated Type-2 alkene. Chemicals in this class are defined by a conjugated system composed of an electrophilic group linked to an alkene carbon (Kemp and Vellaccio, 1980). Since the pi electrons in a conjugated structure are highly polarizable, the α,β-unsaturated carbonyl of ACR is a soft electrophile. Characteristically, soft electrophiles will preferentially form adducts with soft nucleophiles, which in biological systems are sulfhydryl groups on cysteine residues (Friedman, 2003; Hinson and Roberts, 1992; LoPachin and DeCaprio, 2005). Indeed, results from recent molecular and proteomic studies suggest that ACR selectively forms adducts with sulfhydryl groups on cysteine residues of several presynaptic proteins (Barber and LoPachin, 2004; LoPachin et al., 2004, 2007). Sulfhydryl addition, in turn, has significant neurotoxicological implications since the regulatory functions of certain synaptic proteins (e.g., SNAP-25, v-ATPase) are determined by the redox states of specific sulfhydryl groups (LoPachin and Barber, 2006). Accordingly, we have hypothesized that ACR alkylation of essential sulfhydryl groups impairs the function of presynaptic proteins that regulate neurotransmission (LoPachin et al., 2002, 2003).

In a recent in vitro study, we determined the structure–toxicity relationships for ACR and a series of α,β-unsaturated carbonyl analogs (Fig. 1B). Results showed that these conjugated Type-2 alkenes produced parallel, concentration-dependent decreases in synaptosomal function that were highly correlated (r2 ≥ 0.91) to reductions in corresponding sulfhydryl content. In contrast, nonconjugated alkene or aldehyde analogs (Fig. 1B) did not affect synaptosomal function or thiol content (LoPachin et al., 2007). Whereas the conjugated alkenes were equivalent with respect to in vitro neurotoxic efficacy (i.e., ability to produce a maximal effect), these toxicants differed significantly in relative potency. The marked differences in Type-2 alkene potency could be due to variations in the degree of pi electron polarizability or relative softness, which determines the ability (rate of reaction) of the conjugated alkenes to form adducts with soft nucleophilic sulfhydryl groups on proteins (Pearson and Songstad, 1967). Therefore, softness is likely to have significant implications for nerve terminal nucleophilic targets and...
mechanisms of Type-2 alkenes. The interaction of a soft electrophile with a nucleophile is governed by the shape and energy of the respective frontier molecular orbitals (Chattaraj, 2001). Consequently, the propensity of these chemicals to form adducts can be defined by quantum mechanical parameters such as hardness (η), softness (σ), and chemical potential (μ) (Chattaraj et al., 2006; Pearson, 1990). In the present study, we calculated these and other quantum mechanical descriptors for ACR and the structurally related Type-2 alkenes used in the present study (left column). Also shown are the nonconjugated structural analogs used (right column).

MATERIALS AND METHODS

Chemicals and materials. Unless otherwise indicated, all reagents were high performance liquid chromatography grade or better and water was doubly distilled and deionized. ACR (99% purity), N-ethylmaleimide (NEM), acrolein, methylvinyl ketone (MVK), methyl acrylate (MA), N-acetyl-L-cysteine (NAC), N-acetyl-L-lysine (NAL), Krebs-Henseleit buffer, and Percoll were purchased from the Sigma/Aldrich Chemical Company (Bellefonte, PA). 3H-DA (specific activity 23.5 Ci/mmol) was obtained from American Radiolabeled Chemicals (St Louis, MO). Whatman GF/F filter paper and Whatman GF/B filter disc were purchased from the Brandel Company (Gaithersburg, MD).

Animals. All aspects of this study were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the Montefiore Medical Center Animal Care Committee. Adult male rats (Sprague-Dawley, 300–325 g; Taconic Farms, Germantown, NY) were used in this study. Rats were housed individually in polycarbonate boxes, and 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.

Preparation of striatal synaptosomes. The striatum was chosen as a source for synaptosomes based on our previous research (LoPachin et al., 2006, 2007). Rat brain striatal synaptosomes were isolated by the Percoll gradient method of LoPachin et al. (2004). In brief, bilateral striata (100–120 mg wet wt. tissue) were rapidly removed and minced in cold (4°C) buffer containing sucrose 0.32M, ethylenediaminetetraacetic acid 1mM and dithiothreitol (SED; 0.25mM, pH 7.4). Tissue was gently homogenized in SED buffer (10 passes in a Teflon-glass homogenizer; 700 RPM) and the resulting homogenate was centrifuged at 1000 × g (10 min, 4°C). The pellet (P1) was washed once and supernatants (S1 and S2) were combined. Protein content of the pooled supernatant was determined by the Bradford assay using bovine serum albumin as standard. The protein concentration of the supernatant was adjusted to 5 mg/ml and then layered on top of a freshly prepared 4-step discontinuous Percoll gradient (3%, 10%, 15%, and 23% Percoll in SED, pH 7.4). Gradients were centrifuged at 32,000 × g for 6 min and synaptosomes were collected at the last interface (15%/23%). Synaptosomes were washed twice in Krebs buffer containing NaCl 140mM, KCl 5mM, NaHCO3 5mM, MgCl2 1mM, Na2HPO4 1.2mM, glucose 10mM, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) 10mM (pH 7.4), pelleted and then resuspended.

Synaptosomal membrane transport. Striatal synaptosomes (10 μg protein) were incubated with graded concentrations of toxicant or Krebs–Hepes buffer for 15 min at 30°C (LoPachin et al., 2004). Synaptosomes were then washed, filtered trapped by rapid filtration through a cell harvester (see above) and superfused (3 min) with Krebs–Hepes buffer containing [3H]DA (0.30μM). To correct for low affinity Na+ independent transport, uptake was measured in the presence and absence (equimolar choline chloride substitution) of sodium ions. Synaptosomes were then washed and corresponding radioactivity was measured by scintillation counting. To investigate nucleophilicity as a determinant of type-2 alken amino acid targets, synaptosomes were preincubated (1 min) with either NAC (500μM) or NAL (500μM) and then exposed (15 min) to graded concentrations of NEM, acrolein, or MVK. In these studies, NAL was chosen for comparison with NAC because (1) the pKa of NAL is comparable to that of NAC; (2) the lysine side chain of NAL is a primary amine and therefore is structurally comparable to the primary thiol of cysteine; and (3) data exist suggesting lysine as a target for ACR, acrolein, and MVK (e.g., see Kaminskas et al., 2005; Uchida et al., 1998). Synaptosomal uptake of [3H]DA was determined as described above. The concentration–response data for transport were fitted by nonlinear regression analysis and the IC50’s (95% confidence intervals) were calculated by the Cheng–Prusoff equation (Prism, GraphPad Software, San Diego, CA).

Measurement of free sulfhydryl groups. The concentration-dependent effects of Type-2 alken analogs on total free sulfhydryl content in synaptosomes were determined by the method of LoPachin et al. (2004). Following incubation (15 min) with graded concentrations of toxicant or control buffer solutions, synaptosomes (200 μg protein) were solubilized with 1% sodium dodecyl sulfate (5 min). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB; 3mM) was added and, following equilibration (5 min, 25°C), absorbance was read at 412 nm using FIG. 1. (A) This figure illustrates the conjugated α,β-unsaturated carbonyl structure of chemicals in the Type-2 alken class. (B) This figure presents the corresponding line structures for ACR and the structurally related Type-2 alkenes used in the present study (left column). Also shown are the nonconjugated structural analogs used (right column).
**Quantum mechanical parameters:** lowest unoccupied molecular orbital, highest occupied molecular orbital, $\eta$, $\mu$, $\sigma$, $\omega$, and $\omega'$. The lowest unoccupied molecular orbital (LUMO) energy ($E_{\text{LUMO}}$) and highest occupied molecular orbital (HOMO) energy ($E_{\text{HOMO}}$) were calculated using Spartan04 (version 1.0.3) software (Wavefunction Inc., Irvine, CA). Single-point energies for each structure were calculated at the density functional level of theory using a B3LYP-6-31G* basis set from 6-31G* geometries. Global (whole molecule) hardness ($\eta$) was calculated as $\eta = (E_{\text{LUMO}} - E_{\text{HOMO}})/2$ and chemical potential ($\mu$) was calculated as $\mu = (E_{\text{LUMO}} + E_{\text{HOMO}})/2$ (Chattaraj et al., 2006; Madjarova et al., 2005). Softness ($\sigma$) is used as defined in Pearson (1990) and Maynard et al. (1998) and is calculated as the inverse of hardness or $\sigma = 1/\eta$. The electrophilicity index ($\omega$) was calculated as $\omega = \mu^2/2\eta$ (Chattaraj et al., 2006) and the nucleophilicity index ($\omega'$) as $\omega' = \eta_A/(\mu_A - \mu_B)^2/(\eta_A - \eta_B)^2$, where $A$ = reacting nucleophile and $B$ = reacting electrophile (Jaramillo et al., 2006). Quantum mechanical parameters were computed for both s-cis and s-trans conformers for all Type-2 alkenes. Since these two conformations exist in equilibrium and all combinations of data gave essentially the same results (i.e., correlation coefficient), the values used (Tables 1–3) represent the best fit to the experimental data. Linear regression analysis was used to assess the relationship between the calculated quantum mechanical parameters and neurotoxic potency (IC$_{50}$’s; Table 1) or kinetic data ($k$, $k_2$; Tables 1 and 3). Corresponding coefficients of determination ($r^2$) were calculated from the Pearson correlation coefficient (InStat 3.0, GraphPad Software) and are provided in the text.

**Determination of rate constants.** Rate constants for the reactions of Type-2 alkenes with cysteine sulfhydryl groups were calculated for comparisons with quantum mechanic parameters. Sulfhydryl-containing compounds were incubated with a molar excess of a given Type-2 alkene at either pH 7.4 or 8.8 and sulfhydryl concentrations were determined over time by the DTNB method (see above). For each Type-2 alkene–sulfhydryl analog combination, the corresponding graph of $\log [\text{SH}]/[\text{H}]$ versus time ($\text{SH} = $ concentration of sulfhydryl at time $t$; $\text{H} = $ initial concentration at $t_0$) was straight (regression analysis; $r^2$ range = 0.87–0.99) indicating that the reaction followed pseudo-first order kinetics (see example provided in Fig. 3). Pseudo-first order rate constants ($k_1$) were derived directly from these graphs and second-order rate constants ($k_2$) were calculated according to Friedman et al. (1965). The rate constant ($k$) for the reaction of sulfhydryl thiolates with the Type-2 alkenes was calculated from the previously described relationship: $\log (k - k_2) = \log k_1 + pK_a - pH$ (Friedman et al., 1965; Whitesides et al., 1977).

**Results**

**Calculated Softness of Type-2 Alkenes and their Nonconjugated Analogs**

To determine how electrophilic softness was related to sulfhydryl reactivity, we compared selected quantum mechanical parameters to kinetic data. The LUMO energies ($E_{\text{LUMO}}$) and the HOMO energies ($E_{\text{HOMO}}$) for the Type-2 alkenes and their nonconjugated analogs (Fig. 1B) were calculated using commercially available software (Spartan04; Wavefunction, Inc.). The calculated energy values were then used to compute the softness ($\sigma$) of each electrophile (Table 1). Softness is considered the ease with which electron redistribution takes place during covalent bonding and thus, the softer the electrophile, the more readily it will form an adduct by accepting outer shell electrons from a nucleophile such as the sulfur atom. Based on the respective $\sigma$ values presented in Table 1, NEM, acrolein, and MVK are softer electrophiles than MA and ACR. To establish the relationship between $\sigma$ and sulfhydryl reactivity, we determined the respective second-order rate constant ($k_2$) for the reactions of Type-2 alkenes with the sulfhydryl group of NAC. Results (Table 1) show that ACR and MA have very slow reaction rates relative to the

**Table 1**

<table>
<thead>
<tr>
<th>Conjugated alkenes</th>
<th>$E_{\text{LUMO}}$ (eV)</th>
<th>$E_{\text{HOMO}}$ (eV)</th>
<th>$\sigma$ (eV)</th>
<th>$\omega$ (eV)</th>
<th>$\omega'$ (eV)</th>
<th>$k_2$ (log IC$_{50}$) (pH = 7.4)</th>
<th>$k_2$ (log IC$_{50}$)</th>
<th>$k_2$ (log IC$_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEM</td>
<td>2.36</td>
<td>7.30</td>
<td>0.406</td>
<td>4.73</td>
<td>2.170</td>
<td>4.60</td>
<td>4.33</td>
<td></td>
</tr>
<tr>
<td>Acrolein</td>
<td>1.70</td>
<td>6.98</td>
<td>0.379</td>
<td>3.57</td>
<td>0.332</td>
<td>4.01</td>
<td>4.28</td>
<td></td>
</tr>
<tr>
<td>MVK</td>
<td>1.33</td>
<td>6.71</td>
<td>0.372</td>
<td>3.00</td>
<td>0.037</td>
<td>3.57</td>
<td>3.48</td>
<td></td>
</tr>
<tr>
<td>MA</td>
<td>1.01</td>
<td>7.36</td>
<td>0.315</td>
<td>2.76</td>
<td>1.893</td>
<td>1.10</td>
<td>0.336</td>
<td></td>
</tr>
<tr>
<td>ACR</td>
<td>0.69</td>
<td>6.77</td>
<td>0.329</td>
<td>2.30</td>
<td>3.651</td>
<td>0.44</td>
<td>0.359</td>
<td></td>
</tr>
<tr>
<td>Nonconjugated analogs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propanal</td>
<td>– 0.33</td>
<td>– 6.858</td>
<td>0.307</td>
<td>1.981</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Allyl alcohol</td>
<td>+ 0.51</td>
<td>– 6.933</td>
<td>0.269</td>
<td>1.386</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

\(^*\)Second-order reaction rates ($k_2$) were determined for Type-2 alkene interactions with NAC at pH 7.4 and pH 8.8 (n = 4–6 experiments).

\(^*\)Sulfhydryl (–SH) loss was determined in Type-2 alkene exposed striatal synaptosomes (n = 4–6 experiments).

\(^*\)Inhibition of $^3$H-DA uptake was determined in Type-2 alkene exposed striatal synaptosomes (n = 4–6 experiments).
kinetics of NEM and acrolein. Furthermore, $\sigma$ was well correlated to $\log k_{2}$ ($r^2 \geq 0.81$), which indicated the degree of softness (increasing $\sigma$ value) determined the ability (rate of reaction) of the conjugated alkenes to form adducts with soft nucleophilic sulphydryl groups.

At the molecular level, the rate of sulphydryl adduct formation will determine the neurotoxic potency of the Type-2 alkenes. Therefore, the relationship of $\sigma$ to several *in vitro* neurotoxic parameters was defined. Results (Table 1) show that the rank order of softness was directly related to corresponding neurotoxic potency (IC50); i.e., the softest electrophiles (NEM, acrolein, MVK) were the most potent (lowest IC50 values) conjugated alkenes with respect to depletion of synaptosomal thios and impairment of function, whereas the relatively harder MA and ACR were least potent (higher IC50 values). Regression analysis showed that the $\sigma$ values for the Type-2 alkenes were highly correlated to the log of the respective IC50's for synaptosomal uptake inhibition ($r^2 = 0.93$) and sulphydryl loss ($r^2 = 0.91$). In contrast, the nonconjugated analogs had lower $\sigma$ values and, correspondingly, did not cause synaptosomal toxicity (Table 1).

Studies investigating aromatic compounds have used quantum mechanical indicators other than softness (e.g., $E_{\text{LUMO}}$) as determinants of toxicity (Cronin *et al.*, 2001). Additionally, recent chemical research has suggested that an “electrophilic index” ($\omega$), which combines softness with chemical potential, is a good descriptor of reactivity (Chattaraj *et al.*, 2001; Parr, 1999). In our study, respective $E_{\text{LUMO}}$ for the Type-2 alkenes was highly correlated to kinetic measurements ($r^2 = 0.93$), but somewhat less correlated ($r^2 = 0.83$) to thiol loss and only qualitatively ($r^2 = 0.75$) related to uptake inhibition (Table 1). Likewise, calculated $\omega$ values (Table 1) were well correlated ($r^2 = 0.87$) to the kinetic data and qualitatively related with neurotoxic potency ($r^2 = 0.74$ for thiol loss, $r^2 = 0.64$ for uptake inhibition). Overall, our results suggest that $\sigma$, which describes the property of electrophilic softness, is a better descriptor for the interactions of Type-2 alkenes with their soft nucleophilic targets; i.e., sulphydryl groups.

### Calculations of Chemical Potential ($\mu$) and the Nucleophilic Index ($\omega$) for Putative Cysteine Targets

Soft electrophiles, such as the Type-2 alkenes, preferentially form adducts with soft nucleophiles, which in biological systems are primarily sulphydryl groups on cysteine residues (see introductory section). Free sulphydryl groups can exist in the thiol or thiolate state. To investigate the activities of these sulphydryl states in the formation of adducts with the Type-2 alkenes, we calculated the relevant orbital energy values ($E_{\text{HOMO}}$) for a series of cysteine analogs in the thiol and thiolate states. These values were used to compute the chemical potential ($\mu$), which is the ability of a nucleophilic species to transfer electrons, or more precisely, electron density to the electrophile. Values of $\mu$ are independent of pH and reflect the inherent electronic nature of the structural moiety upon which the computations are based (i.e., the thiol–thiolate structures shown in Fig. 2). The molecular orbital energy was also used to calculate $\omega$, which is a recently developed “index of nucleophilicity” (Jaramillo *et al.*, 2006). Regardless of the quantum mechanical parameter considered ($\mu$ or $\omega$), the respective values for the cysteine analogs show that the sulphydryl group in the thiol state (Table 2) is considerably less capable of transferring electron density and is, therefore, less nucleophilic than the corresponding thiolate state (Table 3). This indicates that the thiolate state is a better nucleophile than the thiol state and is the preferred target for alkene electrophiles. As demonstrated in Equation 1, the thiolate concentration is determined by $K_a$ and contributions from

### TABLE 2
Calculated Quantum Mechanical Parameters for Cysteine Analogs in the Thiol State

<table>
<thead>
<tr>
<th>Thiol</th>
<th>$E_{\text{LUMO}}$ (eV)</th>
<th>$E_{\text{HOMO}}$ (eV)</th>
<th>$\mu_{\text{thiol}}$ (eV)</th>
<th>$\omega$ (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys ester (+ 1)</td>
<td>-4.45</td>
<td>-11.00</td>
<td>-7.73</td>
<td>0.644</td>
</tr>
<tr>
<td>Cys (0)</td>
<td>0.14</td>
<td>-5.87</td>
<td>-2.87</td>
<td>0.031</td>
</tr>
<tr>
<td>GSH (- 1)</td>
<td>2.85</td>
<td>-2.29</td>
<td>0.28</td>
<td>0.701</td>
</tr>
<tr>
<td>NAC (- 1)</td>
<td>4.56</td>
<td>-1.35</td>
<td>1.61</td>
<td>1.700</td>
</tr>
</tbody>
</table>

*For each cysteine analog, the corresponding ionization state is presented in parentheses (see Fig. 2).

### TABLE 3
Calculated and Experimental Parameters for Cysteine Analogs in the Thiolate State

<table>
<thead>
<tr>
<th>Thiolate</th>
<th>$pK_a$</th>
<th>$E_{\text{LUMO}}$ (eV)</th>
<th>$E_{\text{HOMO}}$ (eV)</th>
<th>$\mu$ (eV)</th>
<th>$\omega$ (eV)</th>
<th>Log $k_2$ (pH = 7.4)</th>
<th>Log $k$ (pH = 7.4)</th>
<th>Log $k_2$ (pH = 8.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cys ester (0)</td>
<td>6.53</td>
<td>-0.08</td>
<td>-4.19</td>
<td>-2.14</td>
<td>0.11</td>
<td>-2.21</td>
<td>-2.15</td>
<td>-1.77</td>
</tr>
<tr>
<td>Cys (- 1)</td>
<td>8.15</td>
<td>4.76</td>
<td>-0.35</td>
<td>2.21</td>
<td>1.44</td>
<td>-2.50</td>
<td>-1.67</td>
<td>-1.15</td>
</tr>
<tr>
<td>GSH (- 2)</td>
<td>8.56</td>
<td>6.68</td>
<td>2.08</td>
<td>4.41</td>
<td>2.65</td>
<td>-2.80</td>
<td>-1.61</td>
<td>-1.93</td>
</tr>
<tr>
<td>NAC (- 2)</td>
<td>9.52</td>
<td>8.39</td>
<td>3.58</td>
<td>5.99</td>
<td>3.83</td>
<td>-3.65</td>
<td>-1.53</td>
<td>-1.62</td>
</tr>
</tbody>
</table>

*For each cysteine analog, ionization state is presented in parentheses (see Fig. 2). Second-order rate constants ($k_2$) for the interaction of ACR with the cysteine compounds were determined experimentally ($n = 4–6$ experiments). The $k_2$ values at pH 8.8 were corrected for the corresponding thiolate concentration for each cysteine analog according to the algorithm: $\log (k - k_2) = \log k_2 + pK_a - \text{pH}$. 

Expressed as $E_{\text{LUMO}}$ and $E_{\text{HOMO}}$, these parameters describe the electronic properties of the cysteine analogs. The $\mu$ (intrinsic chemical hardness) and $\omega$ (stability index) values are calculated using quantum mechanical methods and provide a quantitative measure of the electrophilic and nucleophilic properties, respectively. The $pK_a$ values indicate the dissociation of the thiolate to the thiol form. Overall, these results provide insight into the mechanisms of electrophilic attack on sulphydryl groups and the factors that influence their reactivity.
If, as the calculated nucleophilic descriptors suggest, the thiolate state is the preferred Type-2 alkene target, this should be reflected in corresponding chemical reaction rates. The thiol–thiolate equilibrium of the cysteine analogs is a function of respective pK_a’s (range 6.53–9.54; see Table 3) and, therefore, second-order rate constants (k_2) were determined at pH 7.4 and pH 8.8. For each nucleophile at pH 8.8, the thiolate concentration will increase relative to that at pH 7.4. If the thiolate is the preferred adduct target, the respective reaction rate for ACR and a given cysteine analog will increase in proportion to the enlarging thiolate concentration as determined by the pK_a. For example, the pK_a of cysteine ethyl ester is 6.53 and, therefore, at pH 7.4, about 88% of sulfhydryl groups will be in the thiolate state versus 99% at pH 8.8. This differential will equate to a relatively small change in velocity. In contrast, the pK_a of NAC is 9.52 and, at pH 7.4, slightly less than 1% of sulfhydryl groups are in the thiolate form, whereas at pH 8.8, 16% are in the thiolate state. This larger change in thiolate concentration will lead to a correspondingly large increase in rate. Accordingly, results show that at pH 8.8 the k_2 values for ACR with the selected cysteine analogs were considerably increased relative to pH 7.4 (Fig. 3; Table 3; see also Cavins and Friedman, 1968; Friedman et al., 1965). Corroborative studies showed that this pH-dependent rate increase occurred for all Type-2 alkenes evaluated with NAC (data not shown). When the k_2 rates of reaction were corrected for the thiolate concentration [log (k _2) = log k _2 + pK_a – pH], the resulting thiolate rate constants (k; Table 3) were directly related to the respective chemical potentials; i.e., μ values were highly correlated to log k (r^2 = 0.94). Similarly, ω^- was well correlated to log k (r^2 = 0.82). Our combined data provide

FIG. 2. Thiol and thiolate structures for each cysteine analog are presented and corresponding ionization states are provided in parentheses.

RSH (thiol) as a nucleophile can be neglected (see also Whitesides et al., 1977).

\[
RSH \xrightarrow{K_a} H^+ + RS \xrightarrow{k} RS + x \quad (1)
\]
evidence that the sulfhydryl thiolate state is the preferred target for the conjugated alkene neurotoxicants.

Application of Quantum Mechanical Descriptors to In Vitro Neurotoxicity

In this series of studies, we determined the relative abilities of NAC (500 μM) and NAL (500 μM) to prevent the in vitro neurotoxicity of Type-2 alkenes; i.e., inhibition of 3H-DA membrane transport and loss of total free thiols in rat striatal synaptosomes. Results indicate that NAC, but not NAL, reduced Type-2 alkene in vitro neurotoxicity. This is illustrated in Figures 4 and 5, which show that the respective IC50s derived from the concentration curves for acrolein-, NEM-, and MVK-induced in vitro neurotoxicity were significantly increased by NAC. In contrast, the addition of NAL did not influence the neurotoxic expression of these Type-2 alkenes. This is consistent with our quantum mechanical computations, since NAL (μ = 1.8, pKa = 9.6) would be a predictably poor nucleophile (compare with NAC: μ = 6.0, pKa = 9.5) and under these conditions the free amino group would be present only in low concentration (~0.5%). Based on our σ calculations (Table 1), ACR and MA are weaker electrophiles and, in accordance with mass action kinetics, require higher concentrations of NAC [a good nucleophile, but present in low (~1%) thiolate concentration at pH = 7.4] for adduct buffering. Accordingly, we found that 500 μM NAC did not affect the induction of in vitro neurotoxicity by either ACR or MA. However, 100–500 mM NAC potentiated the neurotoxicity induced by these toxicants, whereas NAL at the same concentration range did not influence the development of in vitro neurotoxicity (data not shown). In separate studies, these higher in vitro concentrations of NAC were found to be neurotoxic, which precluded further studies with ACR and MA.

DISCUSSION

We have recently shown that ACR, acrolein, and other structurally similar α,β-unsaturated carbonyl derivatives...
produced concentration-dependent synaptosomal dysfunction accompanied by parallel decreases in free sulfhydryl content (LoPachin et al., 2007). Although these Type-2 alkenes were equivalent with respect to in vitro neurotoxic efficacy (i.e., ability to produce a maximal effect), they differed significantly in relative potency. This means that although higher concentrations of ACR (and MA) were needed to drive the in vitro adduct reaction, once formed, these adducts produced neurotoxic consequences that were comparable to those caused by other Type-2 alkene adducts. The marked differences in potency could be due to variations in the degree of pi electron polarizability or softness among the Type-2 alkenes. Softness reflects the ability (rate of reaction) of the conjugated alkenes to form adducts with soft nucleophilic sulfhydryl groups (Pearson and Songstad, 1967). Therefore, relative electrophilic softness has significant implications for in vivo molecular mechanisms of Type-2 alkenes, their sites of action, and nucleophilic targets. Nucleophilic softness is determined by the polarizability of valence electrons. Accordingly, the softest biological nucleophiles are sulfhydryl groups on cysteine residues. This is because sulfur has a relatively large atomic radius with highly polarizable valence electrons. Amino groups on lysine or histidine residues are also nucleophilic and are potential sites for Type-2 alkene adduction. However, these groups are harder nucleophiles (less polarizable) and, consequently, are less favorable targets (reviewed in LoPachin and DeCaprio, 2005). According to the Frontier Orbital Theory in its simplest form, adduct formation occurs when the soft nucleophile donates its highest energy electrons into the empty LUMO of the soft electrophile (Chattaraj, 2001). Therefore, the most relevant frontier orbital for electrophiles is presumably the LUMO, whereas the HOMO is most important for nucleophiles. The propensity of an electrophile and a nucleophile to form an adduct should, therefore, be predictable based on knowledge of the relevant orbital energies (\(E_{\text{LUMO}}, E_{\text{HOMO}}\)). Accordingly, quantum mechanical parameters that include these energies in their respective algorithms might be reliable descriptors of electrophile–nucleophile interactions (Pearson, 1990); e.g., hardness (\(\eta\)), softness (\(\sigma\)), chemical potential (\(\mu\)), and the recently developed higher order parameters such as indices of electrophilicity (\(\omega\); Chattaraj et al., 2006) and nucleophilicity (\(\omega^-\); Jaramillo et al., 2006). In the present study, we calculated these parameters for a congener series of \(\alpha,\beta\)-unsaturated carbonyls and their potential sulfhydryl targets.

To determine how electrophilic softness might be related to the induction of neurotoxicity, we calculated \(E_{\text{LUMO}}, E_{\text{HOMO}}\), \(\sigma\), and \(\omega\) for several Type-2 alkenes and their nonconjugated structural analogs (Table 1). These and other molecular descriptors have been used in previous structure–toxicity studies of \(\alpha,\beta\)-unsaturated derivatives to identify reactive toxicants and putative mechanisms of action (e.g., Maynard et al., 1998; Schultz et al., 2005, 2006). Here we found softness (\(\sigma\)) to be most important. Based on the respective values for \(\sigma\), NEM and acrolein were determined to be softer electrophiles than MA and ACR. The corresponding rank order of softness was directly related to the neurotoxic potency order for these chemicals. Accordingly, NEM, acrolein and MVK produced synaptosomal neurotoxicity at micromolar concentrations,
whereas ACR and MA required higher in vitro concentrations (mM) to drive reactions in the direction of sulfhydryl adduct formation (see respective IC50’s of Table 1). The pi electrons of nonconjugated alkene (alkyl alcohol) or aldehyde systems (propanal) are not mobile and are, instead, restricted to the alkene or carbonyl functions, respectively. The carbonyl carbon of the aldehyde is a relatively hard electrophile and neither it nor a nonconjugated alkene like alkyl alcohol functions as a Michael acceptor for thiol addition. This is consistent with their corresponding quantum mechanical descriptors and, predictably, these structural controls did not alter synaptosomal transport or free thiol content (Table 1). Our calculations of σ therefore suggest that the in vitro neurotoxic potencies of the Type-2 alkene electrophiles are directly related to their respective abilities to form sulfhydryl adducts (softness).

Covalent bonding is involved in the soft–soft interactions that occur when electrophiles form adducts with nucleophilic targets. As stated previously, this type of chemical bonding is mediated by the transfer of highest energy electrons from the soft nucleophile into the empty lowest energy orbital of the soft electrophile (Chattaraj, 2001). The chemical potential (µ) of a nucleophile is a measure of how readily electron density can be donated to the soft electrophile acceptor (Maynard et al., 1998; Pearson, 1990). Therefore, calculations of µ should be a viable measure of relative nucleophilicity. Based on the respective µ values, cysteine thiolis (Table 2) are less capable of transferring electrons than the corresponding thiolate state (Table 3). Our analyses also showed that for each cysteine nucleophile the respective µ values were closely correlated to the ACR rate data, provided that the second-order rate constants (k2) were corrected (k) to reflect the actual concentration of thiolate (see Table 3). These data confirm and extend prior experimental work (Friedman et al., 1965; Whitesides et al., 1977) and demonstrate that the thiolate state is significantly more nucleophilic and, therefore, the preferred target for conjugated alkene electrophiles (see ahead). Our findings also demonstrate that the cysteine thiolate state is a softer nucleophile than lysine. Thus, the µ values for Cys, glutathione (GSH), and NAC (range = 2.21–5.99; Table 3) were substantially higher than that of NAL (µNAL = 1.8), indicating that cysteine thiolate groups were better nucleophiles than the nonionized amine group of lysine.

If target nucleophilicity plays a role in Type-2 alkene neurotoxicity in vitro, then the cysteine analogs in Table 3 should modify in vitro Type-2 alkene neurotoxicity by acting as sulfhydryl adduct buffers. NAL, as a harder nucleophile with an extremely low concentration of the free amino groups at physiological pH (see above), is a less favorable target and should, consequently, have less impact on neurotoxicity. To test this hypothesis, we determined the relative abilities of NAC and NAL to prevent Type-2 alkene neurotoxicity in vitro. Our findings (Figs. 3 and 4) show that NAC, but not NAL, significantly reduced the loss of sulfhydryl groups and synaptosomal dysfunction induced by exposure to the α,β-unsaturated carbonyls. This confirms the neurotoxicological relevance of the sulfhydryl group nucleophilic-state and is consistent with previous research demonstrating a neuroprotective role for NAC in Type-2 alkene neurotoxicity (Arakawa et al., 2006; Benz et al., 1990; however, see Wispryono et al., 1999).

How do differences in the chemical potential (µ) of nucleophilic sulfhydryl targets and the relative softness of electrophiles relate to in vivo mechanisms and sites of action? In the first case, we have proposed that soft electrophilic neurotoxicants preferentially form adducts with highly nucleophilic thiolate sites within cysteine catalytic triads of functionally critical nerve terminal proteins such as NEM sensitive factor (NSF; LoPachin and Barber, 2006; LoPachin et al., 2007). This supposition is supported by our observation that the thiolate state of sulfhydryl groups is a better nucleophile than the parent thiol (compare respective µ values in Tables 2 and 3) and is, therefore, the preferred target of Type-2 alkenes. Furthermore, our findings suggest that softness is likely to be a key determinant of the toxicological manifestations of these electrophiles. Specifically, systemic toxicity (e.g., respiratory, hepatic) is the most notable consequence of environmental exposure to very soft, highly reactive electrophiles such as acrolein and MVK (e.g., see Morgan et al., 2000; Saillenfait et al., 1999). It is probable that a large systemic thiolate sink (both protein and nonprotein) and inherent electrophile defense mechanisms functionally limit the in vivo disposition of these chemicals (e.g., see He et al., 1998). In contrast, ACR, which is recognized as a significant neurotoxicant in humans and experimental animals (Gold and Schaumburg, 2000; LoPachin et al., 2003), is a harder electrophile and, therefore, reacts more slowly with thiolate sites (Table 1). The slower addition rate for ACR (and probably MA) reduces buffering by systemic sulfhydryl groups and, thereby, proportionately expands tissue disposition to the central nervous system. Weaker electrophiles such as ACR will nonetheless slowly react with protein thiolate sites throughout the body, which seems inconsistent with our findings that nerve terminals are a primary site of action (see introductory section). However, nerve terminals are highly vulnerable to electrophile-induced damage since many resident proteins have exceptionally slow turnover rates and are, therefore, susceptible to cumulative addition and dysfunction (see Barber and LoPachin, 2004). Furthermore, nerve terminals are spatially separated from the cell body and, as a result, repair/replacement processes are delayed and transcription-based electrophile defense mechanisms such as the Keap1/Nrf2 pathway are not available (reviewed in LoPachin and Barber, 2006; LoPachin et al., 2002, 2003).

In summary, our previous proteomic studies have identified cysteine sulfhydryl groups as selective targets for adduction by ACR and other Type-2 alkene congeners (Barber and LoPachin, 2004; LoPachin et al., 2007). The present findings show that the selective interactions of these putative neurotoxicants with
their protein sulphydryl targets are governed by the relative softness (σ) of the nucleophile, the chemical potential (μ) of the nucleophile and the existing concentrations of biologically available nucleophilic species (e.g., thiol vs. thiolate vs. amino groups). Whereas all of the calculated descriptive parameters derived from quantum mechanical theory provided at least qualitative agreement, our data suggest that σ and μ are primary determinants of adduct formation and the subsequent induction of Type-2 alkenne neurotoxicity. Calculation of relative electrophile softness (σ) might, therefore, be predictive of the neurotoxic risk potential associated with occupational/environmental exposure to ACR and other related conjugated Type-2 alkenes. In addition, the approach used here could be used to characterize the chemical interactions of other neurotoxic electrophiles (e.g., organophosphate insecticides, hex- carbon solvents) and their nucleophilic targets (e.g., serines, lysines, respectively).

FUNDING

National Institutes of Health grant from the National Institute of Environmental Health Sciences (ROI ES03830-20).

ACKNOWLEDGMENTS

The authors would like to express their sincere thanks to Dr Joseph Ross (Ross Toxicology) for his helpful comments and criticisms during the preparation of the manuscript.

REFERENCES


