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Molecular Mechanisms of 4-Hydroxy-2-nonenal and Acrolein Toxicity: Nucleophilic Targets and Adduct Formation

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Acrolein and 4-hydroxy-2-nonenal (HNE) are byproducts of lipid peroxidation and are thought to play central roles in various traumatic injuries and disease states that involve cellular oxidative stress, for example, spinal cord trauma, diabetes, and Alzheimer's disease. In this review, we will discuss the chemical attributes of acrolein and HNE that determine their toxicities. Specifically, these aldehydes are classified as type 2 alkenes and are characterized by an α,β-unsaturated carbonyl structure. This structure is a conjugated system that contains mobile π-electrons. The carbonyl oxygen atom is electronegative and can promote the withdrawal of mobile electron density from the β-carbon atom causing regional electron deficiency. On the basis of this type of electron polarizability, both acrolein and HNE are considered to be soft electrophiles that preferentially form 1,4-Michael type adducts with soft nucleophiles. Proteomic, quantum mechanical, and kinetic data will be presented, indicating that cysteine sulfhydryl groups are the primary soft nucleophilic targets of acrolein and HNE. This is in contrast to nitrogen groups on harder biological nucleophiles such as lysine or histidine residues. The toxicological outcome of adduct formation is not only dependent upon residue selectivity but also the importance of the targeted amino acid in protein function or structure. In attempting to discern the toxicological significance of a given adduct, we will consider the normal roles of cysteine, lysine, and histidine residues in proteins and the relative merits of corresponding adducts in the manifestations of diseases or toxic states. Understanding the molecular actions of acrolein and HNE could provide insight into many pathogenic conditions that involve initial cellular oxidative stress and could, thereby, offer new efficacious avenues of pharmacological defense.

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1. Introduction

There is now general agreement that the pathogeneses of many diseases, xenobiotic intoxications, and traumatic injuries (e.g., Alzheimer's disease, spinal cord injury, and alcoholism) are characterized by a common pathophysiological cascade involving cellular oxidative stress and membrane lipid peroxidation (*1*−*5*). The peroxidative destruction of membrane lipids has direct toxic effects on the structural integrity of cellular membranes. In addition, the fragmentation of polyunsaturated fatty acids during lipid peroxidation generates highly electrophilic α,β-unsaturated carbonyl derivatives including acrolein, 4-hydroxy-2-nonenal (HNE), and 4-oxononenal (ONE) (*6*−*8*). These lipid byproduct are capable of modifying nucleophilic side chains on amino acid residues (Cys, His, Arg, and Lys) primarily through 1,4-Michael type conjugate reactions (e.g., see refs *1*, *9*−*12*, *15*, and *16*). That the generation of these electrophilic aldehydes and subsequent adduction of protein nucleophiles might have pathophysiological significance is evidenced by the elevated tissue levels of HNE, acrolein, and their respective protein adducts in disease processes that involve cellular oxidative damage (e.g., see refs *17*−*21* reviewed in refs *14* and *22*). Furthermore, the formation of adducts by these reactive aldehydes has been linked to numerous cytotoxic consequences including the disruption of cell signaling, inhibition of enzyme activity, and mitochondrial dysfunction (e.g., see refs *23*−*25* reviewed in ref *26*). Finally, substantial research indicates that protein adduction, as opposed to the depletion of glutathione and other cellular reducing equivalents, is the primary mechanism of acrolein and HNE toxicity (reviewed in refs *16* and *27*−*30*).

Thus, a large database suggests that the liberation of acrolein, HNE, and other α,β-unsaturated aldehyde derivatives during

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membrane peroxidation could mediate, at least in part, many disease processes that involve cellular oxidative stress. However, despite the obvious pathogenic relevance of these aldehydes, a detailed understanding of their cytotoxicities is missing. Clearly, the corresponding molecular mechanism is a multistep process that is initiated by the covalent interaction of an electrophilic aldehyde, such as acrolein, and a target nucleophile, such as a cysteine sulfhydryl group. The rate of this reaction is dependent upon the electrophilic reactivity of the aldehyde and the corresponding receptivity of the nucleophile. The consequences of adduct formation at the protein level (e.g., enzyme inhibition or altered tertiary structure) can then lead to defective cellular processes (e.g., reduced energy metabolism and loss of cytoskeletal structure) and eventual cytotoxicity. However, it is not known whether such toxicity involves adduction of multiple amino acids or the selective targeting of a specific residue. The mechanistic relationship between amino acid adduct formation and subsequent cellular damage also has not been established. Finally, it is not clear whether these toxicants act via a common molecular mechanism or whether different mechanisms are involved on a cell- and/or disease-specific basis. The goal of this review is to address these data gaps. To accomplish this, we will discuss how electrophilicity determines the relative abilities of acrolein and HNE to form adducts with different biological nucleophiles. We will also discuss how the role of a given amino acid residue in protein function determines the toxicological relevance of subsequent adduct formation. On the basis of our interpretation, we propose a unified mechanistic hypothesis of aldehyde toxicity; that is, acrolein and HNE, like other conjugated α,β-unsaturated carbonyl derivatives, produce cytotoxicity by forming Michael type adducts with highly nucleophilic sulfhydryl thiolate groups on cysteine residues of functionally critical proteins. Because acrolein and other structurally related chemicals [e.g., acrylamide (ACR), acrylonitrile, and methylvinyl ketone (MVK)] are significant environmental pollutants, we will also present the possibility that exogenous exposure to these toxicants can accelerate the onset and development of diseases mediated by endogenous aldehyde generation.

2. Nucleophilic Targets and Adduct Chemistry of Conjugated α,β-Unsaturated Carbonyl Derivatives

2.1. Physiochemical Principles Governing Electrophile−Nucleophile Interactions. Acrolein and HNE are α,β-unsaturated carbonyl derivatives, and as such, they are classified as type 2 alkenes (Figure 1; see also ref *31*). Most chemicals in this class are composed of an alkene (a carbon to carbon double bond) linked to an electron-withdrawing group, which in this case is a carbonyl group (carbon to oxygen double bond). The resulting structure (an α,β-unsaturated carbonyl) is a conjugated system that contains mobile outer shell π-electrons. Normally, an alkene functional group is electron rich. However, the combination of polarizable mobile electrons and the electron-withdrawing capacity of the carbonyl group creates an area of electron deficiency at the alkene β-carbon atom of the α,β-unsaturated carbonyl derivatives (see Figure 2 for a detailed explanation). Therefore, acrolein, HNE, and other type 2 alkenes are electrophiles (electron-deficient species) that form adducts with nucleophiles (electron-rich species). Although notable exceptions exist, many toxicants are, in fact, electrophiles that covalently interact with biological nucleophilic targets (*32, 33*).

Electrophiles, however, do not simply react with nucleophiles; rather, such interactions occur along a continuum of relative reactivity. As explained in the following discussion, there is a

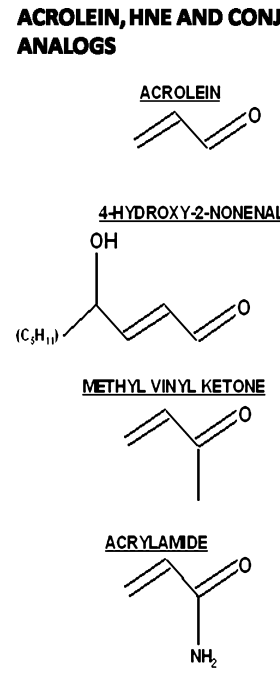

Figure 1. This figure presents line structures for acrolein, HNE, and several structurally related α,β-unsaturated carbonyl derivatives of the type 2 alkene chemical class.

significant degree of selectivity in electrophile−nucleophile interactions, which is predicted by the hard and soft, acids and bases (HSAB) theory of Pearson (reviewed in refs *26*, *33*, and *34*). According to this theory, electrophiles and nucleophiles are classified as either relatively "hard" or "soft", based on inherent electronic characteristics. Hard electrophilic toxicants (e.g., chloroethylene oxide, dimethylnitrosamine) have high positive charge densities (carbocation) at their electrophilic centers and, consequently, these chemicals are characterized by low electron polarizability. In contrast, soft electrophiles (e.g., quinones) have low charge density and their electrons are highly polarizable. The α,β-unsaturated carbonyl structures of acrolein and HNE are, therefore, considered soft electrophiles based on the mobility of their corresponding *pi* electrons. Similarly, the softness of a nucleophile is determined by the polarizability of corresponding valence electrons. Sulfur has a large atomic radius with highly polarizable valence electrons and is the softest nucleophile in biological systems. In contrast, harder biological nucleophiles such as nitrogen and oxygen have small atomic radii and are highly electronegative with low electron polarizability. Based on the HSAB model, soft electrophiles preferentially form adducts with nucleophiles of comparable softness, whereas hard electrophiles form adducts with hard nucleophiles (reviewed in refs *26*, *32*, *34*, and *35*). This model therefore predicts that the preferred nucleophilic targets of acrolein, HNE and other type 2 alkenes are sulfur atoms, as opposed to harder nucleophiles such as nitrogen or oxygen. Consistent with the selectivity principle of this theory, the reaction rate between a soft electrophile and a hard nucleophile is expected to be relatively low (*36, 37*).

2.2. Quantum Mechanical Descriptors of Electrophile−Nucleophile Interactions. HSAB principles are grounded in the frontier molecular orbital (FMO) theory, which, in its most simplistic form, describes covalent bond formation as the overlap occurring exclusively between the respective outermost (frontier) orbitals of the reacting molecules. The FMO of the nucleophile consists of the highest energy orbital holding electrons, known by the acronym HOMO (highest occupied molecular orbital). In contrast, the FMO for the electrophile is the lowest energy

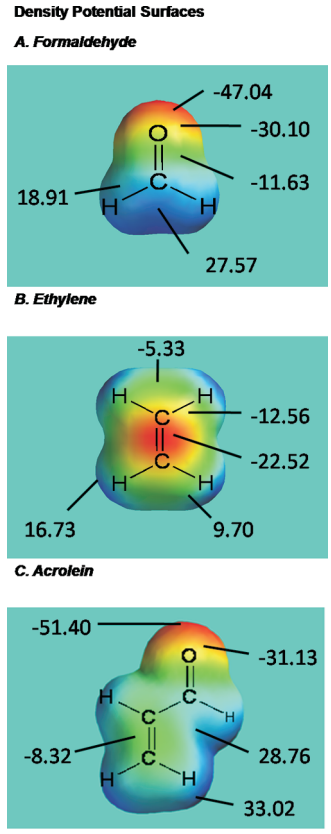

Figure 2. Concept of soft electrophilicity is illustrated in this figure, which shows the color-coded electrostatic potential maps for formaldehyde (A), ethylene (B), and acrolein (C; calculated using Spartan '04, Wavefunction Inc., Irvine, CA). The line structures for each chemical are inserted within the corresponding potential figure. The color gradient for each map illustrates how charge is distributed across the molecule and, therefore, indicates the relative degree to which the corresponding atoms attract oppositely charged atoms. Accordingly, red signifies the most negative electrostatic potential and is used for regions that attract positively charged molecules most strongly. Blue denotes areas with the most positive electrostatic potential and is used for regions that attract negatively charged molecules most strongly. The orange−yellow−green spectrum indicates intermediate (from negative to positive, respectively) electrostatic potential. For each chemical, a numerical example of local electron density or distribution (expressed as kJ/mol) is provided for each color. If we first consider the relatively simple polar covalent bond of formaldehyde (A), it is clear that the highly electronegative oxygen atom (Pauling electronegativity = 3.44) draws electron density as indicated by the localized red-colored zone (−47.04) from the less electronegative carbon (2.55) and hydrogen (2.20) atoms. Here, the resulting electron deficiency of the carbon−hydrogen bonds is reflected in the green−blue gradient, that is, the respective electron density from −11.63 to 27.57. We next consider the carbon−carbon double bond of ethylene (B). A red−yellow gradient (from −22.52 to −12.56, respectively) is centered over the double bond, which indicates the covalent sharing of electron density between two atoms (carbon) of equal electronegativity. Acrolein (C) combines the carbonyl of formaldehyde and the carbon−carbon double bond of ethylene, which is considered to be a conjugated system. As the corresponding red color coding indicates, the electronegative carbonyl oxygen atom has withdrawn electron density (−51.40) from the normally electron-rich carbon−carbon double bond (see the ethylene double bond; B). As a result, the β-carbon atom becomes an electron-deficient or electrophilic center (green = −8.32). Such electron delocalization is possible because the π-orbitals of the conjugated α,β-unsaturated carbonyl structure overlap. Consequently, the respective π-electrons are mobile or polarizable and can, therefore, relocate to the electronegative oxygen atom. The quantum mechanical parameter softness (σ) is an index of π-electron mobility, and on the basis of their respective σ values (Table 1), acrolein and HNE are relatively soft electrophiles that will rapidly form adducts with sulfhydryl groups.

Table 1. Calculated Quantum Mechanical Parameters for α,β-Unsaturated Carbonyl Derivatives and Nonconjugated Analogues[a]

	E_{LUMO} (eV)	σ (eV^{-})	ω (eV)
conjugated alkenes			
NEM	−2.36	0.406	4.73
acrolein	−1.70	0.379	3.57
HNE	−1.53	0.381	3.29
MVK	−1.33	0.372	3.00
MA	−1.01	0.315	2.76
ACR	−0.69	0.329	2.30
nonconjugated analogues			
propanal	−0.33	0.307	1.98
allyl alcohol	+0.51	0.269	1.39

[a] The LUMO energy (E_{LUMO}) and HOMO energy (E_{HOMO}) were calculated using Spartan04 (version 1.0.3) software (Wavefunction Inc.). Global (whole molecule) hardness (η) was calculated as $\eta = (E_{LUMO} - E_{HOMO})/2$, and softness ($\sigma$) was calculated as the inverse of hardness or $\sigma = 1/\eta$. The electrophilicity index (ω) was calculated as $\omega = \mu^2/2\eta$, where μ is the chemical potential of the electrophile and was calculated as $\mu = (E_{LUMO} + E_{HOMO})/2$ (for details, see ref *42*).

orbital that is vacant or LUMO (lowest unoccupied molecular orbital). Covalent bonding, such as adduct formation, occurs when the nucleophile donates high-energy HOMO electrons into the empty LUMO of the electrophile. Hence, the propensity of an electrophile and a nucleophile to form adducts should be predictable if knowledge of the relevant frontier orbital energies is available. Values for these FMO energies have been derived from computer-based quantum mechanical calculations and are the basis of algorithms used to compute several previously mentioned HSAB parameters, for example, hardness [$\eta = (E_{LUMO} - E_{HOMO})/2$]. These quantitative parameters have been demonstrated to be reliable descriptors of electrophile−nucleophile interactions (*33, 34, 38−40*).

Softness (σ), defined as the inverse of hardness or $\sigma = 1/\eta$, measures the ease with which electron redistribution takes place during covalent bonding. Therefore, with respect to electrophilic species, the softer the electrophile (i.e., higher σ value), the more readily it will form adducts by accepting electrons from a nucleophile. The electrophilic index ($\omega = \mu^2/2\eta$) is an important higher order parameter that combines softness ($1/\eta$) with chemical potential [$\mu = (E_{LUMO} + E_{HOMO})/2$] and, it is believed, represents a more sensitive measure of electrophilic reactivity. In previous studies, these quantum mechanical parameters were calculated for a series of structurally related type 2 alkenes (*41−43*) to determine how softness and electrophilicity were related to the induction of nerve terminal (synaptosomal) toxicity. The data presented in Table 1 show that, among the type 2 alkenes tested, *N*-ethylmaleimide (NEM) is the strongest electrophile; that is, both σ and ω are numerically larger (more positive) than those of other class members. On the basis of their respective quantum mechanical parameters, ACR and methyl acrylate (MA) are substantially weaker electrophiles, whereas acrolein, MVK, and HNE have intermediate electrophilic reactivity. In this type 2 alkene series, relative differences in electrophilicity were determined by the substituent functional groups and their contribution to the electron density of the α,β-unsaturated carbonyl structure; for example, the amide group of ACR contributes electron density to the conjugated system, which increases the respective E_{LUMO} and decreases softness. The nonconjugated analogues, allyl alcohol and propanal, are not Michael acceptors and, therefore, have substantially lower values of softness and electrophilicity (Table 1). The respective quantum mechanical values indicate the following rank order of type 2 alkene electrophilicity: NEM $\gg$ acrolein > HNE > MVK $\gg$ MA $\geq$ ACR. In general, this spectrum of electrophi-

licity was closely correlated ($r^2 \geq 0.95$) to the rank order of the corresponding rate constants (k_2) and in vitro neurotoxic potencies [IC_{50}; (*41*−*43*)]. For HNE, however, steric hindrance imposed by the alkane tail (*43*) slowed the corresponding adduct reaction (*59*). Thus, although the respective values of σ and ω reflect electrophilicity equivalent to that of acrolein (Table 1), the rank order of the respective k_{RS^-} and IC_{50} values indicate reactivity less than that of MVK (Table 4). Such disagreement between direct chemical measurements and calculated parameters should be expected, since the algorithms for σ and ω do not consider steric factors. These data nonetheless demonstrate that the type 2 alkenes are electrophiles of varying softness and that the degree of softness determines their relative abilities to impair function and cause toxicity. As compared to other type 2 alkenes, acrolein and HNE are moderately reactive soft electrophiles and their corresponding toxicity is commensurate with their electrophilicity.

According to the selectivity principle of the HSAB theory, soft electrophiles should preferentially react with soft nucleophiles. Although several nucleophilic amino acids are present in biological systems, sulfhydryl groups on cysteine residues are the softest (discussed above). Alternatively, the imidazole side chain of histidine and the ε-amino group of lysine contain nucleophilic nitrogens that are also potential sites for acrolein or HNE adduction. Indeed, there is abundant in vitro evidence that these toxicants can form adducts with lysine and histidine residues; for example, see refs *44*−*52*. However, substantial proteomic data indicate that cysteine sulfhydryl groups are the preferential targets for acrolein, HNE, and other type 2 alkenes [*12, 15, 23, 41, 45, 50, 51, 53*−*57*; also see early studies (*58, 59*)]. This cysteine preference is consistent with the fact that the side chain amino nitrogen groups of lysine and histidine are harder nucleophiles and, therefore, have inherently lower reactivity for soft electrophiles such as acrolein and HNE. These electronic and structural restrictions are reflected in the corresponding second-order rate constants (mean $k_2 \pm$ SD M^{-1} s^{-1}), which demonstrate that Cys (1.33 ± 0.083) is approximately 1000-fold more reactive toward HNE than His ($2.14 \pm 0.312 \times 10^{-3}$) or Lys [$1.33 \pm 0.050 \times 10^{-3}$ (*12, 45*); see also refs *57* and *60*]. The relatively high toxicant-to-protein molar ratios (50:1) and long incubation times (≥24 h) necessary to produce Lys and His adducts during in vitro acrolein/HNE experiments (e.g., see refs *9*, *10*, *47*, *49*, and *61*−*63*) are a reflection of the correspondingly slow rate of adduct formation.

To assess quantitatively the relative nucleophilicity of Cys, Lys, and His residues, the respective FMO energies (E_{HOMO} and E_{LUMO}) can be used to calculate nucleophilic softness (σ) and chemical potential (μ). The latter parameter represents the ability of a nucleophilic species to transfer electron density to the electrophile. Calculated values of μ are independent of pH (i.e., $\mu = E_{LUMO} + E_{HOMO}/2$) and, therefore, reflect the inherent electronic nature of the structural moiety upon which the computations are based. At physiological pH (7.4), the sulfhydryl side chain of cysteine is protonated and, therefore, exists primarily as the neutral (0) thiol. Also, at this pH, the imidazole secondary amine of histidine is mostly deprotonated (0) based on a corresponding pK_a of 6.0, and the primary ε-amino group amine of lysine ($pK_a = 10.5$) is protonated (+1). As reflected in the μ and σ values (Table 2), the respective nucleophilicities of these residues are surprisingly low; that is, the corresponding HOMO energies are relatively low, and the μ values are negative. However, at physiological pH, a small but significant fraction (~10%) of the cysteine sulfhydryl groups is deprotonated and exists in the anionic (−1) thiolate state. In fact, the thiolate fraction is more prevalent than predicted due to the existence of low pK_a cysteine sulfhydryl groups within highly specialized amino acid sequences known as catalytic triads (see below). As the quantum mechanical descriptors indicate, thiolates are much softer nucleopilies than lysine, histidine, or the corresponding thiols; that is, the E_{HOMO} energy is more positive, and the corresponding σ and μ values are larger. This analysis indicates that thiolates are the preferred nucleophilic targets of acrolein, HNE, and other type 2 alkenes. Furthermore, the likelihood that a given nucleophile will form an adduct with a type 2 alkene can be predicted by calculating the nucleophilicity index [$\omega^- = \eta_A(\mu_A - \mu_B)^2/2(\eta_A - \eta_B)^2$]. This recently developed higher order parameter considers the hardness (η) and chemical potential (μ) of both the electrophilic (type 2 alkene) and the nucleophilic (cysteine, histidine, or lysine) reactants (*64*). As suggested by the respective ω^- values (Table 3), acrolein, HNE, and the type 2 alkenes preferentially form adducts with cysteine thiolate sites as opposed to histidine, lysine, or thiol residues, which have significantly lower ω^- values.

If, as the calculated nucleophilic descriptors suggest, the thiolate is the preferred target for acrolein and HNE, this should be reflected in a correspondingly faster rate of adduction. Thus, rates of sulfhydryl adduct formation with type 2 alkenes are known to be dependent upon the pH of the solution. For example, in a recent study, L-cysteine ($pK_a = 8.15$) was shown to react with acrolein 15 times faster when the pH was increased from 7.4 to 8.8. Corroborative studies showed that this pH-dependent increase in rate occurred for the reactions of

Table 2. Calculated Quantum Mechanical Parameters for Nucleophilic Amino Acids

amino acid residue	E_{HOMO} (eV)	μ (eV)	σ (eV)
cysteine thiol (0)[a]	−5.87	−2.87	0.330
histidine (0)	−5.75	−2.75	0.331
lysine (+1)	−10.39	−6.69	0.270
cysteine thiolate (−1)[b]	−0.35	2.21	0.391
histidine (+1)	−10.03	−7.31	0.368
lysine (0)	−5.59	−2.60	0.334

[a] For each nucleophile, quantum mechanical parameters were calculated based on the predominant ionization state (in parentheses) at pH 7.4. [b] For each residue, quantum mechanical parameters were calculated based on the predominant ionization state (in parentheses) in a catalytic triad with cysteine as the central nucleophile. E_{LUMO} (not shown) and E_{HOMO} values were used to calculate the chemical potential (μ) of the nucleophile and corresponding softness (σ). Global (whole molecule) softness (σ) was calculated as the inverse of hardness or $\sigma = 1/\eta$. The chemical potential (μ) was calculated as $(E_{LUMO} + E_{HOMO})/2$ (see ref *42* for details).

Table 3. Calculated Nucleophilic Indices (ω^-) for Type 2 Alkene Reactions with Possible Nucleophilic Targets

electrophile[a]	ω^- Cys (−1)	ω^- Cys (0)	ω^- His (0)	ω^- Lys (+1)
NEM	2.51	0.194	0.250	0.277
acrolein	2.03	0.103	0.123	0.253
HNE	1.93	0.083	0.102	0.287
MVK	1.83	0.064	0.081	0.319
MA	1.59	0.069	0.063	0.332
ACR	1.50	0.036	0.048	0.346

[a] The nucleophilicity index (ω^-) was calculated as $\omega^- = \eta_A(\mu_A - \mu_B)^2/2(\eta_A - \eta_B)^2$, where $\eta = (E_{LUMO} - E_{HOMO})/2$, $\mu = (E_{LUMO} + E_{HOMO})/2$, A = reacting nucleophile and B = reacting electrophile (see ref *42* for details). For each nucleophile, the respective ionization state is presented in parentheses. The nucleophilicity index is a higher order parameter that considers the respective hardness and chemical potential of the electrophilic (type 2 alkene) and nucleophilic (cysteine, histidine, or lysine) reactants and is, therefore, a measure of the likelihood of subsequent adduct formation. As suggested by the respective ω^- values, the type 2 alkenes preferentially form adducts with cysteine thiolate sites as opposed to histidine, lysine, or thiol residues.

Table 4. Type 2 Alkene Reactivity: Comparisons of Nucleophilic Indices (ω^-), Thiolate Rate Constants (k_{RS^-}), and Neurotoxic Potencies (IC_{50} Values)[a]

electrophile	ω^- Cys (−1)	log k_2	log k_{RS^-}	log IC_{50}
NEM	2.51	6.536	7.912	−4.33
acrolein	2.03	2.596	3.417	−4.28
HNE	1.93	0.938	1.759	−3.40
MVK	1.83	2.048	2.953	−3.48
MA	1.59	−0.936	1.011	−0.34
ACR	1.50	−1.804	0.767	−0.36

[a] Second-order rate constants (k_2) were determined for type 2 alkene reactions with L-cysteine at pH 7.4 (n = 4−6 experiments). The k_2 values at pH 7.4 were corrected for the corresponding cysteine thiolate concentration (k_{RS^-}) according to the algorithm: log ($k_{RS^-} - k_2$) = log k_2 + pK_a − pH. Inhibition of membrane ^{3}H-dopamine transport was determined in rat striatal synaptosomes exposed in vitro to graded concentrations of each type 2 alkene. The concentration−response data for transport were fitted by nonlinear regression analysis, and the respective IC_{50} values were calculated by the Cheng−Prusoff equation (see refs *41*−*43* for methodological details).

L-cysteine with all type 2 alkenes evaluated (*42, 43*). The observed changes in second-order rate constant (k_2) obviously reflected the increase in thiolate concentration at the higher pH value and, thus, confirms the notion that the operable reactive species is indeed the anionic thiolate. Moreover, it has been convincingly demonstrated that experimentally determined rate constants like k_2 can be used to derive an anionic rate constant (k_{RS^-}) as a quantifiable measure of inherent nucleophilic strength (*58*). Using the expression log ($k_{RS^-} - k_2$) = log k_2 + pK_a − pH (where k_{RS^-} represents the anionic rate constant), a representative series of thiolate rate constant were calculated. For each α,β-unsaturated carbonyl derivative, the corresponding thiolate rate constants (k_{RS^-}) were highly correlated to μ (r^2 = 0.96; Table 2) and ω^- (r^2 = 0.91; Table 3). Furthermore, the fact that ω^- and k_{RS^-} were closely correlated to the neurotoxic potencies (IC_{50} values; Table 4) provides evidence that thiolate targeting by acrolein and HNE has toxicological relevance (*42, 43*).

2.3. Adduct Chemistry of Acrolein and HNE. It is clear that acrolein and HNE are relatively soft electrophiles that target the soft nucleophilic thiolate state of cysteine residues. The soft−soft interaction between these type 2 alkenes and their sulfhydryl target occurs via a 1,4-Michael type conjugate reaction, that is, nucleophilic attack at the β-carbon of the α,β-unsaturated carbonyl structure with subsequent addition across the carbon−carbon double bond. The resulting intermediate product, a saturated aldehyde, then undergoes an intramolecular reaction with the hydroxyl group to form a cyclic hemiacetal, which is the predominant adduct form (reviewed in refs *1* and *16*). That acrolein, HNE, and other type 2 alkenes preferentially form stable 1,4-adducts with cysteine sulfhydryl groups has been demonstrated by isolation of corresponding protein adducts and subsequent quantitation using mass spectrometry and other proteomic approaches [(*12, 23, 41, 45, 50, 51, 53*−*57*); also see early studies (*58, 59*)].

As an alternative to 1,4-Michael addition, the carbonyl carbon atom of acrolein and HNE could form adducts with primary amines (e.g., Lys) via a 1,2-addition. Nadkarni and Sayre (*61*) have provided indirect evidence that HNE forms such adducts with primary amines and that subsequent Schiff base formation is prevalent in solvent-isolated (buried) hydrophobic protein microenvironments. However, the corresponding kinetics are inherently slow, and the Schiff base product is reversible (*10, 11, 16*). That reactions with carbonyl groups (Schiff base formation) on acrolein and other type 2 alkenes are not neurotoxicologically relevant is suggested by recent in vitro studies, which showed that graded exposure of striatal synaptosomes to propanal (an aldehyde) did not affect function (*41*). Alternatively, because α,β-unsaturated carbonyls are bifunctional (i.e., electrophilic reactivity at the β-carbon and carbonyl carbon atoms), HNE, acrolein, and other chemicals in this class could cause toxicity by cross-linking proteins rather than by forming monoadducts (*1, 62, 65*). However, the results of recent immunoblot analyses and studies with pharmacological cross-link blockers (*41*) did not support a mechanistic role for protein cross-links in the production of alkene toxicity.

3. Histidine, Lysine, and Cysteine Residues: Respective Roles in Protein Structure and Function

The chemical and proteomic data presented thus far indicate that the soft−soft interactions between, for example, acrolein and cysteine thiolates is a kinetically favored reaction that occurs rapidly. In contrast, soft−hard interactions between, for example, acrolein and lysine residues are unfavored and, therefore, significantly slower reactions. It is important to realize that, among potential amino acid targets, differences in nucleophilic reactivity and, therefore, selectivity are not necessarily related to the relative degree of toxicological relevance. That is, it cannot be assumed that rapid adduct formation with a given residue has inherent toxic significance. Rather, it is the role of the residue in protein structure or function and the resulting disruptive consequences of adduction that determine the relevance of an amino acid adduct. In the following subsections, we provide a brief overview of the respective roles that histidine, lysine, and cysteine play in determining protein activity. For more comprehensive discussions, the reader is referred to several relevant reviews, that is, refs *29*, *71*, *74*, and *79*. In a subsequent section, we will discuss how amino acid function might be related to the toxicological consequences of adduct formation.

Histidine is a basic amino acid that plays an important role in cellular buffering. At physiological pH, the imidazole side chain (pK_a = 6.04) is mostly deprotonated (0); therefore, free histidine has limited buffering capacity. Nonetheless, in combination with other amino acids (e.g., alanine), the pK_a of the imidazole can increase, which makes histidine residues in proteins and peptides a dominant buffering system in many cells. When paired with an acidic amino acid (e.g., aspartic acid), histidine residues can participate in the protonation/deprotonation of central nucleophiles in catalytic triads (e.g., see ref *66*). As will be discussed, these acid−base motifs determine the nucleophilic state of, for example, cysteine sulfhydryl groups, which in turn regulates the activity of critical proteins. Histidine residues can also play more direct roles in modulating protein activity. For example, several functionally critical histidine residues are present within the active site of creatine kinase (CK). In a transition state of this enzyme, His 66 forms a salt bridge with the carboxyl group of Asp 326 and thereby locks two flexible loops (residues 60−70 and 323−332) over the active site. Latching of these loops brings together two hydrophobic residues (Ile 69 and Val 325), which presumably creates a binding pocket for the N-methyl group of creatine and imparts specificity for this substrate (*67*). His 191 and His 296 are also located in the active site of CK and are involved in orienting the adenosine rings. Specifically, His 296 and the six-membered adenine ring undergo a stacking interaction, whereas His 191 forms a hydrogen bond with the 2′-hydroxyl group of the ribose ring (*67*). Mutation of these active site histidine residues substantially decreased enzyme activity (*68*).

The ε-amino group of lysine is protonated (+1; pK_a = 10.5) at neutral pH and can participate in the electrostatic interactions

of amino acids (e.g., lysine−glutamate) that mediate protein−protein associations. Similar to histidine, lysine residues are often the basic amino acid component of many catalytic triads (*69*). The primary amine side chain of lysine is also subject to substantial posttranslational modifications (PTMs), for example, acetylation, methylation, sumoylation, and ubiquitylation (*70*−*73*). These reactions are not favored thermodynamically and are, therefore, mediated by specific enzymes (e.g., lysine acetyltransferases and deacetylases). Lysine PTMs can influence protein function by changes in charge density or by reversible interactions with crucial lysine residues of, for example, structural proteins (e.g., Lys 40 of α-tubulin) or enzymes (e.g., Lys 609 of acetyl-CoA synthase). Such modifications alter the activities of many protein classes, for example, histones, cytoskeletal proteins, energy metabolism, mitochondrial enzymes, and plasma membrane-associated receptors (*72, 74, 75*), and similar to protein phosphorylation, lysine modulation might represent a signaling program that orchestrates cell processes (e.g., see refs *70*, *72*, *73*, and *76*).

Cysteine is a polar, uncharged amino acid with a pK_a of 8.3; therefore, at physiological pH, the side chain sulfhydryl group is protonated (0). The biological importance of cysteine stems from the unique ability of this amino acid to undergo reversible oxidation/reduction. On the basis of this ability, cysteine residues can act as redox sensors that detect and transduce changes in cellular redox status caused by the generation of toxic reactive oxygen species (ROS). ROS oxidation of these cysteines produces protein conformational changes that stimulate antioxidant pathways through subsequent induction of gene expression, for example, activation of the Nfr2-Keap1 pathway by ROS and electrophiles [(*77*−*79*); reviewed in ref *80*]. In addition to the management of toxic ROS, reactive cysteines are now recognized as acceptors for redox signaling systems, for example, nitric oxide (NO) and hydrogen peroxide (H_2O_2). The redox status of these cysteines regulates the function of proteins that are involved in numerous critical cell processes (reviewed in refs *29* and *81*−*85*). For example, Cys264 is a reactive cysteine residue located within the nucleotide-binding consensus sequence of NEM sensitive factor (NSF). The ATPase activity of this enzyme, which is critically involved in membrane fusion reactions (e.g., exocytosis), is regulated by reversible S-nitrosylation of Cys264 through NO signaling. As alluded to above, not all cysteine residues are sensitive to ROS or redox signaling. Indeed, the nucleophilicity and, therefore, reactivity of most biological cysteine thiols (RSH) with, for example, NO, is too low to be relevant (Table 2). However, as discussed above, the nucleophilicity of sulfhydryl groups is markedly increased in the anionic thiolate state. Because the pK_a of cysteine is 8.3, sulfhydryl thiolates can exist only in protein microenvironments where the pK_a is lowered. Such conditions exist in catalytic triads where proton shuttling between flanking or proximal (≤6 Å) basic amino acid residues (histidine, arginine, and lysine) and their acidic counterparts (aspartate and glutamate) can deprotonate the sulfhydryl group and thereby lower the corresponding pK_a by several units (e.g., see refs *66* and *69*). The highly nucleophilic thiolate groups of catalytic triads (and diads) therefore represent "receptors" for electrophilic transmitters such as NO and H_2O_2 (*29, 85*−*87*). Cysteine-based catalytic triads are found within the active sites of many proteins (e.g., NSF−Cys264, glyceraldehyde-3-phosphate dehydrogenase−Cys525, and vacuolar-ATPase−Cys254) and play a critical role in modulating corresponding function. Through these diverse cysteine effectors, redox signaling can influence almost all aspects of cell physiology.

3.1. Toxicological Significance of Amino Acid Adduct Formation. Clearly, cysteine sulfhydryl thiolate groups are critically involved in the majority of cellular processes; that is, they are essential for enzyme catalytic activity and metal chelation, they function as ROS sensors, and they are acceptors for redox signaling pathways. Lysine and histidine residues also are physiologically important; lysine residues undergo PTMs, histidine residues function as cellular buffers, and both amino acids participate as the basic components of catalytic triads. Given the apparent significance of lysine, histidine, and, especially, cysteine in protein structure and function, it would seem that adduction of these residues by acrolein or HNE could have toxicological implications. Nonetheless, little direct information is available regarding the toxicological consequences of corresponding adduct formation. Proteomic research has identified HNE or acrolein adducts at specific amino acid residues following in vivo or in vitro exposure of proteins, for example, Cys47 of glutathione S-transferase P1-1, His178 in the phosphorylation lip of Erk-2, and Cys29 in the A chain of cathepsin B (*11, 25, 53, 55, 57, 88*−*90*). However, the toxicological significance of adduct formation was inferred from the presumed role of the targeted amino acid in corresponding protein function. Additional research has shown that incubation of purified proteins (e.g., glyceraldehyde-3-phosphate dehydrogenase) with acrolein, HNE, or other type 2 alkenes disrupts function and produces lysine, histidine, or cysteine adducts (*23, 41, 46, 48, 50, 54, 89, 91*). In these studies, it was not determined whether adduct formation was causally related to protein dysfunction.

A few studies, however, have specifically addressed the toxicological significance of adduct formation by HNE and acrolein. Stewart et al. (*92*) used an in vitro model of tubulin polymerization to show that lysine adducts formed by HNE and ONE had limited functional effects. In contrast, these investigators found that adduction of cysteine residues on the α- and β-tubulin subunits (Cys347α, Cys376α, and Cys303β) inhibited polymerization. In another study, Carbone et al. (*93*) reported that in vitro HNE exposure (10 and 100 μM) impaired the chaperone function of Hsp72 and that this inhibition was associated with adduct formation at Cys267 in the ATPase domain of this protein. The relevance of this adduct was suggested by experiments showing that DnaK, a bacterial Hsp70 variant that lacks Cys267, was resistant to HNE inactivation and that malondialdehyde, a nonconjugated aldehyde analogue, did not affect Hsp72 activity. Research by Eliuk et al. (*94*) showed that HNE, at a pathophysiologically relevant concentrations (10−30 μM), selectively formed Michael adducts with Cys 283 in the active site of recombinant human brain CK (10 μM). Adduction of this cysteine residue was associated with 30−40% inhibition of enzyme activity. At higher HNE concentrations (100−300 μM), active site histidine residues (His 66, His 191, and His 296; see preceding section) were also adducted, and enzyme activity was progressively inhibited. Together, these studies provide preliminary evidence that adduction of certain protein cysteine residues by α,β-unsaturated carbonyl derivatives has potential toxicological significance.

From the preceding overview, it is clear that data gaps exist; therefore, more research is needed to establish the toxicological relevance of lysine, histidine, and cysteine adducts in acrolein and HNE toxicity. Nonetheless, the rank order of amino acid reactivity (Cys ≫ His > Lys) and the relative importance of these residues in cell physiology provide insight into the corresponding molecular mechanisms. Thus, as discussed above, lysine and histidine are relatively poor nucleophiles and are,

therefore, unlikely to be immediate targets for soft electrophiles. This also applies to the transient ionization states of these residues that occur in catalytic triads and other microenvironments where the respective pK_a values are modified (compare corresponding data in Table 2). However, the relatively high thiolate reactivity of acrolein and HNE and the well-known critical roles of this sulfhydryl in cell physiology argue that the initial aspects of toxicity are mediated by cysteine adduction. The slower rate of lysine or histidine adduction might be relevant to toxicities involving high concentrations or subchronic exposure durations. Accordingly, at lower cellular toxicant concentrations [nM to very low μM range; (*95*)] that might develop during the early stages of a disease processes or following chronic environmental acrolein exposure, cysteine adduction will predominate due to the higher nucleophilic reactivity of this residue (see refs *23*, *44*, *53*, and *55*). Although Lys is slightly more abundant in proteins than either Cys or His residues [7, 3, and 2%, respectively; (*96*)], this difference is not sufficient to alter the residue selectivity of the type 2 alkenes through changes in mass action kinetics (*23, 53*−*55, 63*). Results from extensive research on type 2 alkene toxicity (e.g., see refs *1*, *11*−*13*, *41*−*43*, *55*, *56*, *92*, *97*, *98*, and *106*) suggest that the acute effects of acrolein and HNE are mediated by a common mechanism involving adduction of sulfhydryl thiolates in the catalytic triads of proteins that regulate critical cellular processes. Because many of the thiolates in these catalytic centers function as NO acceptors, the irreversible formation of HNE/acrolein adducts at these sulfhydryl groups might disrupt NO signaling (reviewed in refs *14*, *29*, *30*, and *99*). As cellular toxicant concentrations rise (low μM range) or as the exposure duration increases, the available cysteine thiolate pool will diminish, and adduct formation will shift to residues with lower reactivity, that is, lysine or histidine. This scenario could account for the detection of Lys and His adducts, as well Cys adducts, in tissues of patients with chronic diseases that presumably involve oxidative generation of acrolein/HNE, for example, Alzheimer's disease, atherosclerosis, and diabetes (see refs *17*, *51*, *52*, *100*, and *101*). The toxicity associated with lysine adduction is likely mediated by disruption of posttranslation modifications, for example, phosphorylation, acetylation, and methylation (see above). With respect to long-term (chronic) exposure conditions, the cell types or regions most susceptible to HNE or acrolein toxicity are those characterized by slower protein turnover rates. In these conditions, adducts are removed slowly, which favors the accumulation of dysfunctional proteins and the development of cumulative toxicity (reviewed in refs *14*, *30*, and *102*).

4. Role of HNE and Acrolein in Disease Processes

Cellular oxidative stress and associated peroxidation of membrane polyunsaturated fatty acids are implicated in the pathogenesis of many diseases including alcoholic liver damage, diabetes, reperfusion injury (e.g., stroke or myocardial infarction), atherosclerosis, and Alzheimer's disease (reviewed in refs *2*−*4* and *103*−*105*). There is considerable evidence that major aspects of these disease processes are mediated by oxidative stress and the subsequent liberation of acrolein, HNE, and other toxic aldehydes during membrane peroxidation (reviewed in refs *1*, *14*, *30*, and *106*−*108*). Whereas some studies have focused on glutathione (GSH) depletion as the critical event (e.g., see refs *109*−*112*), a substantial database suggests that the in vivo toxicity of acrolein and other type 2 alkenes primarily involves protein adduct formation (e.g., see refs *56* and*113*−*117* reviewed in ref *28*). On the basis of a weight of evidence approach, we propose the following hypothesis: Acrolein, HNE, and other lipid byproducts (e.g., ONE and HHE) are soft electrophiles that form irreversible 1,4-Michael type adducts with soft nucleophilic sulfhydryl thiolate groups of cysteine. These anionic residues are the central nucleophilic components of catalytic triads in the active sites of many critical proteins. Reversible redox modulation of these catalytic sulfhydryl groups by, for example, the endogenous NO or H_2O_2 pathways regulates protein activity. Therefore, irreversible adduction of these regulatory thiolate groups by acrolein or HNE will disrupt redox control of protein function and, thereby, produce cytotoxicity (reviewed in refs *14*, *29*, *30*, and *99*). Although lysine and histidine residues are also targets for type 2 alkene chemicals, these residues are relatively hard nucleophiles with significantly slower adduction kinetics. As a result, the toxic consequences of lysine or histidine adduction are more likely to develop during high-dose intoxication or during the late stages of chronic diseases when adduction of the cysteine thiolate pool has saturated.

The proposed pathophysiological scenario has significant implications for disease states and traumatic tissue injuries that involve initial oxidative stress and subsequent liberation of toxic aldehydes in specific cell types. Thus, for example, a large body of evidence indicates that the pathobiology of Alzheimer's disease involves oxidative damage and subsequent generation of toxic aldehydes in nerve cells of the hippocampus and cerebral cortex. It has been hypothesized that acrolein and HNE selectively target regional nerve terminals and that the ensuing disruption of synaptic function promotes problems with declarative memory and cognition (reviewed in refs *14* and *30*). Certainly, pathokinetic differences such as the affected cell type, the rate of acrolein/HNE generation, and resulting cellular levels will distinguish each disease or injury state. Nonetheless, adduction of NO-targeted sulfhydryl thiolate sites on proteins is a rational, common mechanism for pathogenic conditions that involve cellular oxidative stress. As indicated above, acrolein is a member of the type 2 alkene chemical family that includes ACR, MA, and acrylonitrile. These chemicals have extensive industrial utility and are pervasive environmental pollutants (ambient, occupational, and dietary). Therefore, the proposed mechanism is applicable to acquired toxicities that develop as a result of environmental or occupational exposures to acrolein and other α,β-unsaturated aldehyde derivatives (*14*). Because the type 2 alkenes presumably operate via a common mechanism, it is also possible that environmental exposure to these toxicants (e.g., acrolein, acrylonitrile, and MVK) will accelerate the onset and development of chronic conditions such as diabetes or Alzheimer's disease that involve liberation of endogenous toxic aldehydes (e.g., acrolein, HNE, and ONE). Finally, our proposed mechanism of electrophile toxicity suggests that nucleophilic scavengers such as *N*-acetylcysteine (NAC) might be cytoprotective and, consequently, have possible therapeutic value. However, it should be recognized that nucleophiles would also scavenge the electrophilic mediators of endogenous redox pathways (e.g., NO and H_2O_2) and would, therefore, constitute a significant toxic threat (see ref *42*).

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