Risk Assessment of High-Energy Chemicals by in Vitro Toxicity Screening and Quantitative Structure-Activity Relationships

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Hydrazine propellants pose a substantial operational concern to the U.S. Air Force and to the aerospace industry because of their toxicity. In our continuing efforts to develop methods for the prediction of the toxicological response to such materials, we have measured in vitro toxicity endpoints for a series of high-energy chemicals (HECs) that were recently proposed as propellants. The HECs considered are structurally diverse and can be classified into four chemical types (hydrazine-based, amino-based, triazoles, and a quaternary ammonium salt), although most are hydrazine derivatives. We measured the following endpoints in primary cultures of isolated rat hepatocytes: mitochondrial function (MTT), lactate dehydrogenase leakage (LDH), generation of reactive oxygen species (ROS), and total glutathione content (GSH). In several instances, effective concentrations (EC) were indeterminate, and only lower limits to the measured endpoints could be ascertained. Using molecular descriptors calculated with a semiempirical molecular orbital method, quantitative structure-activity relationships (QSARs) were derived for MTT (EC25) and for GSH (EC50). Correlation coefficients for 2- and 3-parameter QSARs of about 0.9 enable us to predict the toxicity for similar compounds. Furthermore, except in one case, predicted EC values for the uncertain endpoints were consistent with experiment. Descriptors comprising the QSARs for MTT were consistent with the biophysical mechanism of toxic response found experimentally for hydrazine derivatives. Application of our derived QSARs will assist in predicting toxicity for newly proposed propellants.

Key Words: high-energy chemicals; risk assessment; in vitro toxicity; QSAR; hydrazine.

Hydrazine and its methylated derivatives are powerful reducing agents with a wide range of uses, including aerospace fuels. The introduction of hydrazine, monomethyl hydrazine, and 1,1-dimethyl hydrazine as propellants grew out of a need for high-energy, noncryogenic, liquid fuels that can be used alone or mixed with other components. The toxicity of hydrazine propellants is a substantial operational concern to the U.S. Air Force as well as to the aerospace industry. The two most likely occupational exposure routes are inhalation and skin exposure (Keller, 1988).

Several hydrazine derivatives occur naturally in tobacco and mushrooms, some are herbicides, and others are pharmacologically active. Hydrazine and its derivatives enter the environment primarily from aerospace emissions and from manufacturing facilities, although exposure also occurs as a metabolite of the drugs isoniazid (an antitubercular agent) and hydralazine (an antihypertensive agent; Delaney and Timbrell, 1995). Toxic effects due to exposure to hydrazines include liver damage (Amenta and Johnston, 1962), hypoglycemia, disorders of the central nervous system (Fortney, 1966), interference with intermediary metabolism (Moloney and Prough, 1983), induction of systemic lupus erythematosus (Jiang et al., 1994), and carcinogenicity (Toth, 1994).

A number of investigators have reported on the toxicity of hydrazine in vivo and in vitro. Hydrazine exposure leads to ATP depletion and megamitochondria formation in vivo (Kerai and Timbrell, 1997; Preece et al., 1990; Teranishi et al., 2000; Wakabayashi et al., 2000). Hydrazine inhibits the mitochondrial enzyme succinate dehydrogenase (Ghatineh et al., 1992), which subsequently reduces mitochondrial function. Hydrazine also produces toxicity by interfering with a number of metabolic processes such as gluconeogenesis (Kleineke et al., 1979) and reactions involving glutamine synthetase (Kaneo et al., 1984; Noda et al., 1987; Šenko et al., 1984; Willis, 1966).

Metabolic studies of hydrazine suggest that the N–N bond is cleaved in vivo (Delaney and Timbrell, 1995). Workers monitoring the disappearance of hydrazine from hepatic microsomes concluded that hydrazine was oxidized by the cytochromes P450, although the product was not identified (Jenner and Timbrell, 1995). A study aimed to ascertain the role of P450 isozymes in the toxicity of hydrazine using rat hepatocytes in vitro suggested that metabolism by three P450 isozymes (1A1/1A2, 2B1, and 2E2) leads to detoxification, and that the cytotoxicity of hydrazine could be due to the parent compound (Delaney and Timbrell, 1995).

Monoalkylated hydrazine derivatives are metabolically ox-
<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Abbreviation</th>
<th>Neutral Species</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrazinium nitrate</td>
<td>HZN</td>
<td>NH$_2$NH$_2$</td>
<td>hydrazine</td>
</tr>
<tr>
<td>2-Hydroxyethyl/hydrazinium nitrate</td>
<td>HEM</td>
<td>NH$_2$NHCH$_2$CH$_2$OH</td>
<td>hydrazine</td>
</tr>
<tr>
<td>1,2-Diethylhydrazinium nitrate</td>
<td>DEHN</td>
<td>CH$_2$CH$_2$NHNHNHNCH$_2$CH$_2$</td>
<td>hydrazine</td>
</tr>
<tr>
<td>Methylhydrazinium nitrate</td>
<td>MHN</td>
<td>CH$_3$NH$_2$</td>
<td>hydrazine</td>
</tr>
<tr>
<td>1,4-Dihydrazinoctetrazenate nitrate</td>
<td>DHTN</td>
<td>$\text{H}_2\text{N}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}$</td>
<td>hydrazine</td>
</tr>
<tr>
<td>Diaminoguanidine nitrate</td>
<td>DAGN</td>
<td>HNO$_2$(NH$_2$)$_2$</td>
<td>hydrazine</td>
</tr>
<tr>
<td>Nitriaminoanisidine nitrate</td>
<td>NAGN</td>
<td>NH$_2$(NH$_2$)(NHNO$_2$)</td>
<td>hydrazine</td>
</tr>
<tr>
<td>Ethanolamine nitrate</td>
<td>LAN</td>
<td>NH$_2$CH$_2$OH</td>
<td>amine</td>
</tr>
<tr>
<td>Histamine dinitrate</td>
<td>HDN</td>
<td>$\text{H}_2\text{N}_2$CH$_2$C$_2$</td>
<td>amine</td>
</tr>
<tr>
<td>Methylxylamine nitrate</td>
<td>MAN</td>
<td>NH$_2$OCH$_3$</td>
<td>amine</td>
</tr>
<tr>
<td>1,2,4-Triazole nitrate</td>
<td>TN</td>
<td>$\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}$</td>
<td>triazole</td>
</tr>
<tr>
<td>4-Amino-1,2,4-Triazole nitrate</td>
<td>ATN</td>
<td>$\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}\text{N}^{-}$</td>
<td>triazole</td>
</tr>
<tr>
<td>2,2-Dimethyltriazanilium nitrate</td>
<td>DMTN</td>
<td>[(NH$_2$)$_2$N(CH$_2$)$_2$]$^+$</td>
<td>quaternary ammonium salt</td>
</tr>
</tbody>
</table>

**FIG. 1.** Proposed high-energy chemicals.

...dized and form carbon-centered radicals in microsomes and hepatocytes, as well as in enzymatic systems such as P450 and horseradish peroxidase (Albano et al., 1993; Gamberini and Leite, 1993; Gamberini et al., 1998). Disubstituted derivatives, however, are oxidized to carbocations as well as to carbon-centered radicals (Hawks and Magee, 1974). Toxicity of hydrazine derivatives has been attributed to carbocations (Fiala, 1975), carbon-centered radicals (Hill and Thormalley, 1983; Leite and Augusto, 1989; Runge-Morris et al., 1994), and reactive oxygen species (Kawanishi and Yamamoto, 1991). Toxicity is metabolically activated, and the genotoxic effects appear to be the basis of the carcinogenicity of these compounds (Toth, 1984).

In view of the toxicity of hydrazine, alternative aerospace propellants were synthesized and are currently being evaluated (by the Propulsion Directorate of the Air Force Research Laboratory, Edwards AFB, California), including those listed in Figure 1. These high-energy chemicals (HECs) may be categorized as hydrazine-based, amine-based, triazole-based, and a quaternary ammonium salt. A preliminary report of some of these HECs suggests that their toxicity is mediated through oxidative stress (Hussain and Frazier, 2001).

To maintain a safe working environment, it is necessary to develop reliable, rapid, and inexpensive methods for predicting health risks of newly developed chemicals. We therefore aim in this study to examine the toxicity of these HECs in primary rat hepatocytes in vitro with reference to viability, mitochondrial function, and oxidative stress markers. The toxicological profiles of these chemicals, in conjunction with quantitative structure-activity relationships (QSARs) derived therefrom, will assist in the design and optimization of chemicals for new propellants.

QSARs are models that attempt to relate chemical structure to biological endpoints such as pharmacological activity or toxicity (Hansch and Leo, 1995). For a congeneric set of compounds assumed to act by a common mechanism, differences in chemical structure are mapped to changes in biological activity (Hansch and Fujita, 1964). Typically, the negative log of the biological activity is expressed as a linear combination of descriptors that are calculated from chemical structure. However, whereas the aim of QSAR modeling of pharmacological endpoints is optimization of the activity, modeling of toxicological endpoints usually does not involve mechanistically well-defined endpoints, i.e., multiple mechanisms can lead to the same toxicity endpoint (Benigni and Richard, 1998). Toxicity QSARs therefore have two goals: toxicity prediction and distinguishing between mechanisms, or, if mechanistic information is lacking, formulation of the biophysical mechanism of toxic response.

The molecular descriptors that are often used can be categorized as constitutional, topological, geometrical, and those derived from quantum chemical calculations, which include not only molecular orbital energies, but also thermodynamic and electrostatic quantities (Katritsky et al., 1994). Quantum chemical descriptors are employed in this study because they are relevant to the mechanism of toxic response for simple hydrazine derivatives. Some of the QSARs derived for the proposed HECs are consistent with this mechanism. The QSARs also provide a screening capability for newly proposed propellants if the QSAR is valid for the new propellants in question. QSARs must be used only for interpolation and not for extrapolation. However, such a distinction is not always straightforward, because common mechanisms of toxic response among chemicals are not always evident.

**MATERIALS AND METHODS**

**Chemicals.** Collagenase was obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), β-nicotinamide-adenine dinucleotide-reduced (NADH), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), reduced glutathione (GSH), rhodamine 123, annexin V, buthionine-(S,R)-sulfoximine (BSO), insulin/transferrin/sodium selenite (ITS) additive, gentamicin, and dexamethasone were purchased from Sigma Chemical Company (St. Louis, MO). Chee media was obtained from Gibco (Grand Island, NY). All HECs were supplied from the Propulsion Directorate of the Air Force Research Laboratory, Edwards Air Force Base, California, and are 99% pure as determined by Galbraith Laboratories (Knoxville, TN).

**Animals.** Male Fischer 344 rats (225–300 g) were obtained from Charles River Laboratories (Wilmington, MA). Rats were anesthetized with 1 ml/kg of a mixture of ketamine (70 mg/l; Parke-Davis, Morris Plains, NJ) and xylazine (6 mg/l; Mobay Corp., Shawnee, KS) prior to undergoing liver perfusion. All animals used in this study were handled in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy Press, 1996) and the Animal Welfare Act of 1966, as amended.
Liver perfusion, hepatocytes enrichment, and culture. Rat livers were perfused, and hepatocytes were isolated and enriched by the two-step Seglen procedure (Seglen, 1976) with minor modifications as previously described (DelRaso and Frazier, 1999).

Treatment. Primary rat hepatocytes were treated with varying concentrations of HECs dissolved in Chee culture media. After a 3-h incubation to allow cells to attach to culture plates, the media was changed and cells were incubated for 21 h before treatment. The cells were exposed to each HEC for 4 h (Fig. 2). At the end of the 4-h incubation period, the following four toxicity endpoints were evaluated using a 96-well plate reader (SpectraMAX; Molecular Devices, Sunnyvale, CA).

- LDH leakage was assessed by spectrophotometrically measuring the oxidation of NADH at 340 nm in both the cells and media, as described by Moldeus et al. (1978).
- Mitochondrial function was determined spectrophotometrically by measuring the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan by succinic dehydrogenase, as previously described (Carmichael et al., 1987).
- Reduced and oxidized glutathione (GSH and GSSG) were measured using the Glutathione Assay Kit from Cayman Chemical Company (Ann Arbor, MI). The assay is based on the enzymatic recycling method, using glutathione reductase and DTNB (5,5′-dithiobis-2-nitrobenzoic acid, Ellman’s reagent) as described by Tietze (1969).
- Reactive oxygen species (ROS) generation was determined using the method described by Wang and Joseph (1999). Fluorescence was detected using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Computational approaches. All HECs were modeled as neutral species except for DMTN, which cannot be realistically modeled as a neutral molecule and was excluded. The molecular geometries of the remaining 12 HECs were optimized in the gas phase employing a semiempirical molecular orbital method, specifically, AM1, the Austin Method 1 Hamiltonian (Dewar et al., 1985). Vibrational frequencies were also calculated using AM1 at the optimal gas-phase geometries. Although it has been shown that somewhat better QSARs are obtained at higher levels of quantum theory (Trohalaki et al., 1990), AM1 is a reasonable approach for an initial definition of the important molecular parameters that characterize the toxicity. All quantum calculations were performed using MOPAC, Version 6.00 (Stewart, 1990). Polarizabilities were calculated using the dipole expansion method (Kurtz et al., 1990).

CODESSA version 2.061 (Katritzky et al., 1994) was used to extract data from the MOPAC output files, calculate the descriptors, and correlate the descriptors with the data (−log EC25 for MTT and −log EC50 for GSH) to derive the appropriate QSARs. In the so-called heuristic method, a preselection of descriptors occurs. Descriptors unavailable for some compounds are discarded, as are descriptors that are invariant for all compounds in the data set. Descriptors that correlate poorly are also discarded. Additional descriptors are discarded when high intercorrelations between descriptors are found. The remaining descriptors are then ranked according to their correlation coefficients. Correlations of all pairs of descriptors (2-parameter QSARs) are calculated, and the best combinations are saved as working sets. The 10 QSARs with the highest $r^2$ and the 10 with the highest $F$ values are then kept. Each working set is combined with the remaining descriptors to derive 3-parameter QSARs. Again, the 10 QSARs with the highest $r^2$ and the 10 with the highest $F$ values are saved. This method is repeated until the maximum number of parameters specified by the user is achieved. A best multilinear regression algorithm is also available from which the best regression model is obtained (using descriptors that are not collinear), i.e., the best 2-parameter QSAR, best 3-parameter QSAR, etc. are found.

The user may, of course, edit the descriptors available for QSAR generation. Constitutional and topological descriptors are in many cases not easily interpretable in terms of a biological mechanism. Although geometrical descriptors are important in protein binding, CODESSA calculates them from the two-dimensional structure. To aid us in interpreting the QSARs in terms of the mechanisms involved, only quantum mechanical descriptors were employed in our study. These include molecular orbital energies, thermodynamic quantities, and electrostatic charges, as well as descriptors that characterize the shape of optimal molecular geometries.

RESULTS

The MTT assay was used to assess the effects of HECs on mitochondrial function of rat hepatocytes. Mitochondrial function of hepatocytes decreased in a dose-dependent manner with increasing HEC concentration. The dose-response curve for hydroxizinium nitrate (HZN) is typical of the hydrazine-based HECs, in that mitochondrial function is reduced in a concentration-dependent manner (Fig. 3a). Two of the amino-containing compounds (EAN and MAN) displayed toxicity at high concentrations (>100 mM), whereas HDN was toxic at 50 mM. Triazole-containing compounds (TN, ATN, and DMTN) did not exhibit significant toxicity, at even the highest concentration tested (150 mM). To maximize the number of data points for development of QSARs for MTT, the effective concentration resulting in a 25% (rather than the typical 50%) reduction in mitochondrial function, EC25_MTT, was determined. Even so, only lower limits to the EC25_MTT could be determined for four of the HECs. According to the EC25_MTT values (Table 1), the hydrazine-containing compounds are the most toxic, with their toxicity decreasing as MHN > DHTN > HZN > DEHN > HEHN > DAGN > NAGN. The amino-containing compounds displayed medium toxicity as estimated by EC25_MTT, decreasing as HDN > EAN > MAN. Triazole-containing compounds exhibited low toxicity, and the relative toxicity ranking cannot be discerned based on EC25_MTT values because these values were indeterminate.

The viability of rat hepatocytes was evaluated by measuring the leakage of LDH into the media. The dose-response curve for HZN shown in Figure 3b is typical of curves for HEHN, DEHN, and EAN. All other curves are relatively flat over the concentration range studied. HZN appears to be most toxic; it induced substantial LDH leakage at a concentration of 25 mM. The effective concentrations resulting in a 25% increase in LDH leakage, EC25_LDH, are reported in Table 1. In most cases, however, only lower limits for EC25_LDH could be determined. The trend of toxicity, as far as can be determined from the
EC25\textsubscript{LDH} values, is consistent with the EC25\textsubscript{MTT} trend, except that EAN appears more toxic than HDN. By compound class, LDH leakage shows that the order of toxicity is as follows: hydrazine-containing compounds $>$ amino-containing compounds $>$ triazole-containing compounds.

Dichlorofluorescein diacetate (DCFH-DA) is widely used to measure reactive oxygen species (ROS) generation in cells. ROS generation following exposure to HZN (Fig. 3c) is the greatest by far among the HECs tested. Concentration-response curves for most other HECs are flat. No appreciable increase in ROS generation was observed for cells treated with triazole-containing compounds. Even a low concentration of HZN (25 mM) produced a significant increase in ROS generation at the end of the 4-h exposure. The HZN treatment at 100 and 150 mM resulted in an approximately 8-fold increase in ROS. The lowest effective concentration was determined for ROS (LEC\textsubscript{ROS}), although in most cases only lower limits to the LEC could be ascertained. Accordingly, using ROS generation as a

#### TABLE 1

Endpoints Representative of Dose-Response Curves

<table>
<thead>
<tr>
<th>Propellants</th>
<th>EC25\textsubscript{MTT} (mM)</th>
<th>LSC\textsubscript{ROS} (mM)</th>
<th>EC25\textsubscript{LDH} (mM)</th>
<th>EC50\textsubscript{GSH} (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HZN</td>
<td>35</td>
<td>5</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>HEHN</td>
<td>68</td>
<td>&gt;150</td>
<td>75</td>
<td>116</td>
</tr>
<tr>
<td>DEHN</td>
<td>50</td>
<td>&gt;150</td>
<td>65</td>
<td>32</td>
</tr>
<tr>
<td>MHN</td>
<td>18</td>
<td>7</td>
<td>&gt;150</td>
<td>58</td>
</tr>
<tr>
<td>DHTN</td>
<td>32</td>
<td>&gt;150</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>DAGN</td>
<td>110</td>
<td>30</td>
<td>&gt;150</td>
<td>145</td>
</tr>
<tr>
<td>NAGN</td>
<td>&gt;150</td>
<td>&gt;150</td>
<td>&gt;150</td>
<td>&gt;150</td>
</tr>
<tr>
<td>EAN</td>
<td>115</td>
<td>105</td>
<td>30</td>
<td>144</td>
</tr>
<tr>
<td>HDN</td>
<td>58</td>
<td>25</td>
<td>&gt;150</td>
<td>80</td>
</tr>
<tr>
<td>MAN</td>
<td>150</td>
<td>&gt;150</td>
<td>&gt;150</td>
<td>&gt;150</td>
</tr>
<tr>
<td>TN</td>
<td>&gt;150</td>
<td>&gt;150</td>
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<td>ATN</td>
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<td>&gt;150</td>
<td>&gt;150</td>
<td>&gt;150</td>
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<tr>
<td>DMTN</td>
<td>&gt;150</td>
<td>&gt;150</td>
<td>&gt;150</td>
<td>&gt;150</td>
</tr>
</tbody>
</table>
measure of toxicity, the relative toxicity is HZN > MHN > HDN > DAGN > EAN.

Glutathione is the principal intracellular nonprotein thiol that is the major source of reducing power in the cell (Sies, 1999). It provides a primary defense against oxidative stress by its ability to scavenge free radicals and thereby maintains cellular oxidation-reduction homeostasis. GSH protects cells against damage by scavenging highly reactive free radicals that can interact with critical cellular components. Monitored changes in GSH homeostasis provide an indicator of cell damage. The ability to scavenge free radicals and thereby maintains cellular oxidation-reduction homeostasis. GSH protects cells against damage by scavenging highly reactive free radicals that can interact with critical cellular components. Monitored changes in GSH homeostasis provide an indicator of cell damage.

We note that the majority of endpoints that are representative of the concentration-response curves for ROS and LDH are uncertain, in that they can only be expressed as lower limits, i.e., greater than the highest concentration tested (>150 mM). Therefore, QSARs were developed only for MTT and GSH. These two endpoints do not correlate very well with each other ($r^2 = 0.531$), as can be seen in the correlation matrix for the four endpoints (Table 2). QSARs for MTT and GSH are therefore not expected to be similar.

The chemical species used in the toxicity studies were the nitrate salts, which in solution consist of the protonated form of the HEC and [NO$_3^-$]. All QSARs, however, were derived using descriptors calculated from the neutral forms of the HECs. It may be that the descriptors for the neutral and protonated forms are similar. In addition, it is reasonable to assume that in solution the protonated and neutral forms of the HECs exist in equilibrium. If the neutral form is metabolically active and undergoes a chemical reaction, the equilibrium would be driven toward the neutral. Descriptors calculated from the neutral species would therefore be appropriate.

Employing lower limits as data is not recommended when deriving a QSAR, because changes in chemical structure will be mapped to a uniform toxic response. Doing so for MTT, however, results in the following, which is the 3-parameter QSAR obtained with the best $r^2$:

$$-\log EC_{25,\text{MTT}} = 18.0 + 0.493 E_{\text{HOMO}} - 1.82 E_C(NH) - 0.944 E_C(N-H),$$

where $E_{\text{HOMO}}$ is the energy of the highest occupied molecular orbital, $E_C(NH)$ is the maximum coulombic interaction (Lowe, 1978) for an N–H bond, and $E_C(N-H)$ is the minimum exchange energy (Lowe, 1978) for an N–H bond (values for descriptors for all QSARs appear in Table 3). The statistics for this equation are given in Table 4.

### TABLE 2

Correlation Matrix for Toxicity Endpoints

<table>
<thead>
<tr>
<th></th>
<th>EC25_MTT</th>
<th>LSC_ROS</th>
<th>EC25_LSD</th>
<th>EC50_GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC25_MTT</td>
<td>1.000</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>LSC_ROS</td>
<td>0.596</td>
<td>1.000</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>EC25_LSD</td>
<td>0.776</td>
<td>0.323</td>
<td>1.000</td>
<td>—</td>
</tr>
<tr>
<td>EC50_GSH</td>
<td>0.531</td>
<td>0.673</td>
<td>0.157</td>
<td>1.000</td>
</tr>
</tbody>
</table>

**Note.** Lower-limit values were included when calculating the $r^2$ values displayed.

### TABLE 3

Descriptor Values

<table>
<thead>
<tr>
<th>HEC</th>
<th>$E_{\text{HOMO}}$ (eV)</th>
<th>$\mu$ (debye)</th>
<th>BO$_{\sigma-\pi}$</th>
<th>BO$_x$</th>
<th>$E_C(N-H)$ (eV)</th>
<th>$E_C(N-H)$ (eV)</th>
<th>$\alpha$ (au)</th>
<th>$\gamma$ (au)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HZN</td>
<td>-9.1622</td>
<td>0.0010</td>
<td>0.0202</td>
<td>1.0198</td>
<td>5.4600</td>
<td>5.4269</td>
<td>11.3534</td>
<td>952.67</td>
</tr>
<tr>
<td>HEHN</td>
<td>-9.8400</td>
<td>2.2440</td>
<td>0.0305</td>
<td>1.0169</td>
<td>5.3482</td>
<td>5.4894</td>
<td>32.4849</td>
<td>1640.4e</td>
</tr>
<tr>
<td>DEHN</td>
<td>-9.5200</td>
<td>1.5460</td>
<td>0.0269</td>
<td>0.9979</td>
<td>5.3510</td>
<td>5.4907</td>
<td>47.4187</td>
<td>9909.3</td>
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<tr>
<td>MHN</td>
<td>-8.9655</td>
<td>0.7780</td>
<td>0.0254</td>
<td>1.0010</td>
<td>5.3615</td>
<td>5.4704</td>
<td>20.6411</td>
<td>2338.4</td>
</tr>
<tr>
<td>DHTN</td>
<td>-9.3835</td>
<td>0.0030</td>
<td>0.0473</td>
<td>1.5398</td>
<td>5.2059</td>
<td>5.5201</td>
<td>81.5196</td>
<td>11355.</td>
</tr>
<tr>
<td>DAGN</td>
<td>-9.6690</td>
<td>2.5970</td>
<td>0.0298</td>
<td>1.8811</td>
<td>5.2399</td>
<td>5.6601</td>
<td>40.2175</td>
<td>2318.2</td>
</tr>
<tr>
<td>NAGN</td>
<td>-10.4533</td>
<td>3.2020</td>
<td>0.0329</td>
<td>1.8500</td>
<td>5.1363</td>
<td>5.6550</td>
<td>51.0642</td>
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<td>5.2500</td>
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<td>0.2000</td>
<td>0.0357</td>
<td>0.9979</td>
<td>5.4442</td>
<td>5.4901</td>
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<td>TN</td>
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<td>5.3300</td>
<td>0.0165</td>
<td>1.5582</td>
<td>5.2208</td>
<td>5.6936</td>
<td>31.2049</td>
<td>2259.2e</td>
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</table>
| ATN | -10.1646               | 5.2460        | 0.0165            | 1.5956 | 5.4064          | 5.5317          | 38.7341     | 2658.2      

**Note.** $E_{\text{HOMO}}$, energy of the highest occupied molecular orbital. $\mu$, dipole moment. BO$_{\sigma-\pi}$, maximum $\sigma-\pi$ bond order. BO$_x$, maximum bond order of a nitrogen atom. $E_C(N-H)$, minimum exchange energy for an N–H bond. $E_C(N-H)$, maximum coulomb energy for an N–H bond. $\alpha$, polarizability. $\gamma$, second hyperpolarizability.
Predicted values of \(-\log EC_{25}\) calculated according to Equation 1 are plotted as a function of the experimental values in Figure 4. Although the statistics are good (Table 4), TN and ATN (two of the three HECs for which only lower-limit endpoints are known) are predicted incorrectly (both points should lie below the slope = 1 line if both the experimental and predicted EC_{25} values are > 150 mM). It is notable that TN and ATN are both triazoles and that the predicted EC_{25} value for NAGN is > 150 mM, which does not contradict experiment.

If the lower-limit data are excluded, the best 3-parameter QSAR obtained for MTT is

\[
-\log EC_{25} = 4.38 + 0.627 E_{\text{HOMO}} - 12.4 BO_{\sigma-\pi} - 0.390 BO_{\text{N}},
\]  

(2)

where \(BO_{\sigma-\pi}\) is the maximum \(\sigma-\pi\) bond order, and \(BO_{\text{N}}\) is the maximum bond order of a nitrogen atom. The statistics for Equation 2 (Table 4) are marginally better than those for Equation 1. More importantly, however, TN, ATN and NAGN (the three HECs for which only lower-limit endpoints are known) are all predicted to be > 150 mM and therefore do not contradict experiment (Fig. 5).

As shown in Figure 6, a 2-parameter QSAR derived excluding the lower-limit data can also acceptably describe MTT as well as predict the EC_{25} values for TN, ATN, and NAGN without contradicting experiment:

\[
-\log EC_{25} = 3.16 + 0.500 E_{\text{HOMO}} - 0.0971 \mu,
\]  

(3)

where \(\mu\) is the dipole moment (relevant statistics are given in Table 4). Equation 3 has the advantage that it describes the endpoint values in terms of well-understood descriptors.

All QSAR derived for GSH exclude lower-limit data (NAGN, MAN, TN, and ATN). MAN is always predicted incorrectly, i.e., EC_{50_{\text{GSH}}} < 150 mM. The two 3-parameter QSARs with the highest \(r^2\) both predict NAGN incorrectly as well as MAN. The 3-parameter QSAR with the third-highest \(r^2\) is

### Table 4

<table>
<thead>
<tr>
<th>Equation</th>
<th>N</th>
<th>(r^2)</th>
<th>F</th>
<th>SE</th>
<th>q</th>
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<td>37.8</td>
<td>0.0248</td>
<td>0.793</td>
</tr>
</tbody>
</table>

*Note.* N, number of HECs used to derive the QSAR. \(r^2\), correlation coefficient. F, Fischer criterion F value. SE, standard error. q, cross-validated correlation coefficient, calculated using a leave-one-out strategy, q characterizes the predictive power of the QSAR.
where $\alpha$ is the polarizability (see Table 4 for relevant statistics). As seen in Figure 7, MAN is predicted incorrectly by Equation 4, whereas the predictions for NAGN, ATN and TN do not contradict experiment. Interestingly, MAN is in an HEC category (amine based) represented in the data set that was used to derive Equation 4 whereas TN and ATN are not.

As in the 3-parameter QSARs for GSH, the two 2-parameter QSARs with the highest $r^2$ both predict NAGN incorrectly as well as MAN. The third-best 2-parameter QSAR for GSH is

$$-\log EC_{50_{GSH}} = -1.48 - 0.319 \mu + 0.00242 \gamma,$$

where $\gamma$ is the second hyperpolarizability (see Table 4 for relevant statistics). As seen in Figure 8, Equation 5 predicts GSH for NAGN, ATN, and TN without contradicting experiment, although the value for MAN is predicted incorrectly.

**DISCUSSION**

In general, our results are consistent with a previous study showing that hydrazine generates ROS and destabilizes mitochondrial function (Kerai and Timbrell, 1997). We also demonstrate that hydrazine-based compounds are generally more toxic than amine- and triazole-containing compounds. However, there are exceptions. For example, hydrazine-based NAGN is less toxic than two triazole-based HECs (EAN and HDN).

Increased generation of intracellular ROS by HECs is likely to contribute to oxidative stress, which may ultimately result in
cytotoxicity. Oxidative stress represents a potentially toxic insult, which if not counteracted leads to membranous dysfunction and protein and DNA damage (Loft and Poulsen, 1999; Preece and Timbrell, 1989). HEC toxicity as measured by EC50GSH, is moderately correlated to LECROS (Table 2), which is an indication of the induction of massive oxidative stress. Previously, it has been shown that the loss of GSH increased endogenous ROS to toxic levels in hepatocytes (Anundi et al., 1979), so it is possible that direct GSH depletion indirectly leads to cellular production of ROS as byproducts of normal cellular function. Although the exact mechanism is not known, the depletion of GSH and generation of ROS suggests that toxicity of HECs may be mediated through oxidative stress.

The E

HOMO descriptor pervades the best QSARs derived for MTT. According to Koopman’s theorem, the negative of the E

HOMO energy of a neutral species is its ionization potential (IP). This is consistent with the experimental evidence that the mechanism of toxic response, at least for hydrazine derivatives, involves oxidation, which, it is assumed, leads to formation of carbon-centered free radicals. A measure of the relative ease with which an HEC is oxidized, i.e., its IP, is therefore expected to be an apt descriptor, as it is in Equations 1–3. The low toxicity displayed by the amine-based HECs (except for HDN) and triazole-based HECs may be due to their high IPs, but this is hardly overwhelming evidence that the amines and triazoles act through the same mechanism as the hydrazines. It is conceivable that the HECs with low-lying HOMOs are not oxidized, and that any toxicity observed is the result of a different mechanism. In either case, however, the QSARs derived for MTT should be valid for all three classes of HEC. Only further testing will bear this out.

Although the statistics for Equation 1 are marginally better than Equation 2, the latter is preferred, because its predictions for all the lower-limit EC25

MTT values do not contradict experiment. Equation 2 contains two bond-order terms: maximum σ–π bond order (BO

σ–π) and maximum bond order involving a nitrogen atom (BO

N). Figure 9 identifies the bonds pertaining to each descriptor. For the HECs used to derive Equation 2, the bonds pertaining to BO

σ–π and BO

N differ for only three HECs (HDN, MAN, and DAGN). The identities of the bonds for BO

σ–π and BO

N differ for all three of the HECs (TN, ATN, and NAGN) for which only lower-limit EC25

MTT data are known. BO

N in Equation 2 can therefore be considered a correction for BO

N, or vice versa. Bond order is a measure of bond strength or resistance to bond breaking. One interpretation of Equation 2 is that toxicity can be mapped in part to the strength of the bonds pertaining to BO

σ–π, unless the bonds pertaining to BO

N are different (Fig. 9), in which case toxicity is mapped to a combination of the strengths of both bonds. The correspondence of EC25

MTT with the maximum σ–π bond order (BO

σ–π) is consistent with the mechanism postulated for alkyl hydrazines: the N–N bond resists breaking, and oxidation results in formation of carbon-centered free radicals.

Almost all the bonds in Figure 9 are associated with the HOMO. For all hydrazine HECs except DHTN, the HOMO is centered on the nitrogen atoms forming N–N bonds, which coincide with the bonds with maximum σ–π bond order (BO

σ–π). The electron density of the HOMO for DHTN is delocalized over the ring, but has maxima on the two nitrogens bonded to the ring. For EAN, TN, and ATN, the HOMO is centered on the nitrogen atom forming C–N bonds, which are also the bonds involving nitrogen with maximum bond order (BO

N). For MAN, the HOMO is centered on the sole nitrogen atom. For HDN, the HOMO is centered mostly on the ring carbons forming the C–C bond and, to a lesser extent, with the ring atoms forming the C–N bond. In general, HECs with higher toxicity have HOMOs associated with N–N bonds, and HECs with lower toxicity have HOMOs associated with C–N bonds.

Although the biophysical significance inferred by Equation 2 for the bonds portrayed in Figure 9 is in agreement with experiment, another explanation is also plausible. Consider the correlation coefficients of the individual descriptors in Equation 2. The HOMO energy correlates well with −log EC25 for
(r² = 0.72), whereas BO_N and BO_N correlate very poorly either individually (r² = 0.002 and 0.007, respectively) or in combination (r² = 0.03). The values BO_N and BO_N might only be fortuitous, in that they serve as corrections for the HOMO energy and therefore do not have any biophysical significance. The other, less statistically significant QSARs for MTT combine the HOMO energy with descriptors like polarity, partial charges, and dipole moment. These descriptors can be interpreted as relating to solubility and/or interaction of the HEC with an active site.

The QSARs derived for GSH (Equations 4 and 5) do not show a correlation with the HOMO energy (the HOMO energy correlates poorly with −log EC50_GSH; r² = 0.16), which infers that the toxic responses represented by EC25_MTT and EC50_GSH are mediated by different mechanisms. One hypothesis is that GSH depletion depends more on the reactivity of the free radicals formed from HEC oxidation and on subsequent metabolites, whereas mitochondrial function is more directly sensitive to initial products of the oxidation. It is known that hydrazine reduces mitochondrial function by inhibiting succinate dehydrogenase (Ghatineh et al., 1992). Oxidation of the hydrazine-based HECs produces NH₂NH²⁺, which may similarly reduce mitochondrial function. The appearance of BO_N in Equation 4 infers that the mechanism does not involve breaking the pertinent bonds (Fig. 9). However, most of the descriptors in the GSH QSARs (polarity, partial charges, dipole moment, etc.) relate to solubility and/or interaction of the HEC with an active site and are not very helpful in hypothesizing a mechanism. Note that although the QSARs for GSH do not offer insight into the biophysical mechanisms involved, they may be useful in predicting toxicity for additionally proposed propellant candidates.

In conclusion, hydrazine-based HECs are generally more toxic in in vitro toxicity assays than amine- and triazole-containing HECs. Our data suggest that one mechanism of HEC toxicity involves oxidative stress. The differences in the QSARs derived for MTT and GSH infer that cellular responses reflected in these endpoints are mediated by different mechanisms. It is notable that our QSARs encompass three chemical classes. Although the chemical species used in the toxicity studies were the nitrate salts, our QSARs were derived using descriptors calculated from the neutral species. Either the descriptors for the neutral and protonated species are similar, or the neutral species are metabolically active. Future QSARs derived using descriptors calculated from the protonated forms of the HECs will allow us to corroborate these suggestions and to further investigate the biophysical mechanism. In particular, the pKₐ of the protonated HECs may prove beneficial. In addition, if reactive metabolites are the actual mediators of the toxic responses, then perhaps better QSARs would result from consideration of chemical descriptors calculated from these metabolites.

Our ultimate goal is to predict human toxicity of HECs, which would necessitate construction of in vivo biokinetic models. Many of the descriptors calculated in this study for the HECs were not discussed because of their inapplicability to in vitro toxicity prediction. However, these descriptors may be useful in estimating the physicochemical properties of HECs and thereby find utility in the prediction of the in vivo kinetic behavior of HECs.

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REFERENCES


