Perspective

Immune-Mediated Agranulocytosis Caused by the Cocaine Adulterant Levamisole: A Case for Reactive Metabolite(s) Involvement

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ABSTRACT:

The United States Public Health Service Administration is alerting medical professionals that a substantial percentage of cocaine imported into the United States is adulterated with levamisole, a veterinary pharmaceutical that can cause blood cell disorders such as severe neutropenia and agranulocytosis. Levamisole was previously approved in combination with fluorouracil for the treatment of colon cancer; however, the drug was withdrawn from the U.S. market in 2000 because of the frequent occurrence of agranulocytosis. The detection of autoantibodies such as antithrombin (lupus anticoagulant) and an increased risk of agranulocytosis in patients carrying the human leukocyte antigen B27 genotype suggest that toxicity is immune-mediated. In this perspective, we provide an historical account of the levamisole/cocaine story as it first surfaced in 2008, including a succinct review of levamisole pharmacology, pharmacokinetics, and preclinical/clinical evidence for levamisole-induced agranulocytosis. Based on the available information on levamisole metabolism in humans, we propose that reactive metabolite formation is the rate-limiting step in the etiology of agranulocytosis associated with levamisole, in a manner similar to other drugs (e.g., propylthiouracil, methimazole, captopril, etc.) associated with blood dyscrasias. Finally, considering the toxicity associated with levamisole, we propose that the 2,3,5,6-tetrahydroimidazo[2,1-b]thiazole scaffold found in levamisole be categorized as a new structural alert, which is to be avoided in drug design.

Agranulocytosis Associated with Cocaine Use: Identification of the Cause

In the summer of 2008, a man and woman, both in their twenties, were separately admitted to a Canadian hospital with unrelenting fevers, flu-like symptoms, and dangerously low white blood cell counts. Their symptoms were consistent with a life-threatening disorder known as agranulocytosis (Chang et al., 2010). Agranulocytosis is an immune disorder, typically caused by chemotherapy or highly individualized and unexpected (idiosyncratic) reaction to certain nonchemotherapy drugs such as the antipsychotic clozapine and the antibiotic combination trimethoprim-sulfamethoxazole (Ibañez et al., 2012). Nonchemotherapy drugs such as the antipsychotic clozapine and the antibiotic combination trimethoprim-sulfamethoxazole (Ibañez et al., 2012) have been associated with agranulocytosis (Macfarlane and Bacon, 1978).

There have now been several dozen cases of cocaine-related agranulocytosis (including one death) reported in Canada (British Columbia, Alberta) and the United States of America (Colorado, Arizona, New Mexico, and Washington) (Waller et al., 2010; Walsh et al., 2010). Similarities in the onset of symptoms in almost all cases included severe neutropenia (absolute neutrophil count <0.5 × 10^9 cells/l), symptoms of infectious illness (fever and malaise or sore throat), and a disproportionally high association with a positive test for cocaine and its metabolites and, surprisingly, the presence of levamisole (6-phenyl-2,3,5,6-tetrahydroimidazo[2,1-b]thiazole) in the blood of those patients who had snorted, injected, or smoked cocaine. In the following year, a few cocaine addicts in San Francisco, mostly crack (a solid smokable form of cocaine) smokers, began displaying even stranger symptoms such as death, darkened skin. In the Canadian case, toxicological analysis of urine specimens from the two patients revealed the presence of cocaine and its metabolites and, surprisingly, the presence of levamisole (6-phenyl-2,3,5,6-tetrahydroimidazo[2,1-b]thiazole; Fig. 1), a drug that was once used to treat colon cancer but is now reserved for veterinary use as an anthelmintic (Zhu et al., 2009). Since the 1970s, clinical use of levamisole has been associated with cases of agranulocytosis (Macfarlane and Bacon, 1978).

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ABBREVIATIONS: HLA, human leukocyte antigen; DEA, U.S. Drug Enforcement Agency; t_{1/2}, plasma elimination half-life; F, oral bioavailability; P450, cytochrome P450; T_{max}, time to attain maximal systemic exposure; MHC, major histocompatibility complex; RM, reactive metabolite; MPO, myeloperoxidase; ACE, angiotensin-converting enzyme.
for human leukocyte antigen (HLA) B27. The “dead darkened skin” (noted in California patients) was the result of epidermal infections related to the patients’ compromised immune function. Most patients recovered fully after treatment with intravenous antibiotics and granulocyte colony-stimulating factor; however, neutropenia reoccurred in a subset of these individuals, presumably caused by cocaine-levamisole use after the initial episode. Above all, the fatality that occurred via complications from opportunistic infections as a result of agranulocytosis, with positive toxicology for cocaine and levamisole, highlights the potential dangers of the phenomenon.

With an estimated two million cocaine users in the United States, this new adverse effect associated with cocaine use has caught the media’s attention (e.g., Time magazine and major news networks). Scientific publications are alerting physicians and public health officials to the potentially life-threatening side effects of cocaine contaminated with levamisole. Several government agencies have responded with alerts highlighting the dangers of levamisole-tainted cocaine. An illustration of such a warning is evident in the nationwide public alert issued in September of 2009 by the U.S. Department of Health and Human Services Substance Abuse and Mental Health Services Administration. The news release warned: "A dangerous substance, levamisole, is showing up with increasing frequency in illicit cocaine powder and crack cocaine. Levamisole can severely reduce the number of white blood cells, a problem called agranulocytosis. This is a very serious illness that needs to be treated at a hospital. If you use cocaine, watch out for: high fever, chills, or weakness, swollen glands, painful sores (mouth, anal), any infection that won’t go away or gets worse very fast, including sore throat or mouth sores, skin infections, abscesses, thrush (white coating of the mouth, tongue, or throat), pneumonia (fever, cough, shortness of breath)."

It is common practice to “cut” cocaine with adulterants to increase the amount of product and increase profits. These adulterants are generally of similar appearance and/or physicochemical behavior, and they are typically inert or relatively harmless chemicals. The adulterants can be devoid of pharmacological activity (e.g., sugars such as mannitol, lactose, sucrose, etc.), or they can be more widely available pharmaceutical agents (e.g., caffeine, benzoic acid, lidocaine, methylphenidate, etc.), which are thought to enhance the feelings associated with cocaine. For example, upon ingestion, cocaine numbs the gums, and because the anesthetic action of lidocaine causes stronger numbness, users get the impression of high-quality cocaine when in reality a “diluted” product is being consumed. More recently, levamisole appears to be added as a cutting agent from the outset, in countries of origin. The adulteration of seized cocaine bricks with levamisole was documented by the U.S. Drug Enforcement Agency (DEA) as early as 2005. Levamisole contaminated less than 10% of cocaine seized by the DEA in 2007, and rose to 30% from July to September 2008 (Casale et al., 2008). This number increased to 69% in July 2009. Seized crack cocaine described in the 2008 DEA report also contained 6% levamisole. DEA unpublished data in 2009 noted an average concentration of approximately 10% levamisole detected in cocaine. As will be discussed later, levamisole’s half-life in blood is short, less than 6 h, which makes it difficult to positively diagnose whether the symptoms associated with agranulocytosis truly arise from levamisole exposure. However, in addition to levamisole detection in urine, Buchanan et al. (2010) recently demonstrated the presence of levamisole (as high as 10%) in a patient’s crack cocaine pipe, which confirms levamisole as a cocaine adulterant.

**Therapeutic Applications of Levamisole**

The racemic form of levamisole, i.e., tetramisole (Fig. 1) was first disclosed as an anthelmintic agent in the 1960s by Janssen Pharmaceuticals. Levamisole is the levo-isomer of tetramisole, and it is several-fold more potent as an antiparasitic than the dextrorotatory isomer, dexamisole (Fig. 1). Levamisole was introduced as a broad spectrum veterinary anthelmintic in 1965 and anthelmintic in humans in 1966. Levamisole causes paralysis and passive elimination of worms, by inhibition of fumarate reductase in nematodes (Janssen, 1976). Presently, its utility in the United States is limited to veterinary use for the eradication of nematode infections. However, in parts of the world...
where parasitic infections are common, levamisole continues to be prescribed in humans (Albonico et al., 2003). Levamisole is also used to treat childhood nephrotic syndrome (excessive levels of protein in the urine) outside the United States (Hodson, 2003). Serendipitous results on de novo pharmacology have been reported with levamisole, including apparent beneficial effects on host defense mechanism and enhanced immunologic protection against bacterial and viral pathogens (Janssen, 1976; Amery and Bruyneels, 1992). In the 1990s, several reviews about levamisole’s efficacy as a human immune system modulator appeared in the literature, which focused on its potential beneficial effects in cancer treatment. Results were mixed with some trials showing enhanced chemotherapeutic activity (Green and Erlich, 1991; De Brabander et al., 1992). Outside the United States, levamisole has been used as an immunomodulator in treating rheumatoid arthritis, AIDS, ulcerative colitis, chronic hepatitis B, nephritic syndrome, malignant melanoma, breast cancer, acute myeloid leukemia, and amyotrophic lateral sclerosis, with inconclusive results. In 1991, the racemic form of levamisole hydrochloride (marketed under the trade name of Ergamisol) was approved by the U.S. Food and Drug Administration for use as adjuvant therapy with fluorouracil in the treatment of colorectal cancer. “However, the drug was voluntarily removed from the U.S. market in 2000 because of the common occurrence of agranulocytosis” (Ullrich et al., 2011).

**Agranulocytosis As a Side Effect of Levamisole Therapy**

As early as 1977, it was observed that agranulocytosis is caused by the therapeutic use of levamisole, leaving patients susceptible to fulminate and opportunistic infections (Macfarlane and Bacon, 1978). Reports of ear lobe and cutaneous necrotizing vasculitis have also been reported in the literature after levamisole use in the treatment of cancers, childhood nephrotic syndrome, and rheumatologic disorders, and in individuals who had ingested levamisole-tainted cocaine (Rongoletti et al., 1999; Buchanan et al., 2011). Over the years, the incidence of mild to serious blood disorders, including hematologic depression, agranulocytosis, and leukopenia, has been noted in several clinical investigations with levamisole (Moertel et al., 1995; Ejlertsen et al., 2010). Agranulocytosis was seen in 2.5 to 13% of individuals using levamisole clinically (Symoens et al., 1978). Furthermore, dose dependence was demonstrated in cancer trials involving 2635 patients, wherein 3.1% of the patients developed agranulocytosis when dosed at 2.5 mg/kg levamisole (approximate translation to a 175-mg daily dose based on a average body mass of 70 kg) for 2 consecutive days every week as opposed to 0.1% of patients developing agranulocytosis when dosed at 2.5 mg/kg levamisole for 3 consecutive days every other week (Amery and Butterworth, 1983). Available clinical data suggests that agranulocytosis is reversible upon discontinuation of levamisole therapy, and it appears to occur more frequently in female patients than in male patients (Symoens et al., 1978; Amery and Butterworth, 1983). As such, female gender has been the subject of discussion in idiiosyncratic drug-induced agranulocytosis, and several studies have noted the preponderance of agranulocytosis in females relative to men (Ibáñez et al., 2005).

**Why Is Levamisole Used as a Cocaine Adulterant?**

Speculation of why levamisole has been used as a cocaine adulterant centers on two factors: namely, availability and enhanced pharmacologic effect. The simplest postulate for the inclusion of levamisole as a cutting agent in cocaine is its low cost, right look (taste and melting point relative to cocaine), and easy accessibility in highly agrarian developing nations, many of which are implicated as the regions in which the tainted cocaine is produced. Multigram quantities of levamisole are required for the treatment of afflicted animals (often over several consecutive days), and, in an effort to avoid costly parasitic helminth infestations in their herds, many farmers have opted for prophylactic treatment of all animals (Waller, 2006), thus increasing quantities being sold in these same developing regions. Emergence of anthelmintic-resistant organisms may have also increased the availability of levamisole as an uninformed farming populace administers progressively larger doses to afflicted herds.

A second hypothesis is that levamisole is intentionally added to street cocaine because it potentiates the effects of cocaine. Mood-elevating effects have been reported in humans as a side effect of adjuvant therapy with levamisole for colon cancer (Goldin et al., 1982). Alternatively, after levamisole administration to race horses, the identification of the stimulant aminorex as a circulating metabolite of levamisole (Fig. 1) suggests that a similar metabolic fate in humans would lead to enhanced hypertensive stimulation, a common sensation associated with cocaine use (Gaine et al., 2000). Studies in animals have shown that the pharmacodynamics of aminorex are similar to those of cocaine and amphetamines with respect to their stimulant effects and indirect sympathomimetic effects in the central nervous system (Young, 1992). As such, the hypothesis is strengthened based on the recent findings of Bertol et al. (2011) on the detection of aminorex as a urinary metabolite of levamisole in humans.

**Disposition of Levamisole in Animals and Humans**

Details on levamisole pharmacokinetics are available in dogs and humans (Table 1). In humans, levamisole is rapidly absorbed ($T_{\text{max}} = 1.5$ h) after oral administration at a dose of 150 mg, with an approximate oral bioavailability ($F$) of 62.5% (Kouassi et al., 1986). The plasma elimination half-life ($t_{1/2}$) of levamisole is estimated to be 5.6 h in humans (Woestenborghs et al., 1981). In dogs, after a 10 mg/kg i.v. dose, levamisole exhibits moderate plasma clearance and steady-state distribution volume of 8.92 ml·min$^{-1}$·kg$^{-1}$ and 1.42 l/kg, respectively, yielding a $T_{1/2}$ of 1.8 h. The corresponding $F$ in dogs ranges from 44% in fed animals to 64% in fasted animals (Watson et al., 1982). Alternatively, after levamisole administration to race horses, the identification of the stimulant aminorex as a circulating metabolite of levamisole (Fig. 1) suggests that a similar metabolic fate in humans would lead to enhanced hypertensive stimulation, a common sensation associated with cocaine use (Gaine et al., 2000). Studies in animals have shown that the pharmacodynamics of aminorex are similar to those of cocaine and amphetamines with respect to their stimulant effects and indirect sympathomimetic effects in the central nervous system (Young, 1992). As such, the hypothesis is strengthened based on the recent findings of Bertol et al. (2011) on the detection of aminorex as a urinary metabolite of levamisole in humans.

**TABLE 1**

<table>
<thead>
<tr>
<th>Species</th>
<th>Gender</th>
<th>Dose</th>
<th>Route</th>
<th>CL$_{p}$</th>
<th>$t_{1/2}$</th>
<th>Vd$_{ss}$</th>
<th>AUC</th>
<th>$C_{\text{max}}$</th>
<th>$T_{\text{max}}$</th>
<th>CL$_{\text{renal}}$</th>
<th>$F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>M and F</td>
<td>150 mg</td>
<td>p.o.</td>
<td>8.04$^a$</td>
<td>5.6</td>
<td>3.80$^a$</td>
<td>3070</td>
<td>717</td>
<td>1.5</td>
<td>1.75</td>
<td>62.5$^a$</td>
</tr>
<tr>
<td></td>
<td>M (n = 7)</td>
<td>150 mg</td>
<td>p.o.</td>
<td>8.25$^a$</td>
<td>5.4</td>
<td>3.79$^a$</td>
<td>3070</td>
<td>672</td>
<td>1.7</td>
<td>1.86</td>
<td>61.5</td>
</tr>
<tr>
<td>F (n = 3)</td>
<td>150 mg</td>
<td>p.o.</td>
<td>7.56$^a$</td>
<td>5.9</td>
<td>3.83$^a$</td>
<td>3070</td>
<td>820</td>
<td>1.1</td>
<td>1.50</td>
<td>64.7</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>M and F</td>
<td>10 mg/kg</td>
<td>i.v.</td>
<td>8.92</td>
<td>1.8</td>
<td>1.42</td>
<td>18,100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>p.o. (fasted)</td>
<td>1.3</td>
<td>1.39</td>
<td>12,200</td>
<td>3330</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>p.o. (fed)</td>
<td>1.6</td>
<td>2.64</td>
<td>8940</td>
<td>1980</td>
<td>4.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

p.o., oral; i.v., intravenous; CL$_{p}$, plasma clearance; Vd$_{ss}$, steady-state distribution volume; AUC, area under the plasma concentration time curve; $C_{\text{max}}$, maximal plasma concentration; CL$_{\text{renal}}$, renal clearance of unchanged parent compound.

$^a$The calculations are based on the estimated oral bioavailability, assuming average human body weight of 70 kg.
al., 1988). Human mass balance studies with orally administered \([^{3}H] \text{levamisole} \) indicate metabolism as a predominant route of elimination (Adams, 1978; Schnieden, 1981; Kouassi et al., 1986). In contrast with the moderate \(t_{1/2}\) of levamisole \((\approx 5.6 \text{ h})\), the \(t_{1/2}\) of circulating levels of radioactivity, which is attributed to levamisole metabolites, is much longer at \(\approx 16 \text{ h}\). Approximately 70% of the radioactivity is excreted by the kidney in the form of metabolites; renal excretion of unchanged levamisole accounts for \(3 \sim 4\%\) of the administered dose. To date, an oxidative metabolite \(p\)-hydroxy-levamisole \((1)\) and its corresponding glucuronide conjugate \(2\) (Fig. 1) have been identified in urine that collectively account for \(\approx 25\%\) of the administered levamisole dose. The identities of the additional urinary components that amount to \(\approx 45\%\) of urinary radioactivity remain uncharacterized. In vitro metabolism studies in human hepatic tissue, however, paint a more elaborate picture of levamisole metabolism. Apart from the formation of \(1\), in vitro incubations of \([^{14}C] \text{levamisole}\) in human hepatocytes indicate the presence of several additional metabolites \(3 \sim 8\) (Fig. 1) (Janssen, 1976; Symoens et al., 1979; Roberts, 1994). With the exception of \(3\), which can arise from a cytochrome P450 (P450)-mediated imidazolidine ring dehydrogenation, the rate-limiting step in the formation of \(4 \sim 8\) appears to be mediated by a ring scission of the thiazolidine motif. Thus, the formation of \(4\) can be rationalized to proceed via P450-mediated \(\alpha\)-carbon hydroxylation (adjacent to the sulfur), followed by a spontaneous ring opening to the aldehyde intermediate, which upon oxidation would generate the carboxylic acid metabolite \(4\). Subsequent desulfurization and/or \(N\)-dealkylation in \(4\) would lead to the corresponding imidazolidin-2-one and imidazolidine-2-thione metabolites \(5\) and \(6\), respectively. The thiazolidine ring opening pathway leading to the free thiol metabolite \(3\) \((2\text{-mercaptoethyl})\) phenylimidazolidine-2-one \((7)\) contrasts the sequence of reactions proposed in the case of \(4\), because one can envision its formation through an oxidative (or hydrolytic) cleavage of the imine bond in levamisole. Metabolite \(7\) can also be generated chemically by exposing levamisole to alkaline media (Symoens et al., 1979). \(S\)-Methylation/\(S\)-oxidation of the free thiol group in \(7\) would generate sulfoxide \(8\). Apart from the role of hepatic metabolism in levamisole elimination, Shu et al. (1991) have also characterized novel hydroxamic acid lactam-type metabolites (compounds \(9 \sim 11\), Fig. 2) from anaerobic incubations of levamisole in human intestinal bacteria.

Recent investigations into the components present in adulterated cocaine have found a change in the purity of levamisole, where it once was pure “pharmaceutical-grade,” and more recent samples have been shown to contain levamisole degradation products such as compounds \(3\) and \(7\) (Casale et al., 2008). These impurities are believed to have originated from either a degradation of the levamisole during the production of crack cocaine or from the use of levamisole created through alternate (nonpharmaceutical) means.

Based on the available information comparing the in vitro and in vivo metabolism of levamisole in humans and preclinical species (Koyama et al., 1983; Roberts, 1994), there appear to be no human-specific metabolites of levamisole. In preclinical species (e.g., rats,
dogs, and monkeys), a novel biotransformation sequence involving imidazoline ring dehydrogenation of \( p \)-hydroxylevamisole (1) (or vice versa) to yield 12 followed by \( S \)-oxidation to metabolites 13 and 14 also has been noted (Fig. 1). The in vivo relevance of levamisole metabolites generated through in vitro systems and the identities of the drug-metabolizing enzymes responsible for levamisole metabolism in human remain to be elucidated. It is possible that the unaccounted urinary radioactivity in the human mass balance study with levamisole consists of the metabolites observed in the in vitro metabolism study in hepatocytes.

**Levamisole Toxicological Findings in Preclinical Species**

Several short- and long-term preclinical safety studies have been conducted with levamisole in rodents, dogs, and nonhuman primates. Results observed in short- and long-term safety studies (Roberts, 1994) in rodents were deemed to be of minimal clinical significance with findings indicative of treatment-related decreases in weight gain, food consumption, increases in liver and kidney weights, and urine pH. Most importantly, no hematological changes consistent with agranulocytosis were observed in rodents in any of the cited toxicity studies. Likewise, single dose of levamisole in primates showed no hematological changes.

The results of short-term toxicity studies (duration <90 days) in dogs reported no hematological changes at levamisole doses up to 20 mg/kg per day. In contrast, chronic studies revealed significant hematotoxicity within 8 weeks of dosing at 5.0 or 20 mg/kg per day, but not at the 1.25 mg/kg per day dose, indicating that hematotoxicity associated with levamisole in dogs is dose-dependent in a manner similar to the observations in humans (Roberts, 1994). Within this study, one female dog dosed at 5 mg/kg per day and all six dogs (male and female) dosed at 20 mg/kg per day exhibited severe hemolytic anemia as measured by decreases in hematocrit, hemoglobin, and red blood cell count along with increases in erythroblasts and immature granulocytes. The hematological parameters returned to normal approximately 2 weeks after withdrawal of treatment. However, upon rechallenge, the onset of hematological toxicities was rapid. In a large, long-term hematological study, 25 of 100 dogs dosed with 20 mg/kg per day for 14 weeks showed hemolytic anemia, suggesting an immune component to the hematological changes. Overall, the preclinical toxicity findings with levamisole suggest that the dog represents a potential model for assessing agranulocytosis potential of drugs. As such, this hypothesis has reasonable credibility considering that the blood dyscrasias associated with cephalosporin antibiotics cefonicid and cefazedone in humans also have been discerned in dogs (Bloom et al., 1987). In addition, preclinical toxicity assessments of an atypical antipsychotic drug candidate DMP406—structurally related to clozapine (Fig. 3)—revealed severe neutropenia in several female dogs over the course of a 3-month study, which led to suspension of this agent from clinical consideration (Lorenz et al., 1999).

**FIG. 4. Illustrations of HLA associations with drug hypersensitivity reactions.**

**Levamisole**

*Daily dose = 150 - 175 mg*

MHC class I associations: HLA B27

- Agranulocytosis

**Abacavir**

*Daily dose = 600 mg*

MHC class I associations: HLA B*57:01

- Hypersensitivity
- Hepatotoxicity

**Carbamazepine**

*Daily dose = 1600 mg*

MHC class I associations: HLA B*15:02

- Agranulocytosis
- Stevens Johnson syndrome
- Toxic epidermal necrolysis

**Sulfamethoxazole**

*Daily dose = 2400 mg*

MHC class I associations: HLA B A30, B13, Cw6

- Agranulocytosis
- Stevens Johnson syndrome
- Toxic epidermal necrolysis

**Flucloxacillin**

*Daily dose = 4000-8000 mg*

MHC class I associations: HLA B*57:01

- Hepatotoxicity
The precise mechanism by which levamisole causes agranulocytosis is unknown; however, it is thought to be immune-mediated as evidenced by the detection of autoantibodies such as antithrombin (lupus anticoagulant) and others. As indicated earlier, the association between levamisole use and agranulocytosis is greater in patients carrying the HLA B27 genotype, thereby indicating a possible genetic predisposition to the condition (Mielants and Veys, 1978; Hodinka et al., 1981). HLA B27 is a class I surface antigen encoded by the B locus in the major histocompatibility complex (MHC) on chromosome 6, and it is involved in the encoding of cell-surface receptors that capture and present self- and pathogen-derived peptides to T cells as part of an immune surveillance. The clinical characteristics of levamisole-induced agranulocytosis (slow onset and a more rapid occurrence upon rechallenge) are all signs of a delayed-type hypersensitivity syndrome, which could potentially arise through levamisole-induced T-cell activation via the HLA B27 genotype. As such, delayed drug hypersensitivity mediated by MHC class I alleles also has been noted with several drugs (Fig. 4), including abacavir, carbamazepine, sulfamethoxazole, and fluclouxacinin (Bharadwaj et al., 2012). In several of these cases, the mechanism for HLA/MHC-dependent, T-cell stimulation is thought to involve formation of an electrophilic reactive metabolite (RM) via bioactivation of the drug. The electrophilic RM reacts with a self-protein or peptide to generate a hapten, which then undergoes antigen processing to a novel MHC ligand that is trafficked to the cell surface, where it activates antigen-specific T cells (Pohl et al., 1988; Pichler et al., 2002). Haptenization via RM formation in peripheral blood cells has also been implicated in agranulocytosis associated with several structurally diverse drugs such as amnopyrine, clozapine, sulfamethoxazole, ticlopidine, methimazole, etc. (Fig. 3) (Uetrecht, 1992). Peripheral blood leukocytes contain a variety of enzymes that are capable of metabolizing xenobiotics including drugs. The enzyme myeloperoxidase (MPO) appears to be the most important in the metabolic activation of drugs to RMs. MPO is a peroxidase and generates the powerful oxidant hypochlorous acid, which is capable of oxidizing a broad range of electron-rich functional groups, especially those that contain oxygen, sulfur, and nitrogen (Hofstra and Uetrecht, 1993).

Other than a temporal relationship with prolonged high dosage, very little is known regarding the pathogenesis of levamisole-induced agranulocytosis. Hapten formation by levamisole or its metabolite(s) has been considered on the basis of evidence of granulocytotoxic activity upon addition of levamisole to sera of patients with agranulocytosis (Parkinson et al., 1977). Considering the volume of evidence linking RM formation with agranulocytosis, we hypothesize that a downstream metabolite(s) of levamisole is metabolized by MPO (or other oxidative enzymes such as P450, NADPH oxidase, or prostaglandin synthase) to yield a protein-reactive metabolite(s) capable of inducing immune-mediated blood dyscrasias. If this were to be the case, we speculate that the thiourea metabolites (i.e., compounds 4 and 6) are prime suspects. This is not unreasonable considering that related thiourea deriva-

FIG. 5. Are the thiourea metabolites of levamisole (compounds 4 and 6) responsible for agranulocytosis? Structure-toxicity relationships with thiourea-based drugs associated with hematotoxicity.
tives such as metiamide, methimazole, and propylthiouracil (Fig. 5) are also associated with significant incidences of immune-mediated agranulocytosis. The histamine H2 receptor antagonist metiamide was suspended from clinical trials for the treatment of peptic ulcers because an unacceptable number of patients developed agranulocytosis (Forrest et al., 1975). From a structure-toxicity perspective, cimetidine (see Fig. 5) does not contain the thiourea motif present in metiamide and is not associated with agranulocytosis, which then implies metiamide’s thiourea functionality as a causative factor in the resulting toxicity.

FIG. 6. Potential oxidative bioactivation of the phenol metabolite of levamisole (i.e., compound 1) by myeloperoxidase or P450 enzymes to an electrophilic quinone-methide species: similarity with the oxidation of eugenol and trimethoprim.

Structures-toxicity relationships in ACE inhibitors

Fig. 7. Is the free thiol metabolite of levamisole (compound 7) responsible for agranulocytosis? Examples of free thiol drugs associated with blood dyscrasias.

(associated with agranulocytosis)

(not associated with agranulocytosis)
Methimazole and propylthiouracil are cyclic thiourea derivatives that are used extensively in the treatment of Grave’s disease. However, their use is associated with hypersensitivity reactions including a significant incidence of agranulocytosis (0.3–0.4%) (Cooper et al., 1983). Oxidative metabolism of both drugs to RMs is believed to be a causative factor for agranulocytosis. Thus, incubation of [15]C-propylthiouracil in human polymorphonuclear leukocytes and/or MPO leads to covalent binding, mainly through disulfide bonds between protein and a RM of propylthiouracil (Lam and Lindsay, 1979; Lee et al., 1990). As shown in Fig. 5, propylthiouracil-sulfenyl chloride, which can be generated from propylthiouracil chlorination by MPO, has been proposed as the RM that adds to cysteinyi residues on proteins to yield disulfide adducts. Further oxidation of the sulfenyl chloride intermediate yields the corresponding propylthiouracil-disulfide, propylthiouracil-2-sulfinate, and the propylthiouracil-2-sulfonate derivatives, which have been detected as metabolites in propylthiouracil incubations with neutrophils and MPO (Waldhauser and Utrecht, 1991). Furthermore, the facile adduction of the propylthiouracil-2-sulfonate metabolite with sulfydryl nucleophiles, such as N-acetylcyesteine and 3-mercaptopropionic acid (Fig. 5), in the in vitro incubations is suggestive of analogous reactivity with sulfhydryl residues on proteins. In a similar fashion (see Fig. 5), methimazole is also subject to oxidation on the thiourea sulfur in the presence of hypochlorous acid, MPO/H2O2/OCl− system, and/or human neutrophils resulting in the formation of an unstable disulfide metabolite (presumably via the sulfenyl chloride intermediate) (Sayo and Saito, 1991).

Visual examination of the structures of levamisole and its metabolites also suggest that the electron-rich phenol metabolite 1 is a potential candidate for MPO- or P450-catalyzed two-electron oxidation sequence to quinonoid species 15, in a manner similar to that noted for eugenol (Thompson et al., 1999) and the antibacterial agent trimethoprim (Lai et al., 1999), which are associated with idiosyncratic agranulocytosis (Fig. 6). Whether 15 possesses sufficient reactivity toward covalent adduction to proteins remains unclear, because 15 could easily tautomerize to yield the stable nonreactive dihydroimidazole metabolite 12 (see Fig. 6).

Finally, it is possible that the free thiol metabolite 7 of levamisole (Fig. 7) can also play a role in inducing agranulocytosis, similar to the thiol derivatives captopril (Amann et al., 1980; Pillans and Koopowitz A, 1991) and penicillamine (Kean et al., 1980; Umeki et al., 1985) (Fig. 7), which are associated with agranulocytosis in the clinic. Captopril was the first marketed angiotensin-converting enzyme (ACE) inhibitor for the treatment of hypertension. When first marketed, captopril was administered at doses up to 1000 mg in severely hypertensive patients. A series of systemic adverse reactions including skin rashes, agranulocytosis, and autoimmune syndromes were reported, and the dose dependence of these effects was observed across studies (Wilkin et al., 1980). Mixed disulfide conjugates of the free thiol group in captopril with reduced GSH and with cellular proteins have been observed and have implicated bioactivation of the sulfhydryl functionality as a plausible cause for toxicity including agranulocytosis (Yeung et al., 1983; Migdalof et al., 1984). From a structure-toxicity relationship standpoint, it is interesting to note that the newer ACE inhibitors such as lisinopril, benazepril, enalapril, and ramipril lack the free thiol group (Fig. 7) and are rarely associated with agranulocytosis.

An empirical correlation has been made for the occurrence of idiosyncratic drug toxicity and the daily dose. Low daily dose drugs (10 mg or less) are rarely associated with idiosyncratic adverse reactions regardless of their ability to form RMs. While the amount of levamisole ingested during cocaine use remains unclear, it is important to note that agranulocytosis with levamisole occurs more frequently at the high dose of 150 mg. The situation is analogous with the high daily dose drugs depicted in Fig. 3, which are associated with agranulocytosis. As such, future studies that will address protein covalent binding and/or RM formation with levamisole in activated neutrophils/monocytes, recombinant myeloperoxidase, and perhaps even human hepatic tissue should confirm (or refute) our hypothesis on levamisole bioactivation to protein-reactive species as a causative factor for the blood dyscrasias. Regardless of the outcome of such future studies on levamisole bioactivation, we suggest that the levamisole scaffold be classified as a “structural alert,” and should be avoided by medicinal chemists in drug design.

Authorship Contributions

Participated in research design: Wolford, McDonald, Eng, Hansel, Chen, Bauman, Sharma, and Kalogutkar.

Performed data analysis: Wolford, McDonald, Eng, Hansel, Chen, Bau- man, Sharma, and Kalogutkar.

Wrote or contributed to the writing of the manuscript: Wolford, McDonald, Eng, Hansel, Chen, Bauman, Sharma, and Kalogutkar.

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