

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

Angela Wolford, Thomas S. McDonald, Heather Eng, Steven Hansel, Yue Chen, Jonathan Bauman,
Raman Sharma and Amit S. Kalgutkar

Pharmacokinetics, Dynamics and Metabolism Department (A. W., T. S. M., H. E., S. H., Y. C., J. B., R. S.), Pfizer Worldwide Research and Development, Groton, CT 06340

Pharmacokinetics, Dynamics and Metabolism Department (A. S. K.), Pfizer Worldwide Research and Development, Cambridge, MA 02139

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Address correspondence to: Amit S. Kalgutkar, Pharmacokinetics, Dynamics, and Metabolism

Department, Pfizer Global Research and Development, Cambridge, MA 02139. Phone: (617)-551-

3336. E-mail: amit.kalgutkar@pfizer.com

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Abbreviations used are: Levamisole, 6-phenyl-2,3,5,6-tetrahydroimidazo[2,1-*b*]thiazole; HLA, human leukocyte antigen; DEA, drug enforcement agency; CL_p , plasma clearance, $V_{d_{ss}}$, steady state distribution volume; $t_{1/2}$, plasma elimination half-life; F, oral bioavailability; T_{max} , time to attain maximal systemic exposure; CYP, cytochrome P450; MHC, major histocompatibility complex; RM, reactive metabolite; MPO, myeloperoxidase; ACE, angiotensin-converting enzyme; GSH, reduced glutathione.

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was previously approved in combination with fluorouracil for the treatment of colon cancer; however, the drug was withdrawn from the US market in 2000 because of the frequent occurrence of agranulocytosis.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 HLA 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 unremitting fevers, flulike 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 non-chemotherapy drugs such as the antipsychotic clozapine and the antibiotic combination trimethoprim-sulfamethoxazole (Ibáñez *et al.* 2005). Neither of the Canadian patients fit that description, but they did have one thing in common—illegal use of cocaine. In April of that same year, a New Mexico laboratory in the United States had also identified a small number of unexplained cases of agranulocytosis in people who had snorted, injected or smoked cocaine. In the following year, a few cocaine addicts in San Francisco, mostly crack smokers, began displaying even stranger symptoms like dead, 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, Figure 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).

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 $< 0.5 \times 10^9$ cells/l), symptoms of infectious illness (fever and malaise or sore throat), and a disproportionately high association with a positive test for human leukocyte antigen (HLA) B27. The “dead darkened skin” noted in California patients were the result of epidermal infections related to the patient’s compromised immune function. Most patients recovered fully following 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 (Zhu *et al.* 2009; Chang *et al.* 2010; Buchanan *et al.* 2011; Ullrich *et al.* 2011). 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 US 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 in order to increase the amount of product and increase profits. These adulterants are generally of similar appearance and/or physicochemical behavior, and 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 can be more widely available pharmacological agents (e.g., caffeine, benzocaine, lidocaine, methylphenidate, etc.), which are thought to enhance the feelings associated with cocaine. For example, upon ingestion, cocaine

numbs the gums, and since 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 United States 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 (a solid smokable form of 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 six hours, 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 (Figure 1) was first disclosed as an anthelmintic agent in the 1960’s by Janssen Pharmaceutica. Levamisole is the levo-isomer of tetramisole, and is several fold more potent as an anti-parasitic than the dextrorotatory isomer, dexamisole (Figure 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 fumerate 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 Bruynseels, 1992). In the 1990's 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 (Greenspan 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 United States Food and Drug Administration for use as adjuvant therapy with fluorouracil in the treatment of colorectal cancer. *However, the drug was withdrawn voluntarily from the 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 following levamisole use in the treatment of cancers, childhood nephrotic syndrome, and rheumatologic disorders, and in individuals who had ingested levamisole-tainted cocaine (Rongioletti *et al.* 1999; Buchanan *et al.* 2011). Over the years, the incidence of mild to serious blood disorders, including hematologic depression, agranulocytosis and leukocytopenia has been noted in several clinical investigations with levamisole (Moertel *et al.* 1995; Ejlertsen *et al.* 2010). Agranulocytosis was seen in 2.5-13% of individuals using levamisole clinically (Symoens *et al.* 1978). Furthermore, dose dependency was demonstrated in cancer trials involving 2635 patients, wherein, 3.1% of the patients developed agranulocytosis when dosed 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 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, appears to occur more frequently in female patients than male patients (Symoens *et al.* 1978; Amery and Butterworth, 1983). As such, female gender has been the subject of discussion in idiosyncratic 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. Multi-gram 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, following levamisole administration to race horses, the identification of the stimulant aminorex as a circulating metabolite of levamisole (Figure 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 ($T_{\max} = 1.5$ hours) following oral administration at a dose of 150 mg, with an approximate 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 hours in humans (Woestenborghs *et al.* 1981). In dogs, following a 10 mg/kg intravenous dose, levamisole exhibits moderate plasma clearance (CL_p) and steady state distribution volume ($V_{d_{ss}}$) of 8.92 ml/min/kg and 1.42 l/kg, respectively, yielding a terminal $t_{1/2}$ of 1.8 hours. The corresponding oral bioavailability in dogs ranges from 44% in fed animals to 64% in fasted animals (Watson *et al.* 1988). Human mass balance studies with orally administered [^3H]-levamisole (150 mg) indicate metabolism as a predominant route of elimination (Adams, 1978; Schnieden, 1981; Kouassi *et al.* 1986). In contrast with the moderate plasma $t_{1/2}$ of levamisole (~ 5.6 hours), the $t_{1/2}$ of circulating levels of radioactivity, which is attributed to levamisole metabolites, is much longer at ~ 16 hours. Approximately 70% of the radioactivity is excreted by the kidney in the form of metabolites; renal excretion of unchanged levamisole accounts for ~ 3-4 % of the administered dose. To date, an oxidative metabolite *p*-hydroxy-levamisole (**1**) and its corresponding glucuronide conjugate **2** (Figure 1) have been identified in urine that collectively account for ~ 25% of the administered levamisole dose. The identities of the additional urinary components that amount to ~ 45% of urinary radioactivity remain uncharacterized. *In vitro* metabolism studies in human hepatic tissue, however, paint a more elaborate picture on levamisole metabolism. Apart from the formation of **1**, *in vitro* incubations of [^{14}C]-levamisole in human hepatocytes indicate the presence of several additional metabolites **3-8** (Figure 1) (Janssen, 1976; Symoens *et al.* 1979; Roberts, 1994). With the exception of **3**, which can arise from a cytochrome P450 (CYP)-mediated imidazolidine ring dehydrogenation, the

rate-limiting step in the formation of **4-8** appears to be mediated by a ring scission of the thiazolidine motif. Thus, the formation of **4** can be rationalized to proceed via CYP-mediated α -carbon hydroxylation (adjacent to the sulfur), followed by 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-mercaptoethyl)-5-phenylimidazolidine-2-one (**7**) contrasts the sequence of reactions proposed in the case of **4**, since 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 **9-11**, Figure 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”, 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 (non-pharmaceutical) 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 (Figure 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 non-human primates. Results observed in acute and chronic safety studies (World Health Organization Series 27, 1991, page 697) 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 acute and/or chronic toxicity studies. Likewise, acute single dose of levamisole in primates showed no hematological changes.

Short-term toxicity studies (duration < 90 days) in dogs reported no hematological changes at levamisole doses up to 20 mg/kg/day. In contrast, chronic studies revealed significant hematotoxicity within 8 weeks of dosing at 5.0 or 20 mg/kg/day, but not at the 1.25 mg/kg/day dose, indicating that hematotoxicity associated with levamisole in dogs is dose-dependent in a manner similar to the observations in humans (World Health Organization Series 27, 1991, page 805). Within this study, one female dog dosed at 5 mg/kg/day and all six dogs (male and female) dosed 20 mg/kg/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. Upon rechallenge, the onset of hematological toxicities, however, was rapid. In a large long-term hematological study, 25 out of 100 dogs dosed with 20 mg/kg/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 (Figure 4), revealed severe neutropenia in several female dogs over the course of a three-month study, which led to suspension of this agent from clinical consideration (Lorenz *et al.* 1999).

Perspective

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 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 (Figure 3) including abacavir, carbamazepine, sulfamethoxazole and flucloxacillin (Bharadwaj *et al.* 2012). In several of these cases, the mechanism for HLA/MHC-dependent T cell stimulation is thought to involve formation of a 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 (Pichler *et al.* 2002; Pohl *et al.* 1988). Haptenization via RM formation in peripheral blood cells has also been implicated in agranulocytosis associated with several structurally diverse drugs such as aminopyrine,

clozapine, sulfamethoxazole, ticlopidine, methimazole, etc (Figure 4) (Utrecht, 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, capable of oxidizing a broad range of electron-rich functional groups, especially those that contain oxygen, sulfur and nitrogen (Hofstra and Utrecht, 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 CYP, 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. Certainly, this is not unreasonable considering that related thiourea derivatives such as metiamide, methimazole and propylthiouracil (Figure 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 since an unacceptable number of patients developed agranulocytosis (Forrest *et al.* 1975). From a structure-toxicity perspective, cimetidine (see Figure 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.

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 [¹⁴C]-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 Figure 5, propylthiouracil-sulphenyl chloride, which can be generated from propylthiouracil chlorination by MPO, has been proposed as the RM that adducts to cysteinyl residues on proteins to yield disulfide adducts. Further oxidation of the sulphenyl 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 sulfhydryl nucleophiles such as *N*-acetylcysteine and 3-mercaptopropionic acid (Figure 5) in the *in vitro* incubations is suggestive of analogous reactivity with sulfhydryl residues on proteins. In similar fashion (see Figure 5), methimazole is also subject to oxidation on the thiourea sulfur in the presence of hypochlorous acid, MPO/H₂O₂/Cl⁻ system and/or human neutrophils resulting in the formation of an unstable disulfide metabolite (presumably via the sulphenylchloride 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 CYP-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 (Figure 6). Whether **15** possesses sufficient reactivity towards covalent adduction to proteins remains unclear, since **15** could easily tautomerize to yield the stable non-reactive dihydroimidazole metabolite **12** (see Figure 6).

Finally, it is possible that the free thiol metabolite **7** of levamisole (Figure 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) (Figure 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 dependency of these effects was observed across studies (Wilkin *et al.* 1980). Mixed disulfide conjugates of the free thiol group in captopril with reduced glutathione (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 (Figure 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 Figure 4, 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,
Kalgutkar

Conducted experiments: Not applicable

Contributed new reagents or analytic tools: Not applicable

Performed data analysis: Wolford, McDonald, Eng, Hansel, Chen, Bauman, Sharma, Kalgutkar

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

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Figure Legends

FIG. 1. Structures of tetramisole, levamisole, and dexamisole, and metabolic profile of levamisole in preclinical species and human.

FIG. 2. Levamisole metabolites obtained from anaerobic metabolism in human intestinal bacteria.

FIG. 3. Illustrations of human leukocyte antigen (HLA) associations with drug hypersensitivity reactions .

FIG. 4. Myeloperoxidase-catalyzed bioactivation of drugs as a causative factor in drug-induced agranulocytosis.

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.

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

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

Table 1. Pharmacokinetic parameters of levamisole in humans and dogs.

Species	Gender	Dose	Route	CL _p (mL/min/kg)	t _{1/2} (h)	Vd _{ss} (L/kg)	AUC (ng*h/mL)	C _{max} (ng/mL)	T _{max} (h)	CL _{renal} (L/h)	F (%)
Human	M & F	150 mg	po	8.04 ^a	5.6	3.80 ^a	3070	717	1.5	1.75	62.5 ^a
	M (n = 7)	150 mg	po	8.25 ^a	5.4	3.79 ^a	3070	672	1.7	1.86	61.5
	F (n = 3)	150 mg	po	7.56 ^a	5.9	3.83 ^a	3070	820	1.1	1.50	64.7
Dog	M & F	10 mg/kg	iv	8.92	1.8	1.42	18100				
		10 mg/kg	po (fasted)		1.3	1.39	12200	3330	1.8		
		10 mg/kg	po (fed)		1.6	2.64	8940	1980	4.5		

^a The calculations are based on the estimated oral bio-availability, assuming average human body weight of 70 kg. Abbreviations used are: po = oral. iv = intravenous. CL_p = plasma clearance. T_{1/2} = terminal half-life. Vd_{ss} = steady state distribution volume. AUC = area under the plasma concentration time curve. C_{max} = maximal plasma concentration. T_{max} = time to reach C_{max}. CL_{renal} = renal clearance of unchanged parent compound. F = absolute oral bioavailability

Figure 1

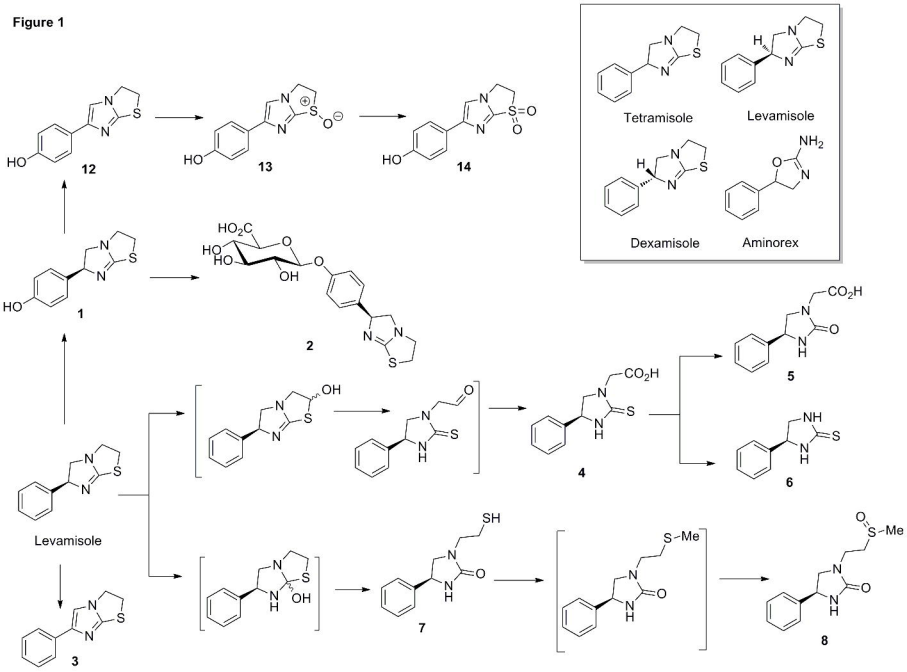
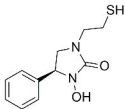
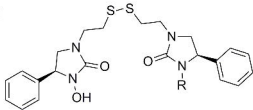


Figure 2



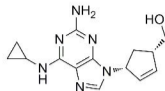
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10: R = H

11: R = OH

Figure 3

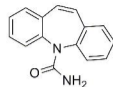


Abacavir

Daily dose = 600 mg

MHC class I HLA B*57:01
associations:

Hypersensitivity
Hepatotoxicity

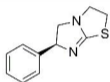


Carbamazepine

Daily dose = 1600 mg

MHC class I HLA B*15:02
associations:

Agranulocytosis
Stevens Johnson syndrome
Toxic epidermal necrolysis

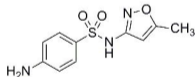


Levamisole

Daily dose = 150 -175 mg

MHC class I HLA B27
associations:

Agranulocytosis

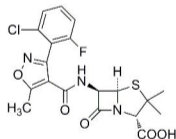


Sulfamethoxazole

Daily dose = 2400 mg

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

Agranulocytosis
Stevens Johnson syndrome
Toxic epidermal necrolysis



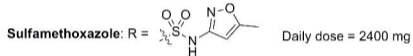
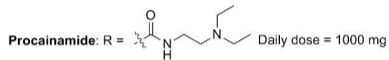
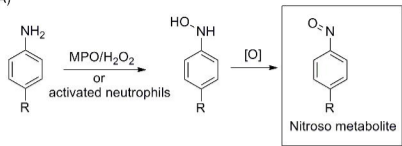
Flucloxacillin

Daily dose = 4000-8000 mg

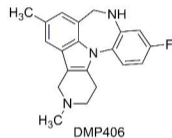
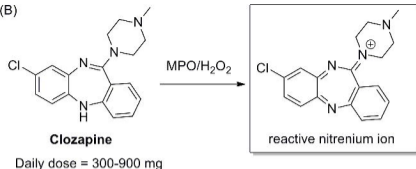
MHC class I HLA B*57:01
associations:

Hepatotoxicity

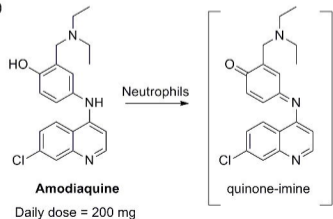
(A)



(B)



(C)



(D)

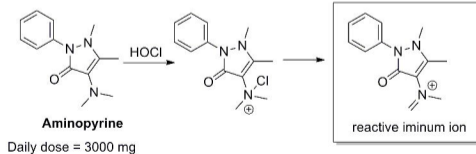


Figure 4

Figure 5

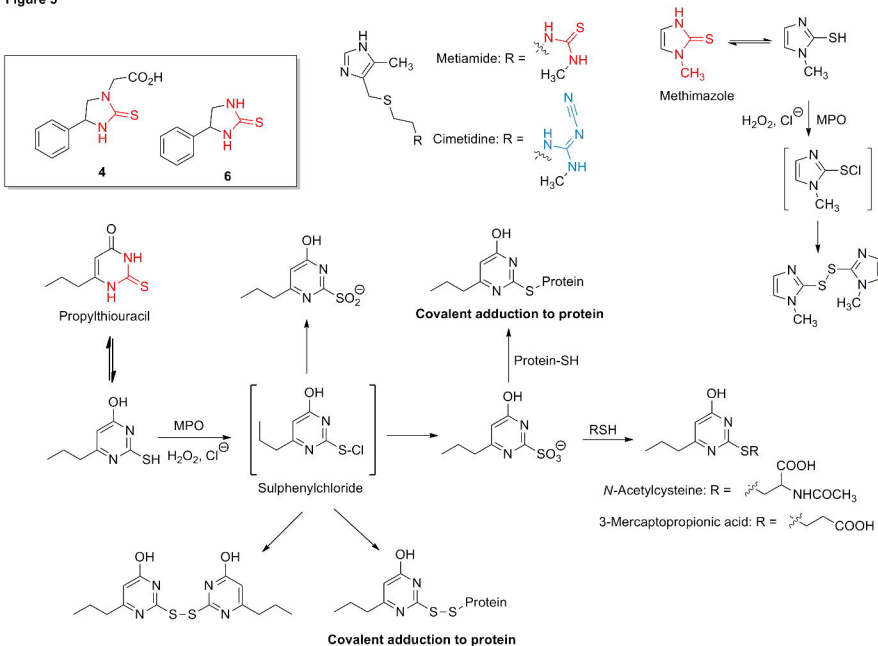


Figure 6

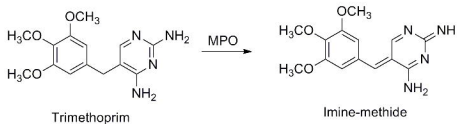
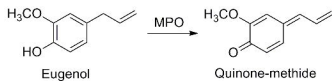
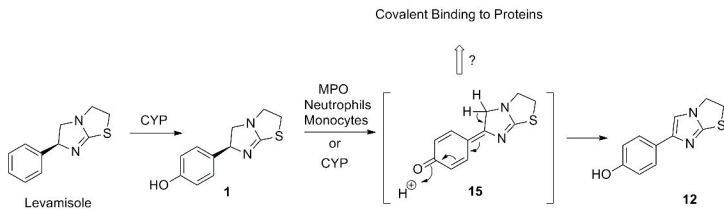
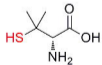
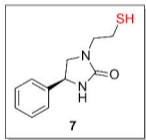
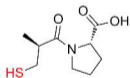


Figure 7

Structure-toxicity relationships in ACE inhibitors

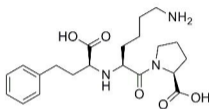


Penicillamine

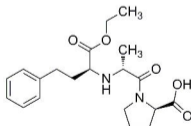


Captopril

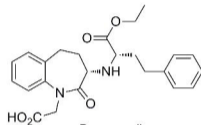
(associated with agranulocytosis)



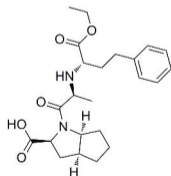
Lisinopril



Enalapril



Benazepril



Ramipril

(not associated with agranulocytosis)