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Transcriptomics provides mechanistic indicators of mixture toxicology for IMX-101 and IMX-104 formulations in fathead minnows (*Pimephales promelas*)

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ABSTRACT

Within the US military, new insensitive munitions (IMs) are rapidly replacing conventional munitions improving safety from unintended detonation. Toxicity data for IM chemicals are expanding rapidly, however IM constituents are typically deployed in mixture formulations, and very little is known about their mixture toxicology. In the present study we sought to characterize the mixture effects and toxicology of the two predominant IM formulations IMX-101 and IMX-104 in acute (48 h) larval fathead minnow (*Pimephales promelas*) exposures. IMX-101 consists of a mixture of 2,4-dinitroanisole (DNAN), 3-nitro-1,2,4-triazol-5-one (NTO), and nitroguanidine (NQ) while IMX-104 is composed of DNAN, NTO, and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). DNAN was the most potent constituent in IMX-101 eliciting an LC50 of 36.1 mg/L, whereas NTO and NQ did not elicit significant mortality in exposures up to 1040 and 2640 mg/L, respectively. Toxic unit calculations indicated that IMX-101 elicited toxicity representative of the component concentration of DNAN within the mixture. Toxicogenomic responses for the individual constituents of IMX-101 indicated unique transcriptional expression and functional responses characteristic of: oxidative stress, impaired energy metabolism, tissue damage and inflammatory responses in DNAN exposures; impaired steroid biosynthesis and developmental cell-signaling in NQ exposures; and altered mitogen-activated protein kinase signaling in NTO exposures. Transcriptional responses to the IMX-101 mixture were driven by the effects of DNAN where expression and functional responses were nearly identical comparing DNAN alone versus the fractional equivalent of DNAN within IMX-101. Given that each individual constituent of the IMX-101 mixture elicited unique functional responses, and NTO and NQ did not interact with DNAN within the IMX-101 mixture exposure, the overall toxicity and toxicogenomic responses within acute exposures to the IMX-101 formulation are indicative of "independent" mixture toxicology. Alternatively, in the IMX-104 exposure both DNAN and RDX were each present at concentrations sufficient to elicit lethality (RDX LC50 = 28.9 mg/L). Toxic-unit calculations for IMX-104 mixture formulation exposures indicated slight synergistic toxicity (ΣTU LC50 = 0.82, 95% confidence interval = 0.73–0.90). Unique functional responses relative to DNAN were observed in the IMX-104 exposure including responses characteristic of RDX exposure. Based on previous transcriptomics responses to acute RDX exposures in fathead minnow larvae, we hypothesize that the potentially synergistic responses within the IMX-104 mixture are related to interactive effects of each DNAN and RDX on oxidative stress mitigation pathways.

Abbreviations: 95% C.I., 95% confidence interval; BDL, below detection limits; cRNA, complementary RNA; DAVID, Database for Annotation, Visualization and Integrated Discovery; DNAN, 2,4-dinitroanisole; HMX, cyclotetramethylene-tetranitramine; IM, insensitive munition; KEGG, Kyoto Encyclopedia of Genes and Genomes; MCs, munition constituents; MOA, mechanism of action; NQ, nitroguanidine; NTO, 3-nitro-1,2,4-triazol-5-one; PCA, Principle Component Analysis; RDX, hexahydro-1,3,5-trinitro-1,3,5-triazine; ROS, reactive oxygen species; TU, toxic unit; ΣTU, total toxic units

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1. Introduction

The Army has a stated goal of replacing traditional munitions with insensitive munitions (IM) that are chemically stable enough to withstand mechanical damage, fire, and impact of projectiles during combat operations while still exhibiting similar explosive characteristics to traditional munitions (Isler, 1998). While IMs are being fielded for use, there are relatively few studies available describing the environmental impact of the mixture formulations compared to the individual constituents. Presently, the IM formulation IMX-101, a mixture of 2,4-dinitroanisole (DNAN), 3-nitro-1,2,4-triazol-5-one (NTO), and nitroguanidine (NQ), is qualified as a replacement for 2,4,6-trinitrotoluene (TNT) in artillery rounds (Lee et al., 2010). The IM formulation IMX-104, a mixture of DNAN, NTO, and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), is qualified as a replacement for Composition B in artillery rounds (Fung et al., 2010). Munitions constituents are manufactured at ammunition facilities, some of which discharge treated wastewater into freshwater environments (Ribeiro et al., 2012; Li et al., 2013). Additionally, IMs have the potential to be released into the environment during live fire use on training ranges and in combat (Taylor et al., 2015a). The IMX-101 and IMX-104 components, NQ and NTO, are orders of magnitude more soluble in water than legacy munitions such as TNT and RDX (Dontsova et al., 2014; Taylor et al., 2015b). Although information on concentrations of IMs in discharges and natural waters are not currently publicly available, and no exposure information can be presently inferred, the manufacturing and use practices of the materials coupled with the chemical properties conducive of environmental transport warrants further investigation. Specifically, effects characterization is critical to establishing the environmental risk posed by IMs, not only for the individual constituents, but also for the mixture formulations that are deployed.

The effects of single IM constituents have been investigated for a variety of aquatic receptors. The aquatic toxicity of DNAN has been reported for bacteria (Dodard et al., 2013), microalga (Dodard et al., 2013), cladocerans (Kennedy et al., 2013, 2015, 2017), tadpoles (Stanley et al., 2015), and fish (Kennedy et al., 2015) where the toxic potency was roughly equivalent to the conventional munition 2,4,6-trinitrotoluene (TNT) across the various acute aquatic exposures (Kennedy et al., 2017). NTO elicits toxicity by increased acidity at high concentrations in aquatic exposures (Pillard et al., 2017), thus pH-buffered NTO assays have been conducted to assess toxic responses not related to acidic stress in microalga (Haley et al., 2009), cladocerans (Kennedy et al., 2017), fish (Sayers, 2009), and tadpoles (Stanley et al., 2015; Pillard et al., 2017). The toxicity of NQ, has been reported for bacteria (Hawari, 2014), microalga (Hawari, 2014), various aquatic invertebrates (van der Schalie, 1985; Burton et al., 1993; Kennedy et al., 2017) and fish (van der Schalie, 1985; Burton et al., 1993; Gust et al., 2017). Results from the NQ and pH-buffered NTO studies indicated low toxic potency (generally > 1000 mg/L) in acute exposures, however both NTO and especially NQ became orders of magnitude more toxic after UV-degradation (van der Schalie, 1985; Kennedy et al., 2017; Gust et al., 2017). Several toxicity studies characterizing RDX exposures in fish and aquatic invertebrates have been summarized in Nipper et al. (2009) and Lotufo et al. (2013) and mechanistic responses in fathead minnow and zebrafish have also been described (Garcia-Reyero et al., 2011; Gust et al., 2011a,b; Warner et al., 2012). This literature indicated that RDX tended to elicit acute toxicity at exposure concentrations similar to TNT and DNAN. Overall, the aquatic toxicity of the individual constituents making up the IMX-101 and IMX-104 formulations have been characterized in at least a few aquatic species, where DNAN and RDX tended to be more toxic than NTO and NQ, as parent compounds.

Although the toxicity of individual IMs have been characterized, the effects of the IMX mixture formulations remain a critical knowledge gap in understanding IM toxicity and environmental risk given that IMs are manufactured and discharged together (Kennedy et al., 2017) and released together when ordinance are fired on training ranges and in theater (Taylor et al., 2015a). Chemicals having similar modes of toxic action are typically hypothesized to elicit “dose-additive” (“concentration-additive” in environmental exposures) effects, whereas chemicals with dissimilar modes of toxic action are generally hypothesized to act in “independence” resulting in less than concentration-additive toxicity (Altenburger et al., 2004; Cassee et al., 1998). Although usually predictive, scores of exceptions to the “independence” hypothesis have been observed where toxicological interactions resulted in antagonistic (reduced) or synergistic (increased) toxic effects (Cassee et al., 1998; Berenbaum, 1989). Given that the mechanisms of action for the IM constituents, DNAN, NTO and NQ are yet to be determined, the appropriate means to summarize the mixture toxicity of the IMX formulations are also unknown. Further, given the relative simplicity of the IMX mixture formulations which are composed of only 3 chemicals, each present at high proportions, the “funnel hypothesis” indicates a greater probability for interactive effects relative to more complex mixtures (Warne and Hawker, 1995). Thus, a considerable knowledge gap exists for IMX-101 and IMX-104 mixture toxicity.

The primary objective of the present study was to address this knowledge gap by characterizing the mixture toxicology of the IM compounds within the IMX-101 and IMX-104 formulations utilizing acute (48 h) fathead minnow (*Pimephales promelas*) larval assays coupled with functional transcriptomics analysis. Experiments were employed comparing and contrasting the toxicity, molecular pathways, and biological functions affected in the individual IM constituent exposures relative to the IMX mixtures. Previous global transcriptomics investigations have provided the initial discovery of toxicity mechanisms for conventional munition constituents (MCs) including dinitrotoluenes (Rawat et al., 2010; Gust et al., 2015) and RDX (Gust et al., 2009; Gust et al., 2011a,b; Warner et al., 2012), each of which have been validated in directed investigations (Wilbanks et al., 2014; Williams et al., 2011) and transitioned to adverse outcome pathways (AOPs) for use in regulatory assessment applications (Collier et al., 2016; Gong et al., 2015). The present transcriptomics investigation represents the hypothesis development phase for mechanism(s) of action (MOA) discovery for the individual IMs and the IMX mixture formulations. The present study serves as the basis for future research efforts to apply directed biochemical and/or cell-biological tests to validate hypothetical MOAs, and provides the first opportunity to establish hypothetical functional responses underlying the mixture toxicology of the IMX-101 and IMX-104 formulations. Given the lack of MOA data for three of the four IM constituents tested, the conservative null hypothesis of no difference in the functional genomic responses and MOA among individual IMs constituents resulting in dose-additive toxicological responses within IMX-101 and IMX-104 mixture formulations was tested.

2. Materials and methods

2.1. Fathead minnow exposures to insensitive munitions

2.1.1. Experimental organisms

Larval (11-d old) *P. promelas* were obtained from aquatic biosystems (Fort Collins, Colorado, USA). all test organisms were within 24-h in age

2.1.2. Chemicals and exposure solutions

Military grade DNAN (> 95% pure, Kennedy et al., 2015), NTO (> 95% pure), and RDX (approximately 10% octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine [HMX] as impurity, Jenkins et al., 2006) and the IM formulations IMX-101 and IMX-104 were obtained from the Holston Army Ammunition Plant (BAE Systems) and used without further purification. NQ for single chemical exposure was obtained from Sigma (Sigma item # N17351). The manufacturer specifications for the NQ indicate that it was 20–25% water for safety purposes. All

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