In silico and *in vitro* analysis of 4,4'-methylenedianiline replacements

Valerie H. Adams[1,2], Emily N. Reinke[2], Lindsay A. Holden[2], and John J. LaScala[3]

[1] presenting author, [2] US Army Public Health Center, 8252 Blackhawk Rd, APG MD 21010, [3] Army Research Laboratory APG MD 21010

ABSTRACT

The first generation PMR (polymerization of monomeric reactants) matrix resin included MDA (4,4'-methylenedianiline) as a component and is currently known as PMR-15. PMR-15 composites have been used for decades to produce a variety of high-quality aerospace and weapon systems structural components. MDA was identified as hepatotoxic, mutagenic, and carcinogenic in animals in the 1980s making it a candidate for replacement. Surrogates for MDA were investigated for this project. A total of 59 MDA-related molecular structures (all provided in methods) were evaluated using in silico methods to estimate physicochemical properties, fate, transport, and toxicity. Of this group, 23 were further evaluated with *in vitro* screening assays for mutagenicity, drinking water safety, skin sensitization, and acute toxicity estimation. As a whole, the MDA replacements were within a biologically active molecular weight range. The average LogKow was 2.44. As a group, the MDA compounds had moderate to low volatility. Using TOPKAT and in vitro cytotoxicity estimation, acute toxicity was predicted as low to moderate. Estimates for acute aquatic toxicity using ECOSAR (**eco**logical **s**tructure-**a**ctivity **r**elationship model, USEPA) and *A. fischeri* bioluminescence were also low to moderate. Based on TOPKAT prediction of skin sensitization, 6 MDA replacements were tested using the h-CLAT assay- 5 of 6 were identified as skin sensitizers with this new approach methodology (NAM). Sixteen replacements were screened for mutagenicity using a modified Ames assay and 9 additional replacements had published mutagenicity data available. The concordance between the TOPKAT and experimental mutagenicity data was >70%. Based on the screening level toxicity data reported here that shows fairly homogeneous categorical hazards, the preferred surrogate(s) should be prioritized by lack of mutagenicity. The development of MDA alternatives with reduced toxicity and PMR resin properties that are similar or improved relative to PMR-15 reduces costs and health effects associated with PMR-15 manufacturing.

INTRODUCTION

MDA is an industrial chemical that has been used for decades in epoxy resins, composites, and polymer applications.

Inhalation and dermal occupational exposure to MDA may occur. Dermal absorption was identified as the primary route of exposure in workers using composite materials to construct helicopter rotor blades (Weiss et al., 2011).

MDA acute oral LD50 = 447 mg/kg (averaged from rat, mouse, rabbit, and guinea pig data). Systemic chronic toxicity occurs in the range of 9-25 mg/kg-day, depending on laboratory species and strain. The critical effects noted include nephropathy and hyperplasia of the liver and thyroid. (ATSDR, 1998).

In a 2-year mouse study, malignant lymphoma and carcinoma of the liver were identified at 19 mg MDA/kg-day in female mice (NTP, 1983). In mice exposed dermally to MDA for 2 years, hepatic tumors were detected at 5 mg/kg-day (ATSDR, 1998).

In both *in vitro* and *in vivo* assessments of mutagenicity, MDA has been identified as genotoxic in the presence of metabolic activation. The mechanism of action for MDA toxicity is attributed to reactive metabolic intermediates that include the generation of DNA damaging nitrosamines.

Fifty-nine MDA replacement candidates (see Table 1 and 2), were evaluated using quantitative structure-activity relationship (QSAR) modeling to predict physical-chemical properties and toxicity.

Twenty-three downselected candidates were tested using *in vitro* toxicity assays (Ames, Microtox™, skin sensitization [h-CLAT], and cell-based acute oral toxicity estimation [CAOTE]) to estimate their potential negative human and environmental effects.

Table 1. MDA compound identification.

Structure ID	CAS No. (nf=not found)	Compound Name	Structure ID	CAS No. (nf=not found)	Compound Name	Structure ID	CAS No. (n-f=not found)	Compound Name
MDA	101-77-9	4,4'-methylenedianiline	MDA19	nf	2,2',6,6'-tetramethyl-(-1,1'-biphenyl)-(-4,4'-diamine	MDA39/ AC49-1413	nf	(E)-5-(4-aminosytyl)benzene-1,3-diamine
MOA	101-80-4	4,4'-oxydianiline	MDA20	nf	5,5'-methylenebis(2-methoxy-4-methylaniline)	MDA 40; 2,5-dMAC	5339-30-0	4,4'-methylenebis(2,5-dimethylaniline)
MDA1	nf	5,5'-ethane-1,2-diyl)bis(benzene-1,3-diamine)	MDA21	nf	5-(4-aminophenethyl)-benzene-1,3-diamine (NOT resveratrol)	MDA 41	142252-05-9	4,4'-methylenebis(3,5-dimethylaniline)
MDA2/ ARL-MDA-3; 2MAC	838-88-0	4,4'-methylenebis(2-methylaniline)	MDA22	nf	5,5'-ethane-1,2-diyl)bis(benzene-1,3-diamine)	MDA 42 (MAS-1981-169C); 2,6-dMAC	4073-98-7	4,4'-methylenebis(2,6-dimethylaniline)
MDA3	nf	6,6'-methylenebis(2-methoxy-4-methylaniline)	MDA23/ ARL MDA-2; 2-MeOAC	1223-2-0-7	4,4'-methylenebis(2-methoxyaniline), Benzenamine	MDA 43	nf	5,5'-(propane-2,2-diyl)bis(2-methoxyaniline)
MDA4	nf	4,4'methylenebis(5-isopropyl-2-methylaniline)	MDA29/ polyaniline 2	69563-88-8	4,4'-((perfluoropropane-2,2-diyl)bis(4,1-phenylene))bis(oxy))dianiline	MDA 44	nf	4,4'-methylenebis(2,6-dimethoxyaniline)
MDA5	nf	3(4-aminophenyl)-1-ethyl-2-methyl-2,3-dihy dro-1H-inden-5-amine	MDA27/ polyaniline 1	nf	4,4'-((5-(4-(4-aminophenoxy)phenethyl)-1,3-phenylene)bis(oxy))dianiline	MDA 45	80-08-0	4-Aminophenyl sulfone; dapsone
MDA6	nf	4,4'-(2-methylpentane-1,3-diyl)dianiline	MDA28/ DFDA	90398-91-7	(5,5'-methylenebis(furan-5,2-diyl))dimethanamine	MDA 46	nf	4,4'-((3,3'-dimethoxy-(-5,5'-dimethyl-(1,1'-biphenyl-(-2,2'-diyl))bis(oxy))dianiline
MDA7	nf	4,4'-(butane-1,4-diyl)bis(2-methoxyaniline)	MDA29/ CH3-DFDA	nf	(5,5'-(ethane-1,1-diyl)bis(furan-5,2-diyl))dimethanamine	MDA 47 (V-DFDA)	nf	4-(bis(5-aminomethyl-(furan-2-yl)methyl)-2-methoxy phenol
MDA8	106-50-3	1,4-diaminobenzene	MDA30/ ARL MDA-4; 3-MAC	nf	6,6'-methylenebis(3-methylaniline)	MDA 48 (BPA dimethacrylate)	nf	1,1'-((propane-2,2-diyl)bis(4,1-phenylene))bis(oxy))bis(2-methylprop-2-en-1-one)
MDA9	611-98-3	bis(4-aminophenyl)methanone	MDA31	2479-46-1	4,4'(1,3-phenylene bis(oxy))dianiline; 4(3)(4-aminophenoxy)phenoxy) aniline; 1,3-Bis(4-aminophenoxy) benzene	MDA 49 (TFTA)	nf	(5,5'-(5'',5''-(1,4-phenylene)bis(methanetriyl))tetrakis(furan-5,2-diyl))tetramethanamine
MDA10	611-79-0	bis(3-aminophenyl)methanone	MDA32	10526-07-5	3,3'-(1,3-phenylenebis(oxy))dianiline	MDA 50 (SY-DFDA)	nf	4-Bis(5-aminomethyl-(furan-2-yl)methyl)-2,6-dimethoxy phenol
MDA11	nf	4×(5-(2-aminobenzyl-furan-2-yl)methyl)-(2-furan-2-yl)methyl) aniline	MDA33	13676-49-8	2-(3-aminophenyl)-1H-benzo[d]imidazol-5-amine	IDMMDA50 (Isocobide dimethacrylate)	nf	hexahydrofuro[3,2-b]furan-3,6-diyl bis(2-methacrylate)
MDA12	nf	3×(5-(4-aminobenzyl-furan-2-yl)methyl) aniline	MDA34	7621-86-5	2-(4-aminophenyl)-1H-benzo[d]imidazol-5-amine	MDA 52 (MAS-1981-169A)	nf	4,4'-((3,3'-dimethoxy-(-5,5'-dimethyl-(1,1'-biphenyl-(-2,2'-diyl))bis(oxy))dianiline
MDA13	143396-58-1	(3R,3R,6S)-hexahydrofuro[3,2-b]furan-3,6-diamine (diamino isosorbide)	MDA35	13676-47-6	2-(4-aminophenyl)benzo[d]oxazol-5-amine	TMB1, MDA53 (MAS-1981-169B)	54827-17-7	3,3',5,5'-Tetramethylbenzidine
MDA14	2213-51-6	furan-2,5-diyldimethanamine	MDA36	84-67-3	2,2'dimethyl-(1,1'-biphenyl-(-4,4'-diamine; m-tolidine, 2,2'dimethyl benzidine	IAMMDA51 (Isocobide diacrylate)	nf	hexahydrofuro[3,2-b]furan-3,6-diyl diacrylate
MDA15	nf	(oxybis(4,1-phenylene)) dimethanamine; (4-(4-(aminomethyl)phenoxy-)phenyl)methanamine	MDA35	13676-47-6	2-(4-aminophenyl)benzo[d]oxazol-5-amine	IAMMDA52 (Isocobide acrylate methacrylate)	nf	6-(acryloyloxy)-hexahydrofuro[3,2-b]furan-3-yl methacrylate
MDA16	nf	2,2'-dimethoxy-(1,1'-biphenyl-(-4,4'-diamine; 2,2-dimethoxy benzidine	MDA36	84-67-3	2,2'dimethyl-(1,1'-biphenyl-(-4,4'-diamine; m-tolidine, 2,2'dimethyl benzidine	MDAB-m (1,3-benzenediamine)	108-45-2	m-phenylenediamine
MDA17	nf	3,3'-dimethoxy-5,5'-dimethyl-(1,1'-biphenyl-(-2,2'-diamine	MDA37	119-93-7	3,3'-dimethyl-(1,1'-biphenyl-(4,4'-diamine; 3,3'-dimethylbenzidine, O-tolidine, 3,3'-dimethylbenzidine	MDAB-o (1,2-benzenediamine)	95-54-5	o-phenylenediamine
MDA18	2050-89-7	[1,1'-biphenyl]-3,3'-diamine	MDA38	346-88-3	3,3-bis(difluoromethyl)-(1,1-biphenyl)-(4,4'-diamine	Bisaniline M	2687-27-6	Bis-M; 4,4'-(1,3-Phenylenediisopropylidene)bisaniline

METHODS/RESULTS

Table 2. Toxicity data available via literature search.

Compound	Oral LD50 (mg/kg)	Oral LOAEL (mg/kg-d)	Inhal LC50 (mg/m3-h)	Skin Irritation	Skin Sensitization	Ocular Irritation	Developm't	Mutagen	Carcinogen
MDA	447 (ave. DB value)	7.35 (DB value)	ND	Negative (DB value)	ND	Indeter (DB value)	ND	Positive (DB value)	Positive (DB value)
MOA	725 (DB value)	10 (DB value); 9.5 (High)	ND	Mod/Irritat (High)	ND	ND	ND	Positive (DB value)	Positive (DB value)
MDA2/ ARL-MDA-3; 2-MAC	ND	ND	ND	Negative (DB value)	ND	Positive (DB value)	ND	Positive (DB value)	Positive (DB value)
MDA8-p	80 (DB value)	37.4 (DB value)	230 (DB value)	mild (Mod)	respiratory sens (DB value)	mild (DB value)	Negative (DB value)	Positive (DB value)	Positive (DB value)
MDA37	ND	ND	ND	ND	ND	ND	ND	Positive (DB value)	Positive (DB value)
MDA 42 (MAS-1981-169C); 2,6-dMAC	ND	ND	ND	ND	ND	ND	ND	Negative (Rao 1982)	ND
MDA 45	1000 (DB value)	60 (DB value)	ND	ND	ND	ND	ND	Negative (DB value)	Negative (DB value)
TMB, MDA53 (MAS-1981-169B)	ND	ND	ND	ND	ND	ND	ND	Negative (DB value)	Negative (DB value)
MDA-m (1,3-benzenediamine)	160 (mouse); 280 (rat), 786.6 (Mod)	ND	3.2 (mg/L)	dermal LD50 1100 (rat)	ND	ND	ND	Positive (DB value)	ND
MDA-o (1,2-benzenediamine)	510-1070 (rat); 698.1 (High)	ND	>91	>5000 mg/kg (rat), Negative (High)	ND	ND	ND	ND	ND
Bisaniline M	ND	ND	ND	GHS 2	ND	severe (DB) GHS 2	ND	ND	ND

In Silico METHODS

- QSAR systems are approaches to estimating physico-chemical properties and biological activity (toxicity) of a chemical based on its molecular structure (OECD, 2019).
- EPISuites® (USEPA 2013) was used to estimate physical-chemical properties listed below for the MDA compounds

Molecular weight (MW)	Octanol-water partition coefficient (log K_{OW})	Bioconcentration factor (BCF)
Boiling point (bp)	Organic carbon partition coefficient (log K_{OC})	Bioaccumulation factor (BAF)
Water solubility	Henry's Law constant (K_H)	Biodegradation
Melting point (mp)	Vapor pressure (vp)	Fugacity

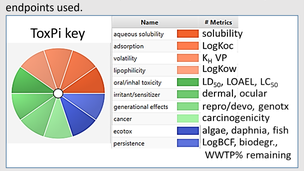
- TOPKAT (BIOVIA 2015) QSAR models evaluated:

RAT ORAL LD50 (MG/KG)	Carcinogenicity	
CHRONIC LOAEL (MG/KG-D)	NTP MALE RAT	NTP MALE MOUSE
RAT INHALATION (MG/M3/H)	NTP FEMALE RAT	NTP FEMALE MOUSE
DEVELOPMENTAL TOX	FDA M-RAT (NON V CARC)	FDA M-MOUSE (NON V CARC)
SKIN IRRITATION	FDA M-RAT (SINGLE V MULT)	FDA M-MOUSE (SINGLE V MULT)
SKIN SENSITIZATION	FDA F-RAT (NON V CARC)	FDA F-MOUSE (NON V CARC)
OCULAR IRRITATION	FDA F-RAT (SINGLE V MULT)	FDA F-MOUSE (SINGLE V MULT)
AEROBIC BIODEGRADABILITY	CARCINOGENICITY (weight of evidence)	AMES MUTAGENICITY

- ECOSAR (USEPA 2012) models provided acute and chronic toxicity for fish, Daphnia, algae. Annotations in the data output included chemical class specific estimations, effects occurring above aqueous solubility limit, and class-specific LogK_{OW} cutoffs for effects at saturation.

In silico data integration

ToxPi™ (Toxicological Prioritization Index v2.3; toxpi.org) was used to evaluate and integrate *in silico* data for MDA replacements to assess potential toxicity and environmental persistence. The parent compound, MDA is indicated with a blue dashed box (see **Figure 1**). Physical and chemical properties are depicted with orange radar segments, predicted toxicity is in green, and predicted ecotoxicity is in blue; see the key below for specific endpoints used.



For each compound, numerical data for selected physicochemical properties (EPISuites) and predicted endpoints (TOPKAT or ECOSAR) were log-transformed (e.g., aqueous solubility, Henry's law constant, vapor pressure, oral LD50, oral LOAEL, inhalation LC50, green algae EC50, daphnia LC50 LOAEL, and fish LC50) and all data were scaled for radar segment creation. Where appropriate, the inverse of some endpoints were used so that **for all radar segments, toxicity or negative effects increases with the distance from the origin (center).** The TOPKAT endpoints for irritant/sensitizer, generational effects, cancer, and biodegradation half-life are categorical (e.g. negative, indeterminate, positive) and have estimates of quality (confidence) based on the similarity of the queried structure to the structural parameters for each model. These categorical endpoints were ranked both the value and confidence for a score of 0 (i.e. negative or nonpersistent with high confidence) to 5 (i.e. positive or recalcitrant with high confidence). Boundary circles provide a visual indicator of the maximal radius for each ToxPi.

From these data MOA, MDA2, and MDA30 are examples of substitutions that may be regrettable (**Figure 1**-red dashed box). The rows below MDA are projected to have fewer toxicity and environmental concerns (e.g., MDA4, MDA 14, and MDA48; **Figure 1**-pink dashed box). As less toxic alternatives, these compounds should be prioritized for additional performance testing and evaluation.

Figure 1. ToxPi visualization tool for MDA compounds.

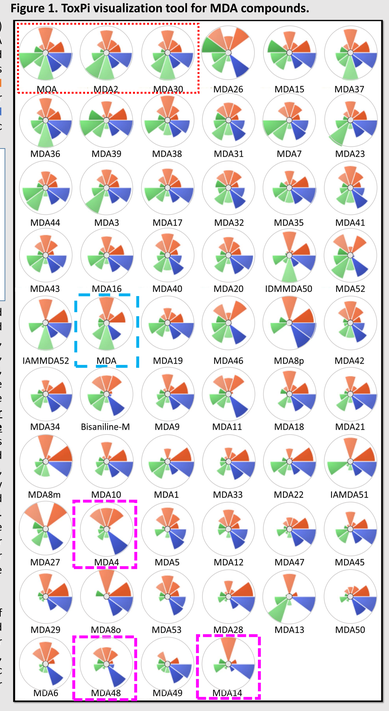


Table 3. TA100 mutagenesis.

Compound	Ames Test TA100 -S9	Ames Test TA100 +S9	Solubility limit ug/mL	highest conc. ug/mL
MDAparent	neg	pos >=3.2 ug/mL	~1000	1000
MDA2	pos DB		n/a	n/a
MDA39	neg	pos >=0.3 ug/mL	~200	~100
MDA40	neg	pos >1.1 ug/mL	~200	100
MDA21	neg	pos >=3.2 ug/mL	~200	200
MDA23	neg	pos 3.2 ug/mL	~1000	1000
MDA30	neg	pos 3.2 ug/mL	~1000	1000 (ppt)
MDA42/54	neg DB		400	400
MDA13	neg	neg	miscible	2000
MDA43	neg	neg	>/= 2000	2000
MDA53	neg	neg	400	400
MDA4	neg	neg	~200	~100
MDA28	neg	neg	100	100
MDA29	neg	neg	100	100
MDA52	neg	neg	~100	80
MDA50	neg	neg	~100	80
IDMMDA50	neg	neg	~100	80
IAMMDA52	neg	neg	~100	80
MDA48	neg	neg	80	80
MDA27	neg	neg	~10	10
MDA47	insoluble		n/a	n/a
MDA49	insoluble		n/a	n/a
IAMDA51	insoluble		n/a	n/a
n/a=not applicable				

Ames Assay. A liquid based Ames test was used to identify compounds mutagenic to *Salmonella* TA98, TA100, TA1535, TA1537, and a composite mix of *E. coli* pKM101/*uvrA* strains. The tests were conducted in both the presence and absence of S9 fraction- a rat liver extract that simulates *in vivo* liver metabolism. Each compound was tested in triplicate and the scores for each treatment were averaged. A compound was scored as mutagenic if the number of revertants exceeded the background by three-fold and demonstrated a dose-dependent increase in revertants. To verify MDA mutagenesis, it was tested on strains TA100 and TA98. The data for the TA100 test are in **Table 3**. MDA47, MDA49 and IAMMDA52 were insoluble and could not be tested in this assay. MDA21, MDA23 and MDA30 were mutagenic at approximately the same concentration as MDA. MDA39 was mutagenic at 10-fold lower concentration and MDA40 was mutagenic at 1/3 lower concentration than MDA. MDA27 was negative in the Ames assay; however, due to poor solubility, the highest dose tested was 10 ug/mL.

Table 4. h-CLAT predicted skin sensitization

Compound	CD54 EC200 (mg/mL)	CD86 EC150 (mg/mL)	Decision
MDA: 40	>0.046	<0.013	Positive
MDA: 43	0.028	0.11	Positive
MDA: 48	0.18	0.22	Positive
IDMMDA50	0.2	0.066	Positive
MDA: 52	0.037	0.013	Positive
IAMMDA52	Negative	Negative	Pending

Six MDA-replacement candidates that were predicted to be skin sensitizers by TOPKAT analysis were assayed with h-CLAT; see **Table 4**. Prior to testing, THP-1 cells were checked and verified for reactivity to DNCB, NiSO4 and lack of reactivity to lactic acid. The relative fluorescence intensities (RFI) of the labeled THP-1 cells were analyzed by flow cytometry (BD FACSVerse; BD FACSuite v1.0.5. software). Two independent experiments were completed for each compound. Where the RFI exceeded the positive criteria (CD54 ≥ 200 and CD86 ≥ 150), the EC200 and EC150 were calculated according to OECD Test Guideline 442E (OECD, 2016).
CD54 and CD86 cell surface expression were stimulated in all of the tested compounds except IAMMDA 52. MDA 40, MDA 43, MDA 48, IDMMDA 50, and MDA 52 are all considered to be skin sensitizers using the h-CLAT.

Table 5. Microtox data and hazard estimation.

Structure ID	15-min EC50 (mg/L) Mean [95% CI)]	GHS Acute Aquatic Toxicity Category
MDA:	14.42 [11.15-18.66]	3
MDA2:	17.3 [6.998-42.78]	3
MDA: 4:	267.6 [143.6-498.9]	No Category
MDA: 13:	19.85 [17.54-22.56]	3
MDA: 21:	58.58 [40.11-85.6]	3
MDA: 23:	42.81 [30.44-60.2]	3
MDA: 27:	>2000	No Category
MDA: 28:	22.47 [12.27-41.16]	3
MDA: 29:	6.03 [1.46-25.36]	2
MDA: 30:	42.32 [30.72-58.29]	3
MDA: 39:	1057 [841.8-1329]	No Category
GHS Category 1: ≤ 1 mg/L		
GHS Category 2: > 1 mg/L ≤ 10 mg/L		
GHS Category 3: > 10 mg/L ≤ 100 mg/L		

Structure ID	15-min EC50 (mg/L) Mean [95% CI)]	GHS Acute Aquatic Toxicity Category
MDA: 40:	41.54 [28.01-61.61]	3
MDA: 42:	38.64 [29.36-50.84]	3
MDA: 43:	47.86 [44.69-51.24]	3
MDA: 47:	insoluble	N/A
MDA: 48:	>2000	No Category
MDA: 49:	insoluble	N/A
MDA: 52*:	127.5 [87.7-185.4]	3
IDMMDA50:	13.61 [9.095-20.38]	3
MDA: 52**:	>500	No Category
MDA53:	1.18 [0.69-2.01]	2
IAMDA51:	insoluble	N/A
IAMMDA52:	6.29 [4.09-9.73]	2

*Extrapolated due to cytotoxicity/solubility issues.
**The compound was non-toxic at the solubility limit of testing, testing concluded after initial range-finding.

Microtox Assay. The 15 minute EC50 of marine bacteria, *A. fischeri*, treated with MDA replacements were used for estimating aquatic hazard. For each test compound, three individual experiments were performed in duplicate. The toxicity data (EC50 and the 95% Confidence Interval) and risk assessment are presented in **Table 5**. Using the GHS categorization scheme, 6 MDA compounds had EC50 above 100 mg/mL (insufficient toxicity to be categorized), 11 were GHS 3, and three were GHS 2. Three were insoluble. None of the MDA compounds tested were estimated to have high aquatic toxicity (i.e., GHS 1).

Table 6. Estimated acute mammalian oral toxicity.

Compound	Acute Oral Rat LD50 Prediction (mg/kg)	Predicted GHS Acute Oral Toxicity Category	TOPKAT LD50 GHS Category	DB Value
MDA	944	4	4	4
MDA 2:	532	4	5	nd
MDA: 4:	725	4	Not Classified	nd
MDA: 13:	>2000	5	4	nd
MDA: 21:	629	4	Not Classified	nd
MDA: 23:	781	4	Not Classified	nd
MDA: 27:	568	4	Not Classified	nd
MDA: 28:	197	3	3	nd
MDA: 29:	765	4	3	nd
MDA: 30:	684	4	5	nd
MDA: 39:	301	4*	4	nd
MDA: 40:	655	4	5	nd
MDA: 42:	>5000	Not Classified	5	nd
MDA: 43:	475	4	Not Classified	nd
MDA: 45:	Insoluble.	N/A	5	4
MDA: 47:	Insoluble.	N/A	3	nd
MDA: 48:	>5000	5	5	nd
MDA: 49:	Insoluble.	N/A	4	nd
MDA: 50:	448	4	3	nd
IDMMDA50:	>5000	Not Classified	Not Classified	nd
MDA: 52:	782	4	Not Classified	nd
MDA53:	>5000	Not Classified	4	nd
IAMDA51:	Insoluble.	N/A	Not Classified	nd
IAMMDA52:	890	4	Not Classified	nd
GHS Category 1: ≤ 5 mg/kg				
GHS Category 2: > 5 mg/kg ≤ 50 mg/kg:				
GHS Category 3: > 50 mg/kg ≤ 300 mg/kg				
GHS Category 4: > 300 mg/kg ≤ 2000 mg/kg				
GHS Category 5: >2000 mg/kg ≤ 5000 mg/kg				
Not Classified				

Cell-based Acute Oral Toxicity Estimation (CAOTE). Mammalian acute oral toxicity was predicted using data collected as part of the MDA replacement studies and the Neutral Red Uptake Assay for selected MDA compounds (ICCVAM 2006). The THP-1 IC50 for each compound was calculated and used to predict the acute oral rodent toxicity using this equation: log LD50 (mg/kg) = 0.372 log IC50 (µg/mL) + 2.024. Prior to 2016, MDA compounds available at the time were screened using neutral red uptake as a measure of cytotoxicity.
The majority of the MDA replacements tested were predicted to be GHS acute category 4 or 5 using CAOTE and were category 5 or not categorized (i.e. LD50>5000 mg/kg) with TOPKAT. MDA 28 was predicted to be the most toxic as a category 3. Several compounds were not classifiable with predicted toxicities greater than 5000 mg/kg or were not testable due to poor solubility. Read across between the compounds and the development of a general alignment of categorical toxicity was present. In no instances were the categories more than 1 step different between the CAOTE and TOPKAT results. For example, CAOTE was GHS 5 and TOPKAT estimated GHS 4. Of note, MDA (parent compound) scored as Cat.4 by all three approaches. and compared to the TOPKAT estimate and database values (if available), see **Table 6**.

DISCUSSION/CONCLUSIONS

High temperature resins for use in advance composites are necessary for military applications; however, the use of MDA has become problematic due to its liver toxicity and carcinogenicity. The replacement of MDA with less hazardous alternatives is a green initiative and promotes occupational safety and environmental health by reducing exposure to chemicals that may be carcinogenic.

Minimal experimental data were located for the MDA replacements. QSAR modeling was used to evaluate all of the MDA candidates and is the basis for much of the toxicity and hazard evaluation in this report.

As a group, the MDA compounds are relatively small and within the molecular weight range of biologically active molecules (average MW 268; range 104.1-518.5). Using QSAR (EPISuites, ECOSAR and TOPKAT), estimates for physical chemical properties, environmental fate and transport, and toxicity were developed. These data were evaluated and the MDA compounds were ranked for level of toxicity and ecological hazards compared to MDA, the baseline compound, and visualized with TOXPi (**Figure 1**).

Synthesized MDA compounds were tested in a battery of *in vitro* assays (Microtox, Ames, CAOTE). Compounds that were identified in TOPKAT as potential skin sensitizers were screened in the h-CLAT NAM.

When the Ames experimental data were compared to the TOPKAT predicted mutagenicity, 6 of the 25 compounds tested had nonconcordant data. MDA 4, 13, 27, 43, and 52 were predicted to be positive using TOPKAT but were negative in the Ames assay. MDA 40 was predicted to be negative by TOPKAT but was positive in the Ames assay. These discrepancies could be due to solubility or sensitivity in the Ames assay (a recently identified concern for the liquid based Ames assay platforms.)

All of the compounds tested were negative without S9 activation. These data parallel the finding that metabolic activation is needed for MDA to be mutagenic. Metabolic activation to a mutagen is a hazard as human endogenous detoxifying systems could be ineffective at reducing toxicity andmay enhance toxicity. Additionally, sensitive subpopulations may be more vulnerable to MDA toxicity due to disease related liver function.

Six MDA compounds were predicted to be skin sensitizers (MDA 40, 43, 48, 52, IDMMDA50 and IAMMDA52). Recent updates to the OECD *in vitro* skin sensitization test prioritizes the h-CLAT and a positive in the h-CLAT is sufficient to identify sensitization.

Five of these six MDAs were scored as positive using h-CLAT. IAMMDA52 was negative in the h-CLAT and is pending further testing in the direct peptide reactivity assay (DPRA).

In reviewing the available data, conducting in silico and in vitro analyses, all of the candidate MDAs have fairly similar characteristics, likely due to their structural similarities. As evidenced in **Figure 1**, most of the candidates fall within a moderate to low toxicity hazard level, have moderate to low aquatic toxicity and are persistent in the environment for weeks to months.

Downselected MDAs that meet the performance criteria should undergo additional relevant toxicity and safety testing. Data suggest that the MDA compounds are absorbed through the skin; therefore, dermal toxicity and systemic toxicity from dermal absorption should be evaluated. Occupational exposure to dusts should be avoided and appropriate PPE should be worn to prevent skin exposure.

From this tiered approach, several MDA compounds were identified as having potentially more toxic and/or worse ecological outcomes. For the purposes of replacing MDA with a less toxic alternative, the focus should be on eliminating mutagenicity and reducing chronic toxicity.

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