Original Article

A category approach to predicting the hemolytic effects of ethylene glycol alkyl ethers in repeated-dose toxicity

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ABSTRACT — Categorizing chemicals is an approach with the potential to reduce animal testing for hazard assessment of chemicals. In this study we investigated the category approach for testing the hemolytic effects of ethylene glycol alkyl ethers (EGAEs) for repeated-dose toxicity (RDT). Using mechanistic information on the hemolytic effects of ethylene glycol butyl ether, a toxicologically meaningful category was built on the basis of similarity of metabolism, mode of action and the hemolytic effects of several EGAEs and related chemicals. The developed category was then evaluated for analogs from a different data source. Given all structural information on category chemicals, the category can be finally defined as EGAEs (alkyl chain carbon number: 1-4) and their acetates. Current RDT test data suggest that EGAEs with 3 and 4 alkyl carbons primarily cause hemolytic effects, while EGAEs with 1 and 2 alkyl carbon(s) show toxicity to the testis before demonstrating any hemolytic effects. Hence, the category approach appears to be applicable to hemolytic effects of EGAEs with 3 and 4 alkyl carbons and their acetates to estimate the no observable adverse effect level (NOAEL) for RDT. It consists of three steps: structure-based primary screening of untested chemicals, categorization of compounds that form hemolytic alkoxyacetic acids by predicting how they are metabolized, and finally estimation of hemolytic levels by employing read-across. Our results clearly demonstrate the usefulness of the category approach for predicting the hemolytic effects of untested EGAEs and their acetates in RDT.

Key words: Category approach, Repeated-dose toxicity, Ethylene glycol alkyl ether, Hemolytic anemia

INTRODUCTION

In regulatory toxicology, repeated-dose toxicity (RDT) is an essential item of information for hazard evaluation of industrial chemicals. The number of chemicals for which RDT has been tested, however, is limited, since toxicity testing is costly and time-consuming. However, chemical regulation policies are increasingly requiring that RDT data be made available for marketed but untested chemicals. The category approach therefore has potential as a useful method of hazard evaluation of hitherto untested chemicals. Using this approach, chemicals whose toxicological properties are likely to be similar, or which show a regular pattern of behavior as a result

of structural similarity, are grouped into a chemical category. If an untested chemical falls into this category, its toxicity is estimated using data for tested analogs in this category. This is termed "data gap filling." The category approach is increasingly finding acceptance for several regulatory endpoints (OECD, 2007; Schaafsma *et al.*, 2009; van Leeuwen *et al.*, 2009). However, it is less commonly applied to RDT, since chemicals that are similar as to molecular structure often have dissimilar toxic effects. Hence, chemicals with a common toxicological mechanism need to be grouped into categories to increase reliability, causality and confidence in toxicity prediction. Plausible connections between chemicals, key events and adverse outcome are now proposed as adverse outcome

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pathway (AOP) (Ankley *et al.*, 2010; Schultz, 2010); and AOP is increasingly regarded as valuable for forming toxicologically meaningful categories (OECD, 2011). Several attempts have recently been made to form categories (Fabjan *et al.*, 2006; Sakuratani *et al.*, 2008) and for data gap filling by read-across (Wu *et al.*, 2010; Blackburn *et al.*, 2011) for complicated toxicity endpoints.

Some ethylene glycol alkyl ethers (EGAEs) are high-production-volume chemicals with a wide range of industrial uses, such as in paints, varnishes, resins, engine fuels, and hydraulic fluids; and in many household products, including floor polishes, and glass, leather, and upholstery cleaners. Since the 1980s, concerns over potential human exposure, as well as initial reports suggesting potential toxicity, resulted in extensive research efforts to characterize the toxic effects of EGAEs (NIOSH, 1990; NIOSH, 1991). EGAEs are reported to induce various toxicities in laboratory animals, including hematotoxicity as well as reproductive and developmental toxicities. These are generally dependent on dose and alkyl carbon chain length (Johanson, 2000; Johnson, 2002; OECD, 2004). Ethylene glycol butyl ether (EGBE), which has a straight alkyl chain of 4 carbons, is one of the major marketed EGAEs. Repeated administration of EGBE primarily causes hemolytic anemia, but does not cause reproductive or developmental toxicities (NTP, 1993; CICAD, 2005). The hemolytic mechanism has been studied extensively (Ghanayem *et al.*, 1987; Ghanayem, 1989; Udden, 2002). Some EGAEs appear to be similar to EGBE in terms of hemolytic effects and mechanism of action. However, the hemolytic effects have not been fully evaluated for all existing EGAEs and related chemicals. Because of the potential exposure to various EGMEs and related chemicals through consumer products, filling lack of the toxicity data is desirable.

In this study, category approach was applied to predict the hemolytic effects of EGAEs and related chemicals. A toxicologically meaningful category was built on the basis of similarity of metabolism, mode of action and hemolytic effects of several EGAEs and related chemicals. The developed category was then evaluated with the category candidates from a different data source. Finally, as-yet untested EGAEs and related chemicals were categorized and the hemolytic effects were predicted based on the toxicity data of suitable analog(s). Results of category validation support the reliability of prediction of hemolytic effects of untested EGAEs and related chemicals.

MATERIALS AND METHODS

Data set

One criterion for selection for EGAEs was the carbon

number of the alkyl chain, since the aim was to determine the boundary of the alkyl chain of EGAEs for hemolytic effects. The EGAE-related chemicals were chosen according to similarities in chemical structure, physico-chemical properties, chemical reactivity and metabolism. To develop a category, six chemicals were chosen from our RDT database (Hayashi and Sakuratani, 2011). This database consists of a data set of 500 (mainly industrial) chemicals, most of which are derived from RDT studies conducted under Good Laboratory Practice as defined in the Japanese Chemical Substances Control Law (http://dra4.nihs.go.jp/mhlw_data/jsp/SearchPageENG.jsp) and the National Toxicology Program (NTP) of the National Institutes of Health in the USA (<http://ntp.niehs.nih.gov/>). The database will be published in 2012 and is provisionally termed the RDT DB in this study. For evaluating a category, seven chemicals were selected from RepDose DB, a database on RDT studies of commercial chemicals developed by Fraunhofer Institute of Toxicology and Experimental Medicine, Germany, (Bitsch *et al.*, 2006). Chemicals and their abbreviations, and the data source of the RDT test data are depicted in Table 1.

Development of AOP and category

Literatures on RDT, metabolism and various *in vitro* and *in vivo* toxicological studies were gathered for EGAEs. Using the evidence of nearly 30 years of studies, an AOP was built for the hemolytic effects of EGBE, the most extensively studied EGAE, taking into account strength, consistency, and specificity of association of initiating key events and adverse outcomes. To identify hemolytic chemicals, significant decrease in red blood cell (RBC) counts, hemoglobin concentration and hematocrit were utilized as indicators of primary hemolytic effects, and a significant increase in reticulocyte counts and splenic hemosiderin pigmentation were confirmed as secondary hemolytic effects. Hemolytic levels were evaluated as the lowest observed adverse effect level (LOAEL) and the lower confidence limit on the benchmark dose associated with a 10% response (Benchmark Dose Lower Bound 10%: BMDL₁₀) for RBC decrease. Using US EPA-provided Benchmark Dose Software (Version 2.1.2), BMDL₁₀ for RBC decrease (BMDL_{10 RBC ↓}) was estimated with a 10% reduction as relative deviation of BMR type at the 95% confidence level. Dose, animal number, RBC counts and standard deviation were input to a Continuous Model. For selection of the model, the lowest data was used by visual confirmation of the goodness-of-fit using graphical displays. If hematology data were available for both males and females, the data indicating the more severe hemolytic effects were utilized.

Table 1. Chemicals, their abbreviations and the RDT test data source in this study

Abbreviation	Chemical name	Data source of RDT test (database)
EGME	Ethylene glycol methyl ether	NTP, 1993 (RDT DB and RepDose DB)
EGEE	Ethylene glycol ethyl ether	NTP, 1993 (RDT DB and RepDose DB)
EGiPE	Ethylene glycol isopropyl ether	MHLW, 2002 (RDT DB and RepDose DB)
EGiPEA	Ethylene glycol isopropyl ether acetate	Not available
EGPE	Ethylene glycol propyl ether	Katz <i>et al.</i> , 1984 (RepDose DB)
EGPEA	Ethylene glycol propyl ether acetate	Katz <i>et al.</i> , 1984 (RepDose DB)
EGtBE	Ethylene glycol <i>tert</i> -butyl ether	MHLW, 2001 (RDT DB)
EGiBE	Ethylene glycol isobutyl ether	Not available
EGBE	Ethylene glycol butyl ether	NTP, 1993 (RDT DB and RepDose DB)
EGBEA	Ethylene glycol butyl ether acetate	Not available
EGHE	Ethylene glycol hexyl ether	Klonne <i>et al.</i> , 1987 (RepDose DB)
DGPE	Diethylene glycol propyl ether	Not available
DGBE	Diethylene glycol butyl ether	Johnson <i>et al.</i> , 2005 (RepDose DB)
PGMEA	Propylene glycol methyl ether acetate	MHLW, 1998 (RDT DB)
PGME	Propylene glycol methyl ether	Landry <i>et al.</i> , 1983 (RepDose DB)
PGPE	Propylene glycol propyl ether	ECETOC, 1995 (RepDose DB)
PGBE	Propylene glycol butyl ether	ECETOC, 1995 (RepDose DB)

A data matrix was constructed and carefully evaluated in terms of category candidates, formation of alkoxyacetic acid and swelling of RBC as key events, and significant decrease in RBC counts as an indicator of hemolytic effects, to develop a toxicologically meaningful category. Finally, category definition was described on the basis of chemical structure and metabolism of the category chemicals.

Prediction of the hemolytic effects of category candidates

The chemical inventory of the Ministry of International Trade and Industry (MITI), Japan was first screened to obtain chemicals containing a EGAE substructure with alkyl chains of 3 and 4 carbons using the OECD QSAR Toolbox Ver. 2.1 (http://www.oecd.org/document/54/0,3746,en_2649_34379_42923638_1_1_1_1,00.html), a freely available software application that incorporates information on chemical and various toxicity data and has a function for grouping chemicals. After dataset chemicals had been removed, the remaining EGAE, acetate ester of EGAE and diethylene glycol alkyl ether were chosen as untested category candidates. The name of the remaining four chemicals is listed in Table 1. Their metabolism was predicted based on documented metabolism information on analog chemicals for categorization. Presumed hemo-

lytic levels were estimated by read-across with $BMDL_{10}^{RBC \downarrow}$ (if available) or $LOAEL_{RBC \downarrow}$ of suitable tested analog(s).

RESULTS

Building an AOP for the hemolytic effects of EGBE

An AOP represents existing knowledge of the linkage between a chemical, the molecular initiating event and the adverse outcome at individual (or population) level (Ankley *et al.*, 2010). Since more studies on EGAE-induced hemolysis have been performed with EGBE, the AOP for EGBE-induced hemolytic effects was built by taking into account the strength, consistency, and specificity of the association with a series of sets of experimental evidence, as shown in Fig. 1.

There is strong evidence to support the proposed AOP. Repeated inhaled and oral administration of EGBE caused acute and subacute hemolytic effects (NTP, 1993, 2000; OECD, 2004). Pretreatment of rats with pyrazole, an alcohol dehydrogenase inhibitor, or cyanamide, an aldehyde dehydrogenase inhibitor, suppressed butoxyacetic acid formation and EGBE-induced hemolysis (Ghanayem *et al.*, 1987). Pyrazole inhibition of EGBE metabolism to butoxyacetic acid was accompanied by an increase in

Fig. 1. AOP of EGBE-induced hemolytic anemia in rats. EGBE is readily absorbed following inhalation, oral and dermal exposure. EGBE is primarily oxidized by alcohol dehydrogenase to yield butoxyaldehyde, predominantly in the liver. Further conversion of the aldehyde by aldehyde dehydrogenase produces butoxyacetic acid. Unlike EGBE, butoxyacetic acid is not rapidly cleared from the blood, and interacts with RBCs. Butoxyacetic acid apparently acts as a possible toxicant that induces swelling of RBCs and subsequent hemolysis. The adverse outcome is identified by hematology data in RDT tests including decrease in RBC count (RBC↓), decrease in hemoglobin concentration (Hb↓) and decrease in hematocrit (Hct↓), as well as increased reticulocyte count (Ret↑) and hemosiderin pigmentation in the spleen.

EGBE metabolism to the glucuronide and sulfate metabolites in urine (Ghanayem *et al.*, 1987). There was a correlation between the amount of butoxyacetic acid in the urine and EGBE-induced hematotoxicity (Ghanayem *et al.*, 1987). Moreover, equimolar amounts of butoxyacetic acid administration induced hemolysis at similar levels of EGBE (Ghanayem *et al.*, 1987). Incubation of rat RBCs with butoxyacetic acid induced swelling and subsequent leakage of hemoglobin in a dose-dependent manner (Ghanayem *et al.*, 1989). The molecular initiating event elicited by butoxyacetic acid is unknown. One possible explanation is that hemolytic effects might start with a specific interaction between the alkoxyacetic acid moiety and an unidentified component of the RBC membrane, causing disruption of the osmotic balance (Ghanayem, 1989). It has also been proposed that butoxyacetic acid causes colloid osmotic swelling of RBCs (Udden and Patton, 2005). Morphological changes in RBCs characterized as having a cup-shaped form, induced by butoxyacetic acid *in vitro*, were similar to those induced by EGBE administration *in vivo* (Udden, 2000). The altered cells may be destroyed by macrophages in the spleen or liver (Udden and Patton, 2005). None of the minor metabolites have been shown to be toxic. AOP identifies a measurable key event linked to an adverse outcome for developing a category. In this AOP, metabolic formation of hemolytic alkoxyacetic acids is critical information associated with hemolysis to form an EGAE category for hemolytic effects.

Development of EGAE category for hemolytic effects

To develop a category for EGAEs, the following six chemicals were selected from the RDT DB, based on variations in the alkyl chain: ethylene glycol methyl ether (EGME), ethylene glycol ethyl ether (EGEE), ethylene glycol isopropyl ether (EGiPE), ethylene glycol *tert*-butyl ether (EGtBE), ethylene glycol butyl ether (EGBE) and propylene glycol methyl ether acetate (PGMEA). Table 2 shows a matrix that compares metabolism, RBC swelling activity and hemolysis in rats for each of these chemicals. EGME, EGEE, EGiPE and EGBE were chiefly metabolized to the corresponding alkoxyacetic acids (Miller *et al.*, 1984a; Sumner *et al.*, 1995; Cheever *et al.*, 1984; Hutson and Pickering, 1971; Ghanayem *et al.*, 1987; Sabourin *et al.*, 1992). EGME showed weak hemolytic activity in RDT (NTP, 1993), which was supported by evidence of slight swelling activity of rat RBCs when exposed to methoxyacetic acid *in vitro* and no significant mean corpuscular volume (MCV) increase *in vivo* (Ghanayem *et al.*, 1989; NTP, 1993). Ethoxyacetic and butoxyacetic acids apparently showed swelling of rat RBCs and hemolysis *in vitro* (Ghanayem *et al.*, 1989). Moreover, repeated administration of EGEE, EGiPE, EGtBE or EGBE to rats induced a significant increase in MCV and hemolysis (NTP, 1993; MHLW, 2001, 2002). Propylene glycol methyl ether acetate (PGMEA) was readily hydrolyzed to PGME, which underwent *O*-demethylation and conjugation to form propylene gly-

Category approach for repeated-dose toxicity

Table 2. A toxicity data matrix for EGAEs and related chemicals from the RDT DB for forming a category

Chemical structure Name CAS No.	Alkoxyacetic acid formation (<i>in vitro</i>) (<i>in vivo</i>)	Swelling of RBC (<i>in vitro</i>) ^{*1} (<i>in vivo</i>) ^{*2}	Hemolytic effects and levels ^{*3} Strain of rat, duration, route Dose
EGME 109-86-4	+	+	LOAEL _{RBC↓} ; 4500 ppm (9.4 mmol/kg/d), BMDL _{10 RBC↓} ; 5.50 mmol/kg/d F344 rat, 13-w, drinking water 750, 1500, 3000, 4500 ppm
EGEE 110-80-5	+	+	LOAEL _{RBC↓} ; 5000 ppm (4.4 mmol/kg/d), BMDL _{10 RBC↓} ; 4.31 mmol/kg/d F344 rat, 13-w, drinking water 1250, 2500, 5000, 10000 ppm
EGtPE 109-59-1	+	+	LOAEL _{RBC↓} ; 125 mg/kg/d (1.2 mmol/kg/d), BMDL _{10 RBC↓} ; 0.74 mmol/kg/d SD rat, 28-d, gavage 30, 125, 500 mg/kg/d
EGtBE 7580-85-0	No data found	+	LOAEL _{RBC↓} ; 100 mg/kg/d (0.85 mmol/kg/d), BMDL _{10 RBC↓} ; 0.33 mmol/kg/d SD rat, 42-d, gavage 4, 20, 100 mg/kg/d
EGBE 111-76-2	+	+	LOAEL _{RBC↓} ; 750 ppm (0.69 mmol/kg/d), BMDL _{10 RBC↓} ; 0.86 mmol/kg/d F344 rat, 13-w, drinking water 750, 1500, 3000, 4500, 6000 ppm
PGMEA 108-65-6	-	-	No effects on RBC; 1000 mg/kg/d (7.6 mmol/kg/d) SD rat, 44-d, gavage, 30, 100, 300, 1000 mg/kg/d

^{*1} *In vitro* swelling of RBC was referenced from the results of cell volume increase with alkoxyacetic acids by Ghanayem *et al.* (1989).

^{*2} *In vivo* swelling of RBC was referenced from the results of MCV increase in hematology data of RDT tests.

^{*3} Hemolytic level was presented as LOAEL and BMDL₁₀ for significant RBC decrease (LOAEL_{RBC↓} and BMDL_{10 RBC↓}).

col and PGME-glucuronide and -sulfate, respectively. No methoxyacetic acid was detected in rats (Miller *et al.*, 1984b). Repeated administration of PGMEA caused no hemolytic effects or any other pathological changes (MHLW, 1998).

Taken together, EGME, EGEE, EGtPE, EGtBE and EGBE appear to be similar as to metabolism, mode of action and hemolytic effects. Hence, they can be included in the EGAE category for hemolytic effects. PGMEA is not included in this category due to absence of metabolic formation of methoxyacetic acid and lack of hemolytic effects. However, the metabolic features of this chemical

suggest that metabolic convergence to category chemicals needs to be taken into account when forming a category. A previous study demonstrated the rapid hydrolysis of PGMEA *in vivo* to PGME and showed that, once hydrolyzed, the kinetics of PGME derived from PGMEA are identical to those of PGME (Domoradzki *et al.*, 2003). It is thus reasonable to conclude that chemicals such as acetate esters of EGAE are converted to hemolytic EGAE. Examining the chemical structure of category chemicals and the toxicokinetic features of other related chemicals allows the chemical space of the category to be described. The category we propose is the group of EGAE (alkyl

chain carbon number: 1-4) or chemicals with the EGAE substructure, which are metabolized chiefly to the corresponding alkoxyacetic acid. These category members produce hemolytic anemia by acting via the AOP of EGBE.

Evaluation of a proposed category for analogs from a different data source

To evaluate the proposed category, EGAEs and related chemicals were obtained from RepDose DB, a different data source. All EGAEs were initially selected; and acetate ester of EGAE, diethylene glycol alkyl ether and propylene glycol alkyl ether were then selected based on similarities in chemical structure, physicochemical properties, and chemical reactivity. Chemicals already present in the RDT DB, and chemicals whose hematology data in rats are not available in RepDose DB, were then eliminated. The remaining seven chemicals are listed in Table 3.

The first chemical, ethylene glycol propyl ether (EGPE) has a straight alkyl chain with 3 carbons. This chemical appears to be within the boundary of the proposed category. No documented metabolism information was found, but it is reasonable to presume that EGPE is metabolized to propoxyacetic acid by analogy with the metabolism of EGEE and EGBE (Fig. 2A). This suggests that EGPE is a member of this category and thus causes hemolytic effects. The presumed NOAEL for hemolysis was roughly estimated by averaging the $\text{BMDL}_{10 \text{ RBC } \downarrow}$ of EGEE, EGPE and EGBE (interpolation) as 2.0 mmol/kg/d. In the RDT study in rats receiving EGPE for 6 weeks by gavage, dose-related hemolytic anemia was observed at 1.9 mmol/kg/d or more (Katz *et al.*, 1984) and the $\text{BMDL}_{10 \text{ RBC } \downarrow}$ was estimated to be 2.3 mmol/kg/d with default standard deviation (4.5%).

The second chemical, ethylene glycol propyl ether acetate (EGPEA) contains an EGPE substructure linked with an acetyl group via an ester bond. There is no documented metabolism data found for this chemical. The structural analogs, ethylene glycol ethyl ether acetate (EGEEA) and diethylene glycol butyl ether acetate (DGBEA), were reported to be rapidly hydrolyzed to form EGEE and DGBE, respectively, as major metabolites (Stott and McKenna, 1985; Gargas *et al.*, 2000; Deisinger and Guest, 1989). Hence it is reasonable to assume that EGPEA is readily hydrolyzed to EGPE (Fig. 2B) and exerts its hemolytic effects with similar severity to EGPE. Repeated gavage exposure to EGPEA for 6 weeks in rats primarily produced dose-dependent hemolytic changes at 7.5 mmol/kg/d and above (Katz *et al.*, 1984). From this dose-response relationship, $\text{BMDL}_{10 \text{ RBC } \downarrow}$ of EGPEA was calculated to be 3.0 mmol/kg/d (using a default standard deviation of 4.5%), while the $\text{BMDL}_{10 \text{ RBC } \downarrow}$ of EGPE is

already known above to be 2.3 mmol/kg/d. These similar responses may indicate that the same ultimate substance produces hemolysis in spite of slight differences derived from the additional hydrolysis step.

EGHE was shown to be oxidized to hexoxaldehyde in rat liver homogenate (Aasmoe *et al.*, 1998). The aldehyde metabolite is thought to be converted to hexoxyacetic acid (Fig. 2C), in line with the documented metabolism of EGBE to butoxyacetic acid and DGBE to butoxyethoxyacetic acid as previously described. Previous *in vitro* studies have demonstrated that the hemolytic activities of alkoxyacetic acids with straight alkyl chains increased as the length of the alkyl chain extended to a maximum of 4 carbons (butoxyacetic acid), but declined when the chain length increased to 5 carbons (pentoxyacetic acid), and that propoxyacetic acid and pentoxyacetic acids have similar hemolytic potency (Ghanayem *et al.*, 1989). These results suggest that EGHE may have lower hemolytic effects than EGPE *in vivo*. EGHE did not reveal any hemolytic effects under relatively lower inhaled exposure conditions (Klonne *et al.*, 1987).

Diethylene glycol butyl ether (DGBE) has an EGBE substructure on its molecule. Oxidative degradation of DGBE may result in the formation of EGBE. However, a previous metabolism study has demonstrated that the terminal alcohol moiety of DGBE is oxidized to generate butoxyethoxyacetic acid as a major metabolite; and butoxyacetic acid was not detectable (Fig. 2D) (Deisinger and Guest, 1989). Hence DGBE was concluded not to be in this category. Repeated administration of DGBE for 13 weeks showed very slight hemolytic anemia in rats at 7.6 mmol/kg/d (Johnson *et al.*, 2005).

PGME undergoes a conjugation reaction with glucuronide and sulfate, and *O*-dealkylation (Miller *et al.*, 1984a); methoxyacetic acid is not formed (Fig. 2E), suggesting no hemolytic effects of PGME as PGMEA. One study confirms that repeated administration of PGME induces no hemolytic effects (Landry *et al.*, 1983). It is reasonable to presume that methylation of the alpha carbon of EGPE affords a protective effect against hemolysis, probably by changing the metabolic pathway such that no methoxyacetic acid is formed. The metabolism feature and non-hemolytic effects are predicted to be similar in PGPE and PGBE, which can be confirmed by a series of RDT studies of these chemicals (ECETOC, 1995).

Evaluating the proposed category with seven chemicals clearly demonstrated the validity of this category for hemolytic effects of EGAEs and related chemicals. Given all structural information on category chemicals in Tables 2 and 3, the category can be finally defined as EGAEs (alkyl chain carbon number: 1-4) and their acetates.

Category approach for repeated-dose toxicity

Table 3. A toxicity data matrix for EGAEs and related chemicals from the RepDose DB for evaluating a category

Chemical structure Name CAS No.	Alkoxyacetic acid formation (<i>in vitro</i>) (<i>in vivo</i>) (<i>in silico</i>)	Hemolytic effects <i>in silico</i> (predicted hemolytic levels)	Hemolytic effects and levels* Strain of rat, duration, route Dose
EGPE 2807-30-9	+	+	LOAEL _{RBC↓} ; 1.9 mmol/kg/d SD rat, 6-w, gavage 1.88, 3.75, 7.5, 15 mmol/kg/d
EGPEA 20706-25-6	+	+	LOAEL _{RBC↓} ; 7.5 mmol/kg/d SD rat, 6-w, gavage 7.5, 15, 30 mmol/kg/d
EGHE 112-25-4	+	±	No effects on RBC; 71 ppm (0.67 mmol/kg/d) F344 rat, 13-w, 5 d/w, inhalation 20, 41, 71 ppm
DGBE 112-34-5	-	-	LOAEL _{RBC↓} ; 1000 mg/kg/d (6.2 mmol/kg/d) F344 rat, 13-w, drinking water 50, 250, 1000 mg/kg/d
PGME 107-98-2	-	-	No effects on RBC; 3000 ppm (28 mmol/kg/d) F344 rat, 13-w, inhalation 300, 1000, 3000 ppm
PGPE 1569-01-3	-	-	No effects on RBC; 300 ppm (2.8 mmol/kg/d) F344 and SD rat, 98-d, inhalation 30, 100, 300 ppm
PGBE 5131-66-8	-	-	No effects on RBC; 1000 mg/kg/d (7.6 mmol/kg/d) Rat*, 13-w, drinking water 100, 350, 1000 mg/kg/d

*¹Hemolytic level was presented as LOAEL_{RBC↓}. BMDL_{10 RBC↓} was not shown because the standard deviation and number of animals used are not given in most of the RDT reports. *²Strain could not be identified.

Prediction of hemolytic effects of EGAEs and related chemicals without available RDT data

As a trial of the category approach, prediction of hemolytic effects was tested for the following four chemicals without available RDT data: ethylene glycol isopropyl ether acetate (EGiPEA), ethylene glycol isobutyl ether (EGiBE), ethylene glycol butyl ether acetate (EGBEA) and diethylene glycol propyl ether (DGPE) (Table 4). No

documented metabolism information was found for EGiPEA or EGBEA. By analogy to the observed metabolism of EGEEA and DGBEA and predicted metabolism of EGPEA in Fig. 2B, EGiPEA and EGBEA are expected to be almost completely susceptible to metabolic hydrolysis. It is thus reasonable to predict that EGiPEA and EGBEA would be included in the category, and that the presumed NOAEL of hemolysis would be similar to those

Fig. 2. Documented and predicted major metabolic pathways in rats of EGAEs and related chemicals from RepDose DB. EGAE substructures with 3 or 4 alkyl carbons and the other moieties are shown in black and gray, respectively. Solid and dotted arrows respectively represent observed and predicted metabolic reactions. See the Results section for a detailed description.

of the corresponding EGAEs with $\text{BMDL}_{10\text{ RBC}\downarrow}$ of 0.74 and 0.86 mmol/kg/d, respectively. EGtBE has no empiric metabolism data, but it can be predicted to form the corresponding alkoxyacetic acid in the same way as the other category members. It is less likely that the hemolytic level of EGtBE would be different from those of EGPE, EGtPE, EGtBE or EGBE, since molecular size, hydrophobicity and reactivity are apparently similar among the five chemicals, and the alkoxyacetic acid moiety is critical for hemolytic activity. EGAEs with branched alkyl chains of 3 and 4 carbons (EGtPE and EGtBE, respectively) appear to be slightly more hemolytic than those with straight alkyl chains (EGPE and EGBE). Moreover, EGtBE is more hemolytic than EGtPE. The presumed NOAEL of hemolysis of EGtBE can be roughly estimated to be similar to $\text{BMDL}_{10\text{ RBC}\downarrow}$ of EGtBE (0.33 mmol/kg/d) on the protective side. DGPE is presumed not to be a category member, since it does not seem to yield hemolytic alkoxyacetic acids via metabolism like DGBE.

DISCUSSION

New chemical management programs such as the Registration, Evaluation and Authorization of Chemicals (REACH) in the European Union require hazard evaluation for many marketed chemicals and also call for minimizing animal testing. Therefore, several initiatives have emerged to adopt alternative methods for hazard evaluation. A mechanistic interpretation is necessary for regulatory acceptance of toxicity prediction using an alternative strategy. Herein we have attempted to develop and evaluate a mechanism-based category approach for hemolytic effects of EGAEs and related chemicals.

The data quality used in this study is reasonably high because all the data were derived from RDT DB and RepDose DB, where studies were carefully selected by data quality (Hayashi and Sakuratani, 2011; Bitsch *et al.*, 2006). The toxicity endpoint of the category is hemolytic effects, which is one of the endpoints used for regulatory decisions. It can be clearly identified by hematology data, including significant decrease in RBC counts, hemoglobin concentration and hematocrit, accompanied by signif-

Table 4. A data matrix for prediction of hemolytic effects of untested EGAEs and related chemicals

Chemical structure Name CAS No.	Alkoxyacetic acid formation (<i>in silico</i>)* ¹	Hemolytic effects <i>in silico</i> (Predicted hemolytic levels)
EGiPEA 19234-20-9	+	+ (Similar levels to EGiPE)
EGiBE 4439-24-1	+	+ (Similar levels to EGtBE or less)
EGBEA 112-07-2	+	+ (Similar levels to EGBE)
DGPE 6881-94-3	-	-

*¹Metabolism was presumed *in silico* by considering documented metabolism information of structurally related chemicals. See the Results section for a detailed description.

icant increase in reticulocyte counts and splenic hemosiderin pigmentation in RDT test reports.

EGAEs are hemolytic. Certain EGAE analogs are also hemolytic, but some are not (Tables 2 and 3). The current RDT test data can be interpreted in the light of the evidence that alkoxyacetic acid, a major metabolite of EGAEs, is toxic to RBCs and that hemolytic EGAE analogs appear to be metabolized to alkoxyacetic acid (Ghanayem *et al.*, 1987; Ghanayem, 1989; Ghanayem *et al.*, 1989; Udden and Patton, 2005; Starek *et al.*, 2008). Previous *in vitro* studies have demonstrated no hemolytic activity for heptanoic acid, which has a similar chain length but no ether oxygen, or propoxypropionic acid which is isomeric with butoxyacetic acid but has the ether linkage in a different position (Ghanayem *et al.*, 1989). The results strongly indicate that the hemolytic effects might be started by a specific interaction between the alkoxyacetic acid moiety and an unidentified RBC component. The theoretical severity of EGAE-induced hemolytic effects *in vivo* can be influenced by the hemolytic activities of alkoxyacetic acids as well as toxicokinetic factors including the concentrations of alkoxyacetic acids

in the blood during repeated administration. The ranking order of hemolytic activity *in vitro* was butoxyacetic acid > propoxyacetic acid > ethoxyacetic acid > methoxyacetic acid (Ghanayem *et al.*, 1989). Urinary alkoxyacetic acids in rats constituted 50 to 60 % of the dose for EGBE, 25 % to 40 % for EGEE and 34 % for EGME 72 hr post administration (Medinsky *et al.*, 1990). Hence, the carbon number of straight alkyl chains (up to 4) of EGAEs appears to correlate with both hemolytic activity and metabolic formation of alkoxyacetic acids as well as intensity of hemolytic effects. EGAE isomers with branched alkyl chains containing 3 and 4 carbons appear to be slightly more hemolytic than straight-chain versions. Furthermore, EGAE isomers with 4 alkyl carbons appear to be more hemolytic than those with 3 alkyl carbons, although there were variations in the administration period during testing (Table 2).

Given all structural information on category chemicals identified in RDT DB and RepDose DB, the category can be finally defined as a group of EGAEs (alkyl chain carbon number: 1-4) and their acetates (Tables 2 and 3). The applicability domain is described by alkyl chain car-

Table 5. Summary of structure-toxicity relationship of EGAEs and their acetate in RDT

Chemical	Alkyl carbon No.	Critical toxic effects
EGME	1	Testicular degeneration/atrophy
EGEE	2	Testicular degeneration/atrophy
EGPE	3	Hemolysis
EGPEA	3	Hemolysis
EGiPE	3	Hemolysis
EGBE	4	Hemolysis
EGtBE	4	Hemolysis
EGHE	6	Hepatocyte hypertrophy

bon number in the EGAE molecule, since the alkyl carbon number acts as a structural cutoff within the category chemicals. Acetates of EGAE are within the category, since available evidence demonstrates that EGAE acetates are readily hydrolyzed to EGAE counterparts, and that EGAE and their acetates appear to exhibit similar metabolic fate. There is no available metabolic and toxicological data for other fatty acid esters of EGAE. However, the difference in saturated alkyl chain should not have significant impact on chemical reactivity and metabolic hydrolysis (Wu *et al.*, 2010). Hence, series of EGAE esters with different straight saturated fatty chain may yield hemolytic effects via similar metabolic pathway. It should be noted that chemicals often have multiple toxic endpoints in RDT. Both EGME and EGEE are hemolytic, but primarily cause toxic effects in testis at a lower dose where hemolysis is not yet evident (NTP, 1993) (Table 5). Therefore, testicular toxicity needs to be considered rather than hemolytic effects for untested category members with 1 and 2 alkyl chain carbon(s) to be able to estimate the presumed NOAEL for RDT. An *in vitro* structure-toxicity relationship study demonstrated that pentoxyacetic acid (5 carbons) was hemolytic in rat RBCs at about half the potency of butoxyacetic acid (Ghanayem *et al.*, 1989). EGHE was not hemolytic, but the maximum dose was relatively lower in one study (Klonne *et al.*, 1987). Hence, it is possible that EGHE is still hemolytic at higher doses, and the applicability domain might be expanded to 6 alkyl carbons if the hemolytic effects appear at higher doses. However, EGHE appears to be hepatotoxic at doses below which hemolysis is not yet evident (Klonne *et al.*, 1987) (Table 5). Taken together, the category approach in this study can be practically applied to EGAEs with alkyl chain carbon numbers 3 and 4 and their acetates to estimate presumed NOAEL for RDT (Table 5).

A critical step for applying the category approach is to categorize chemicals with a common toxic mechanism. However, untested chemicals often lack mechanistic information: the only available information is chemical structure and physicochemical properties. It is thus necessary to measure or predict whether untested chemicals cause key events linked to an adverse outcome. In the proposed category, metabolic formation of hemolytic alkoxyacetic acids is a measurable or predictable key event associated with hemolytic effects. Fig. 3 illustrates the scheme of category approach for hemolytic effects of EGAEs. Untested category candidates are primarily screened based on the EGAE substructure with alkyl chain carbon numbers of 3 and 4 as well as similar physicochemical properties and chemical reactivity. This step can be supported by computer software equipped with function to search structural analogs with toxicity data, such as Hazard Evaluation Support System (HESS) which we will release soon or OECD QSAR Toolbox (Hayashi and Sakuratani, 2011). The target chemicals are then determined as to whether they are category members by considering hemolytic alkoxyacetic acid formation using documented metabolism data on structural analogs in the metabolism database. Finally the hemolytic levels are estimated by read-across using toxicity data for category chemicals (e.g., $BMDL_{10\text{ RBC}\downarrow}$ or $LOAEL_{\text{RBC}\downarrow}$). Expertise is necessary for interpreting available data and making decision. Results of category validation support the reliability of prediction of hemolytic effects of untested EGAE and their acetates.

Finally, mechanism-based category approach is potentially useful to extrapolate hazard evaluation in experimental animals to human. Results from *in vitro* experiments indicated that rat RBCs are more susceptible than human ones to hemolytic effects to butoxyacetic acid with variation from about 3 to 100-fold (Udden, 2002; Starek *et al.*, 2008). On the other hand, case reports of humans intoxicated by ingesting EGBE suggested that it causes severe toxicity including hemolytic anemia (Gijzenbergh *et al.*, 1989; Rambourg-Schepens *et al.*, 1988). Moreover, differences in the susceptibility and toxicokinetic feature between rat and human have not been fully characterized for other hemolytic alkoxyacetic acids. Hence, more detailed information is needed for mechanism-based extrapolation. If the hemolytic effects prove less relevant to human, this information should be considered for risk assessment to human exposed to the category chemicals.

In summary, we have explored a category approach for hemolytic effects of EGAEs and their acetates in RDT. We conclude that our developed category approach model has potential for regulatory-acceptable prediction of the

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Fig. 3. Scheme of mechanism-based category approach for EGAEs and related chemicals to predict hemolytic effects. Untested category candidates are primarily screened based on the EGAE (sub)structure with alkyl chains with 3 and 4 carbons (untested Chemicals A and B) and similarity of the physicochemical properties and chemical reactivity. The substances are determined as to whether they are category members based on the generation of hemolytic alkoxyacetic acid. Untested Chemical A is included in the category if the alkoxyacetic acid is formed, and untested Chemical B is removed if it is not. Finally, the hemolytic level of untested Chemical A is estimated by read-across using existing toxicity data on category chemicals.

hemolytic effects of the untested analogs.

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