SCIENTIFIC OPINION

PMI COMMENTS AND ASSOCIATED BIBLIOGRAPHY RELATING TO THE APRIL 2015 CLH REPORT "PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELING" FOR NICOTINE BY RIVM

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Acronyms

ANSES	Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail
ATE	Acute Toxicity Estimate
b.w.	Body weight
Cat.	Category
CLP	Classification, Labeling and Packaging
CYP	Cytochrome P450
DAR	Draft Assessment Report
DSD	Dangerous Substances Directive
EC	European Community
ED	Effective Dose
EU	European Union
EFSA	European Food Safety Authority
GLP	Good Laboratory Practice
HPHC	Harmful and Potentially Harmful Constituents
i.v.	Intravenous
LD50	Lethal Dose 50%
LC50	Lethal Concentration 50%
OECD	Organization for Economic Co-operation and Development
PMI	Philip Morris International
RIVM	Rijksinstituut voor Volksgezondheid en Milieu (Dutch National Institute for Public Health and the Environment)
s.c.	subcutaneous
TG	Technical Guideline

1 Executive Summary

PMI disagrees with a proposal submitted by the Rijksinstituut voor Volksgezondheid en Milieu (RIVM) to re-classify nicotine as "acute toxic 1" for oral exposure. In PMI's opinion the current classification of nicotine as "acute toxic 3" for oral exposure is still appropriate and should be maintained.

A comparison of three human exposure scenarios based on elevated but not fatal reports from literature against the LD50 from mice or rats did not indicate that the use of the rat LD50 underestimates the human toxicity.The main reason provided by RIVM to prefer mouse studies is that the metabolic pathways of nicotine in mice and in humans have more similarities to each other compared to metabolic pathways in the rat. However, the toxicity of nicotine is receptor-specific and driven by the parent compound rather than its metabolites. Compared to rats and mice, the rate of metabolism of nicotine is much slower in humans, and is in any case closer to rats than to mice (Matta, et

al., 2007). Considering that metabolism results in detoxification of nicotine, we believe that the rat LD50 data are more relevant than the mouse data. Furthermore, the mouse acute toxicity data is derived from a less reliable source than the rat data, based on the Klimisch rating (Klimisch, Andreae, & Tillmann, 1997) (Segal, et al., 2015).

In conclusion, there is no clear scientific justification that for the acute toxicity the mouse data is more relevant than the rat data, and indeed there is sound justification, based on metabolic rate, that the opposite is more likely to be the case. Therefore, LD50 data from rat are relevant for the acute oral toxicity and the current classification "acute toxic 3" for oral exposure is correct and should be maintained.

2 Quality of available study data for acute oral toxicity

Based on "the information available in the REACH-registration (accessed January 2015), the DAR [Draft Assessment Report] of nicotine (1), EFSA 2009 (2) and other information available in literature" RIVM found overall no reliable human data of acute toxicity (RIVM, 2015).

With regard to animal studies on oral acute toxicity, the RIVM report lists 13 studies using nicotine and 6 studies using nicotine salts; six of the studies on nicotine are categorized as "not acceptable", whilst the remaining 13 are deemed acceptable. Within those studies that RIVM found to be acceptable, they further concluded that "only the rat studies by Van den Heuvel et al. (1990) and Yam et al. (1991) would probably fulfil the OECD TG requirements although the reporting is incomplete". On six studies RIVM concludes that they were "considered acceptable seen the period (pre OECD and GLP) in which they were performed and seen the absence of more recent data from the same species", although the quality of reporting of these studies is limited (RIVM, 2015). This group includes the key studies used by RIVM for the selection of relevant LD50, i.e., the studies by Franke and Thomas for LD50 in dogs and Lazutka et al. for LD50 in mice and support for preference of mice over rat data ((Franke & Earl Thomas, 1932), (Lazutka, Vasilyauskene, & Gefen, 1969), (RIVM, 2015)).

Seven of the oral toxicity studies have also been reviewed by the French Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (ANSES) (Agency for Food, Environmental and Occupational Health & Safety) in the *Opinion on assessing the hazards of nicotine* (Request No. 2014-SA-0130, 2015), including those described in publications by Lazutka et al., Heubner, Ambrose et al., Van den Heuvel et al., and Yam et al.((ANSES French Agency for Food, Environmental and Occupational Health & Safety, 2015) and references therein). Regarding the reliability of these studies ANSES, similarly to RIVM, stated that only two of these studies refer to the guidelines, or to validated experimental protocols. These are the studies by Van den Heuvel et al. (Van den Heuvel, et al., 1990), which according to ANSES complies with the 1981 OECD guidelines, and by Yam et al. (Yam, Reer, & Bruce, 1991), which ANSES considers to conform to the "Up and Down" procedure today advocated by the OECD and the American Society for Testing and Materials (ASTM). These studies, which were both conducted in rats, led to an LD50 of 70 mg/kg (ANSES French Agency for Food, Environmental and Occupational Health & Safety, 2015).

However in contrast to RIVM, ANSES stated that apart from these two studies, the publications available on nicotine acute toxicity are all very old, and none conform to the current guidelines, and that when the European Commission's ToxRTool1 software package was applied to score the studies according to the Klimisch rating, they all obtained a score of 3, which corresponds to "not reliable". This is mainly due to the lack of information and the few details given on the experimental protocols and methods used in these studies (ANSES French Agency for Food, Environmental and Occupational Health & Safety, 2015).

3 Toxicokinetic and toxicodynamic considerations

Of the oral toxicity studies listed in the RIVM report, considerable differences were reported for LD50 values between the investigated species. While the majority of acute oral rat studies showed a comparable range of LD50 values between 50 and 70 mg/kg b.w./day, available acute oral data for dogs and mice show much lower LD50 values: 9.2 mg/kg b.w. in dog and 24 and 3.34 mg/kg b.w in mouse. These differences may be due to toxicodynamic and toxicokinetic species differences and variations in the study protocol, e.g., the method of oral application (RIVM, 2015). For an overview of toxicokinetics, the RIVM report refers mainly to a summary by EFSA (2009) and a review by Hukkanen et al. (Hukkanen, Peyton, & Benowitz, 2005). According to that summary, in humans nicotine metabolism is mediated mostly through hepatic cytochrome P450 CYP2A6 by C-oxidation of nicotine to cotinine as the major detoxification reaction, followed by the hydroxylation of cotinine to *trans*-3'-hydroxycotinine.

While cotinine and *trans*-3'-hydroxycotinine are major urinary nicotine metabolites in all mammalian species studied including mice and dogs; about as much nicotine-*N*'-oxide as cotinine and *trans*-3'-hydroxycotinine is formed by guinea pigs and rats. In rats, nicotine-*N*'-oxide is the main metabolite of nicotine, because CYP2B1/2 is the P450 enzyme metabolizing nicotine in rats, whereas rat CYP2A is inactive in nicotine metabolism ((Hukkanen, Peyton, & Benowitz, 2005), (Tutka, Mosiewicz, & Wielosz, 2005)). According to Tutka et al., the differences in nicotine metabolism observed between rats, rabbits and humans suggest that rabbits are a better model for studying human nicotine metabolism, and RIVM subsequently concludes "that the rat may not be the most relevant species for humans" (RIVM, 2015).

However, there are several indications in the literature that the missing CYP2A activity in rats is less important in the context of acute toxicity. Regarding the human metabolism, Hukkanen et al. mention in their review that studies not only demonstrate the significant role of CYP2A6 in human nicotine metabolism, but also that "they illustrate that other enzymes must also be involved in formation of cotinine and *trans*-3'-hydroxycotinine, at least in subjects lacking the CYP2A6 enzyme. CYP2B6 is the second most active hepatic cytochrome P450 enzyme in nicotine *C*-oxidation when investigated using hepatic tissues or expression systems in vitro, *especially at high nicotine concentrations*" (emphasis added, (Hukkanen, Peyton, & Benowitz, 2005)). Another aspect is that nicotine needs no metabolic activation to exert toxicity and the differences in the discussed metabolic pathways are of a detoxifying nature. The metabolism of nicotine is mediated mostly through the hepatic cytochrome P450 CYP2A6 with the C-oxidation of nicotine to cotinine as the major detoxification reaction, followed by the hydroxylation of cotinine to *trans*-3'-hydroxycotinine, as stated in the RIVM report and the reviews of Hukkanen et al. and Tutka et al. In the same reviews, however, it is reported that neither cotinine nor *trans*-3'-hydroxycotinine have cardiovascular effects while the major nicotine metabolite in the rat, nicotine *N*-1'-oxide is generally regarded as non-toxic ((RIVM, 2015) (Hukkanen, Peyton, & Benowitz, 2005) (Tutka, Mosiewicz, & Wielosz, 2005). Therefore, differences in metabolism rate may be more relevant than differences in species of non-toxic metabolites. According to Matta et al., the plasma nicotine half-life in rodents is generally shorter than in humans: 6–7 min in the mouse and 45 min in the rat vs. 2 h in humans, indicating that the rat is closer to the human than the mouse (Matta, et al., 2007). Kyerematen et al. present in a review the metabolic pattern of nicotine in 5 species as percent of remaining nicotine and formed metabolites (Kyerematen, Vesell, & Vesell, 1991). For remaining nicotine, cotinine and nornicotine the values of man and rat are closer than those for man and mouse; only for nicotine *N*-1'-oxide the mouse is closer to the human value than the rat. Still the difference is so small, that they state in a precedent publication on the metabolism of nicotine by hepatocytes that the percent nicotine *N*-1'-oxide production was similar in incubations with hepatocytes from hamster, mouse, rat and humans (Kyerematen, Morgan, Warner, Martin, & Vesell, 1990).

The general mode of action for nicotine has been summarized in a report of the Health Council of the Netherlands (Health Council of the Netherlands, 2004). Nicotine acts by direct stimulation of the nicotinic cholinergic receptors, which causes a release of neurotransmitters, including acetylcholine, noradrenaline, dopamine, and may result in a myriad of symptoms, i.e., modulation of neurological, neuromuscular, cardiovascular, respiratory, glandular, or gastro-intestinal function. The major effects of nicotine are an initial stimulatory effect on these organs due to parasympathetic ganglionic stimulation at nicotinic receptor sites. At larger doses, the initial stimulatory effect is followed by prolonged ganglionic and neuromuscular blockade, which may result in depression and paralysis of the central nervous system, all peripheral autonomic ganglia, and motor end-plates in skeletal muscles.

Studies investigating nicotine-induced seizure sensitivity and nicotine receptors in rodents have shown that rats are more sensitive than mice: the effective dose to induce seizures in half of the test animals (ED50) is 0.5–1.0 mg/kg in rats, while for mice it is 2–6 mg/kg depending on the strain (Matta, et al., 2007). For the differences seen between mice strains (ED50 2–6 mg/kg) the authors of the study suggest that metabolism differences do not play a major role in determining strain differences in seizure sensitivity. They argue that they neither found relationships between seizure sensitivity and brain or blood levels of nicotine in the parent or first and second filial generations of backcross experiments in mice, nor did they observe any relationship between nicotine metabolism for the investigated strains and their seizure sensitivity. Rather they suggest differences in receptors as the most important parameter that regulates sensitivity to nicotine-induced seizures (Collins, Miner, & Allan, 1989)

4 Proposed oral toxicity LD50 value from mice compared to human exposure scenarios

Clearly the most reliable publications are on oral toxicity studies in rats, showing comparable LD50 values between 50 to 70 mg/kg b.w. However, RIVM appears concerned that based on recently published estimates of the minimal lethal dose of nicotine in humans in the range of 6.5 to 13 mg/kg b.w. (Meyer, 2014) and publications by Hukkanen et al and Tutka et al. reviewing species differences in toxicokinetics the available studies on oral toxicity in rats may underestimate human toxicity (Hukkanen, Peyton, & Benowitz, 2005), (Tutka, Mosiewicz, & Wielosz, 2005). As the acceptable studies in other species are limited to mice and dogs and it is unknown which of these two species is more relevant to humans, it is suggested to take the lowest value in the most sensitive species. Therefore, RIVM proposed to use the acute oral LD50 in the mouse of 3.3 mg/kg b.w. as determined by Lazutka et al. (Lazutka, Vasilyauskene, & Gefen, 1969).

Due to the lack of reliable studies that address the question related to which species would be the best model for oral toxicity in humans, the proposed LD50 is compared to three human exposure scenarios selected from the literature, simulating three kinds of increased but non-fatal exposure to nicotine.

1. The highest dose ingested in the "safety check" of lozenges published by Dautzenberg was 12 mg nicotine; taking into account a range between 20 to 45% for oral bioavailability (RIVM, 2015) this corresponds to a systemic dose of 0.04 to 0.09 mg/kg for a person with 60 kg b.w. (Dautzenberg, Nides, Kienzler, & Callens, 2007)

2. The systemic dose from cigarette smoking is about 1–1.5 mg per cigarette ((Benowitz, Hukkanen, & Peyton, 2009), which corresponds to 40 to 60 mg per day for an intense smoker (40 cigarettes per day) or 0.7 to 1 mg/kg b.w.

3. Brady et al. (Brady, Ritschel, Saelinger, Cacini, & Patterson, 1979) report on the survival of a man after accidental s.c. application of 3.58 mg/kg; bioavailability upon s.c. administration according to modelling in rabbits was 83 % (s.c. versus i.v. application of the same dose), which corresponds to a systemic dose of 2.9 mg/kg b.w.

RIVM proposes the LD50 of 3.34 mg/kg b.w. in mice from Lazutka et al. (Lazutka, Vasilyauskene, & Gefen, 1969) as reference of the acute oral toxicity. To compare the proposed LD50 with the systemic exposure in humans according to the selected exposure scenarios a Margin Of Exposure (MOE) like comparison is performed. Three assessment factors are applied: 10 for intraspecies uncertainty, 10 for interspecies uncertainty and 2 for bioavailability after gavage. Because in this case a comparison to a "toxic dose" was carried out and not to a " safe dose", any value for the MOE below 200 would indicate a high concern of acute (life threatening) toxicity.

MOE "lozenges" = 3.34 mg/kg b.w./0.09 mg/kg b.w. = 37

MOE "smoker" = 3.34 mg/kg b.w. /1 mg/kg b.w. = 3.34

MOE "tranquilizing dart" = 3.34 mg/kg b.w. /2.9 mg/kg b.w. = 1.2

In this simulation, all three MOEs indicate high concern for acute toxicity. In case of changing to the approximately 20-times higher LD50 from the rat studies, only the lozenges model would be outside the high concern level. However, this would be in line with observations from the clinical study, where neither any clinically significant changes in cardiac or laboratory parameters were observed nor serious adverse effects were reported, but only one case of headache during 1 hour in the low and middle dose group and six cases of reported transient stomach heaviness lasting for one hour in the high exposure group.

5 Conclusion

Under Regulation (EC) No. 1272/2008 on the classification, labelling and packaging of substances and mixtures nicotine is currently classified as "acute toxic 3" for oral exposure, "acute toxic 1" for dermal exposures and "aquatic chronic 2". A proposal has been submitted by the Rijksinstituut voor Volksgezondheid en Milieu (RIVM) to re-classify nicotine as "acute toxic 1" for oral exposure and to add "acute toxic 2" for inhalation exposure. Whereas in the past the classification for acute oral toxicity was based on LD50 values for rats, RIVM considers the much lower LD50 values for mice and dogs as more relevant. Based on the lowest available LD50 value of 3.34 mg/kg b.w. (for mice) in their document, RIVM considers warranted a harmonized classification of "acute toxic 1", instead of "acute toxic 3" (RIVM, 2015). PMI disagrees with RIVM's proposal and believes that the current classification of nicotine as "acute toxic 3" for oral exposure is correct and should be maintained.

In PMI's view there is no clear scientific justification that for the acute toxicity the mouse data is more relevant than the rat data, indeed there is sound justification, based on metabolic rate, that the opposite is more likely to be the case. Therefore, LD50 data from rat are relevant for the acute oral toxicity and the current classification "acute toxic 3" for (oral) exposure is correct and should be maintained.

6 References

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