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Letter to the editor

Cyclohexane-1,2-dicarboxylic acid diisononyl ester and metabolite effects on rat epididymal stromal vascular fraction differentiation of adipose tissue (2015) Environmental Research 140: 145–156 Reply to the letter by Otter R.

Dear Dr. Domingo,

Thank you for the opportunity to respond to the comments made by Dr. Otter. First, we would like to note that our manuscript presented data obtained *in vitro*, showing effects of MINCH and not DINCH, on preadipocyte differentiation. In the discussion section, the potential environmental risk associated with DINCH exposure was presented as speculation. Moreover, no publicly available peer-reviewed information was missed since the regulatory information listed in Dr. Otter' letter was cited either directly (European Food Safety Authority, 2006) or indirectly through referencing a critical review of the DINCH literature (Bhat et al., 2014). A point-per-point reply follows:

1. MINCH

MINCH is not a commercially available compound and thus we had to synthesize it. Based on a careful bibliographical search, we established that DINCH is a mixture of different isomers that could be metabolized to various products (Wadey, 2003; Koch et al., 2013). The *cis* form was chosen to reproduce the ratio of 90% *cis* and 10% *trans* isomers reported by BASF (Koch et al., 2013; NICNAS, 2012). Regarding the C9-alcohol "specific branching" mentioned by Dr. Otter, the NICNAS (2012) report he cited does not provide the description of a specific branching but instead refers to the identity of the chemical as "1,2-cyclohexanedicarboxylic acid, diisononyl ester, branched and linear", indicating that it is a mixture relative to the branching of the C9 chains. In the absence of more precision, we synthesized the form with a terminal branching of C8 and C9 (as shown in Figure 1 of our article), corresponding to the structure presented in the toxicology report of Hexamoll® DINCH® (CAS #166412-78-8, 474919-59-0) by ToxServices LLC (http://www.greenchemistryandcommerce.org/documents/Hexamoll-DINCHGS5.28.13.pdf). The synthesized MINCH was analyzed by HPLC-UV, MS (direct infusion to LC-MS) and ^{1}H NMR to determine its purity and chemical structure, and the data obtained have been provided to the journal. Thus, the MINCH used in our study likely represents one of the MINCH species obtained from DINCH, and the statement that we "may have tested the wrong substance" is unfounded because the real number and percentage of DINCH metabolites is still unclear.

It is also unknown whether what is excreted in the urine reflects the metabolism occurring within the various tissues. Bhat et al. (2014) refer in detail to various BASF reports describing lab animal data following single exposure of radiolabeled DINCH. These studies demonstrated that adipose tissue had the longest initial half-life of radioactivity compared to initial half lives in plasma, kidney and liver. These observations would suggest the presence of an adipose-tissue specific metabolism. Unfortunately, in all published toxicological studies performed to assess DINCH toxicity, the adipose tissue evaluation is missing. Moreover, a recent paper by Schütze et al. (2015) reported that a multi-compartment pharmacokinetic model developed to characterize the exposure to DINCH was unable to duplicate the ratio of metabolites seen in 24 h urine sample. The authors concluded that the exposure pattern in the general population did not match the oral exposure in the dosing experiments, or that the toxicokinetics of DINCH is not captured in the controlled dosing experiments. Perhaps a refined physiologically-based pharmacokinetic model that includes an adipose compartment warrants consideration.

It should also be noted that MINCH accounts for 0.72% (0.31–1.26%) of DINCH metabolites. However, as stated in the Koch study, other MINCH metabolites were identified, including OH-MINCH 10.7% (7.7–12.9%), oxo-MINCH 2.0% (1.5–2.6%) and carboxy-MINCH 2.0% (1.8–2.3%), adding up to a total of 15.7% of MINCH species; for this reason, MINCH is indeed a major metabolite of DINCH.

Dr. Otter acknowledges that MINCH is the major metabolite in blood, and states that in contrast to rats it is predominantly glucuronidated in human blood, as suggested by Koch et al. (2013). Glucuronidation is a phase II detoxification pathway in which glucuronic acid is conjugated with xenobiotics/toxicants by hepatic enzymes, creating a more water-soluble compound that can be excreted in the urine (Hayes, 2007). Moreover, a number of glucuronidated chemicals are deglucuronidated by the intestinal flora, leading to the re-absorption of unconjugated chemicals in blood and sometimes a reactivation of the toxic effect (Hayes, 2007). At present no information is available on how much MINCH is going into preadipose cells, prior to its hepatic conjugation and after a possible intestinal deconjugation and portal re-absorption. Furthermore, it is worth noting that our study deals with rat preadipocytes, so the substance used was appropriate to the model studied, which might not express conjugating enzymes.

2. PPARα-agonist

We based our study on the basic pharmacological principle of receptor-ligand (in this case antagonist) interaction. GW6471, a selective PPAR-α antagonist, was capable of blocking both MINCH- and MEHP-induced adipogenesis *in vitro*. The data presented is clear. Whether this is a direct or indirect effect and whether this effect occurs *in vivo* remains to be determined. In his critique, Dr. Otter refers to *in vivo* studies performed with DINCH and assessing PPAR activation in hepatocytes. Indeed, the only available information is in a BASF report, where the levels of cyanide-insensitive palmitoyl-coenzyme A oxidation were found to be

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comparable between control and DINCH-treated Wistar rats up to the dose of 1.6 g/kg-d (Bhat et al., 2014). However, no PPAR ligand binding, induction of PPAR-driven gene expression, or tissue-specific evaluation for PPAR activity for DINCH and any of its metabolites, were reported. In our paper we do not claim that DINCH has a PPAR activity. However, before our study, to our knowledge there was no study available evaluating the PPARα–agonist activity of the metabolite MINCH *in vitro*, and there are no studies evaluating *in vitro* or *in vivo* the effects of MINCH and DINCH on adipocytes. Whether other DINCH metabolites may counteract this effect of MINCH, thereby blocking the MINCH-induced PPARα activation, remains to be examined.

3. Risk to specific populations

Dr. Otter claims that we failed to identify publicly available information (ECHA; NICNAS; SCENIHR; EFSA) regarding bioavailability of the plasticizer. This statement is not accurate since this information was provided in the article of Bhat et al. (2014), included as a reference in our article. Indeed, this critical review reports in detail bioavailability and toxicology studies on DINCH, including those cited by Dr. Otter. Therefore, by citing this review, in essence we provided the readers with the most complete and up-to date description of the regulatory toxicology studies that have been performed on DINCH. Interestingly, the review of Bhat et al. emphasized important aspects of the biological responses to DINCH, such as the "unexplained nonmonotonic thyroid response (TSH levels) in males" as well as "the nonlinear bioavailability in the mid-to high DINCH doses examined for both male and female", properties that "make dose correlations challenging". These types of nonlinear responses are not exclusive to the thyroid, and apply to other endocrines tissues. Moreover, recent studies on other types of environmental chemicals such as BPA have unveiled effects at low doses (relevant to human exposures) that were not observed with the high (exceeding human exposure) doses used until recently in most toxicology (and regulatory) studies. These studies call for caution about extrapolating health risks from very high doses likely to induce different biological pathways than low doses.

The review also pointed at potential gaps in existing studies, such as the lack of thyroid histopathology in adult male F1 rats, and the fact that "studies assessing reversibility were limited to a 4 week study in which thyroid parameters were not assessed". Finally, to the best of our knowledge, none of these studies assessed adipose function. As mentioned earlier, there is no available literature on the effects of DINCH and its metabolites on adipose differentiation.

Compared to the enormous quantity of literature on phthalates, at present there are no scientific reports on potential metabolic and endocrine-disrupting properties of DINCH and its metabolites other than the review of Bhat and colleagues and regulatory agency reports. For this reason, no biological/biochemical data are available on PubMed. Therefore, it is clear that there is a need for more studies, especially to assess lower doses (0.1 and 1 mg/kg/day) than those used in existing studies (20–1000 mg/kg/day) and to perform mechanistic studies in relation to different tissues.

Concerning the calculations used to extrapolate from an active metabolite concentration in cell culture to a corresponding exposure dose level of the plasticizer, we clearly specified in the manuscript that this was only speculation. In response to reviewers request to provide an extrapolation to human exposure levels, we estimated a possible exposure of 20 mg DINCH/kg/day. This value was obtained as follows:

$$AD=\frac{(IVC \times M)\times V}{UEF}$$

$$HED = AD\times\frac{\text{Rat Km}}{\text{Human Km}}$$

M=molecular mass of MINCH (297); IVC: *in vitro* concentration (50 μM); V: rat blood volume/kg (64 ml/kg); UEF: urinary excretion factor (% MINCH found in urine of rats dosed with DINCH; proposed to be present in proportional levels in blood); AD: animal dose; HED: human equivalent dose; Km factor is body weight (kg) divided by BSA (body surface area; m^2) (Reagan-Shaw et al., 2007); rat Km is 6 and human Km is 37.

These calculations suggest that human exposure to 15.4 mg DINCH/kg could result in a 50 μM MINCH blood concentration. If one takes into account a bioavailability of 49% of DINCH (Bhat et al., 2014), then one can extrapolate the DINCH dose to 31 mg/kg. In the article, we averaged the lower and higher extrapolations to 20 mg/kg/day. Evidently this is an extrapolation made based on various assumptions, as indicated in the paper. Moreover, this estimate did not take into account the possibility that a fraction of DINCH or its metabolites might be stored in the body. Indeed, such storage in discreet compartments could explain why DINCH bioavailability was not linear in some of the studies (Bhat et al., 2014) and clearly deserves further investigation. Considering the increasing and broader use of DINCH in recent years, human exposure is likely to increase. In addition, as shown in the phthalate literature, new sources of exposures are often uncovered as more studies are performed in different environments and contexts.

Industry is following strict regulatory rules imposed by governments, and no one questions that the products put on the market comply with these rules. However, science evolves as new technologies become available. This leads to changes in regulatory requirements and regulations. This science-informed and population safety-driven evolutionary process has led compounds once deemed safe, *e.g.* phthalates, BPA, and many drugs, to be revaluated and their use either banned or limited. Ours is an *in vitro* study which may or may not translate *in vivo*. However, *in vitro* studies are the new standard supported by most regulatory agencies, and although imperfect, they represent an economically and ethically sound first step in performing toxicology and risk assessment studies.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.envres.2015.11.002.

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