In vitro cytotoxic effects of secondary metabolites of DEHP and its alternative plasticizers DINCH and DINP on a L929 cell line

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ABSTRACT

Background: Phthalic acid esters are widely used to improve the plasticity of PVC in medical devices (MD). The most famous plasticizer is **DEHP,** whose use in medical devices has been contested by the European authorities since 2008. Several alternative plasticizers are being considered to replace **DEHP,** such as **DEHT, TOTM, DINP** or **DINCH**, but they are also released from the PVC throughout their life cycle and are metabolized in the same way as **DEHP**.

Objectives: Our study focuses on the in vitro cytotoxicity of two alternative plasticizers (**DINCH** and **DINP**) contained in certain medical devices. They are likely to migrate and be transformed in vivo into the primary and secondary metabolites by a metabolism similar to that of **DEHP**. This preliminary study is the first to assess the in vitro cytotoxicity of oxidized metabolites of DINCH and DINP based on the EN ISO 10-993-5 standards documents.

Methods: We have studied the complete multi-step organic synthesis of secondary metabolites of **DINP** and **DINCH** and have performed cytotoxicity tests on L929 murine cells according to the EN ISO 10993-5 standard design for the biocompatibility of a MD. The tested concentrations of obtained metabolites (0.01, 0.05 and 0.1 mg/mL) covered the range likely to be found for DEHP (total metabolism) in biological fluids coming into direct contact with the MD. The concentrations tested in our study were chosen based on a complete transformation of the plasticizers released after direct contact between a MD and the patient's blood.

Results: Only **7-oxo-MMeOCH** is cytotoxic at the highest concentration (0.1 mg/mL) after 7 days of exposure, just like **5-oxo-MEHP** for the same concentration. By contrast, **7-OH-MMeOP**, **7-cx-MMeOP**, **7-oxo-MMeOP**, **7-OH-MMeOCH** and **7-cx-MMeOCH** were not found to be cytotoxic.

Conclusion: The known concentrations of these secondary metabolites in urinary samples are in the µg/L range, i.e. about 100–1000 times lower than the concentrations tested in this study. Cytotoxicity is known to be dose-dependent but it is not always the case for endocrine perturbations and the secondary metabolites could induce endocrine perturbations at very low doses.

1. Introduction

Some phthalate esters present a general concern for public health due to their wide use in the manufacturing of polymeric materials and various consumer products. Some phthalate esters can be classified into two categories according to the number of carbons in the ester chain. On the one hand, there are those with a low molecular weight (number of carbon inferior to 6) which are used as industrial solvents, lubricants, and as components in personal-care products. On the other hand, those with a high molecular weight are commonly used as plasticizers, conferring better flexibility and durability to everyday polyvinyl chloride (PVC) consumer products, such as flooring, food packaging, clothing, children's toys and medical devices (Hauser and Calafat, 2005). These PVC plasticizers can be released from the polymers by volatilization into the air, abrasion of the polymer, leaching into liquids and direct diffusion from the polymer into dust on the polymer surface, resulting in subsequent human exposure which might cause adverse health effects (Giovanoulis G et al., 2018; Zhang, S. et al., 2016; Engel, A. et al., 2017, 2018). With regard to their presence in PVC medical devices, the most famous plasticizers is **DEHP,** whose use has been contested by the European authorities (SCENHIR, 2008, 2016). The toxic potential of **DEHP** is largely due to the metabolic transformation into more toxic metabolites by hydrolysis (**MEHP**) and subsequent oxidation reactions (**5-OH-MEHP**, **5-oxo-MEHP**, **5-cx-MEHP**) (Fig. 1A). Numerous studies have been carried out on **DEHP**, leading to its classification as carcinogenic, mutagenic or toxic for reproduction (CMR1B) because of its toxic effects on reproduction and fertility in rodents. Its use is now highly controlled in medical devices. The European regulation 2017/745 recommends not to exceed a rate of 0.1% in mass fraction in each medical device (Regulation (EU) 2017/745). Two alternative plasticizers (diisononyl phthalate (**DINP**) and 1,2-cyclohexane dicarboxylic acid (**DINCH**)) have been considered by manufacturers as a replacement for **DEHP** in many PVC products, such as medical devices and food packaging, but are limited in toys with concentrations inferior to 0.1% by weight of the plasticized material (European Chemical Agency, 2013; SCENHIR, 2016), due to their physicochemical properties suggesting a lower migration and more favorable toxicological profile

(Fromme et al., 2016; Bhat et al., 2014) than **DEHP**. **DINP** and **DINCH** respectively constitute a complex commercial mixture composed mainly of isomers of phthalate or dialkyl cyclohexanoate with 9 carbon chains. **DINCH** (Hexamoll®) is manufactured by catalytic hydrogenation of the aromatic ring of diisononyl phthalate (Palatinol® N) into the cyclohexane ring existing predominantly in the most stable chair configuration, allowing cis- and trans-configuration. The typical commercial products consist of 90% cis- and 10% trans-isomers. In both molecules, the 4-methyl isomer forms a fraction that represents less than 50% of the total C9 alcohols (Schütze et al., 2017). Like **DEHP**, they are not covalently bonded to the PVC to which they are mixed. As a result, they are released from the products throughout their life cycle (Bernard et al., 2015; Jeon et al., 2016). Because they are a mixture of isomers, their metabolites are also isomeric mixtures (Silva et al., 2013). Both are initially hydrolyzed in rodents and humans into their monoester (**MINP** or **MINCH**), followed by oxidative processes into several metabolites including mono-hydroxy, mono-oxo and mono-carboxymetabolites (Silva et al., 2007a, 2007b; Koch et al., 2007, Koch and Angerer, 2007, 2013) (Fig. 1B).

Some biomonitoring studies carried out in humans have made it possible to demonstrate the presence of **DINCH** and **DINP** metabolites in biological media such as urine, blood and nails (Giovanoulis et al., 2016, 2018, Correia-Sa et al., 2017, Schütze et al., 2012, 2017, Zeman et al., 2013; Koch et al., 2017, Johns et al., 2015). However, despite the animal toxicology data and the in vitro toxicity of the plasticizers, there is almost no information on the *in-vitro* toxicity of the oxidized metabolites of **DINP** and **DINCH** from medical devices except the urinary metabolites of **DINCH,** which seem to have an impact on the activities of the human nuclear receptors ERα, ERβ, AR, PPARα and PPARγ. However, the in vitro data does not support the notion that DINCH or any of the investigated metabolites may exert considerable endocrine effects in vivo at relevant human exposure levels. (Engel et al., 2017, 2018). In a previous study we investigated the cytotoxicity of **DEHP**, **DINCH**, **DINP** and their corresponding primary unconjugated metabolites (**MEHP**, **MINCH**, **MMeOCH**, **MINP**, respectively), and showed that the primary metabolites are more cytotoxic than their corresponding plasticizer at concentrations between 0.01 and 0.1 mg/mL. Only
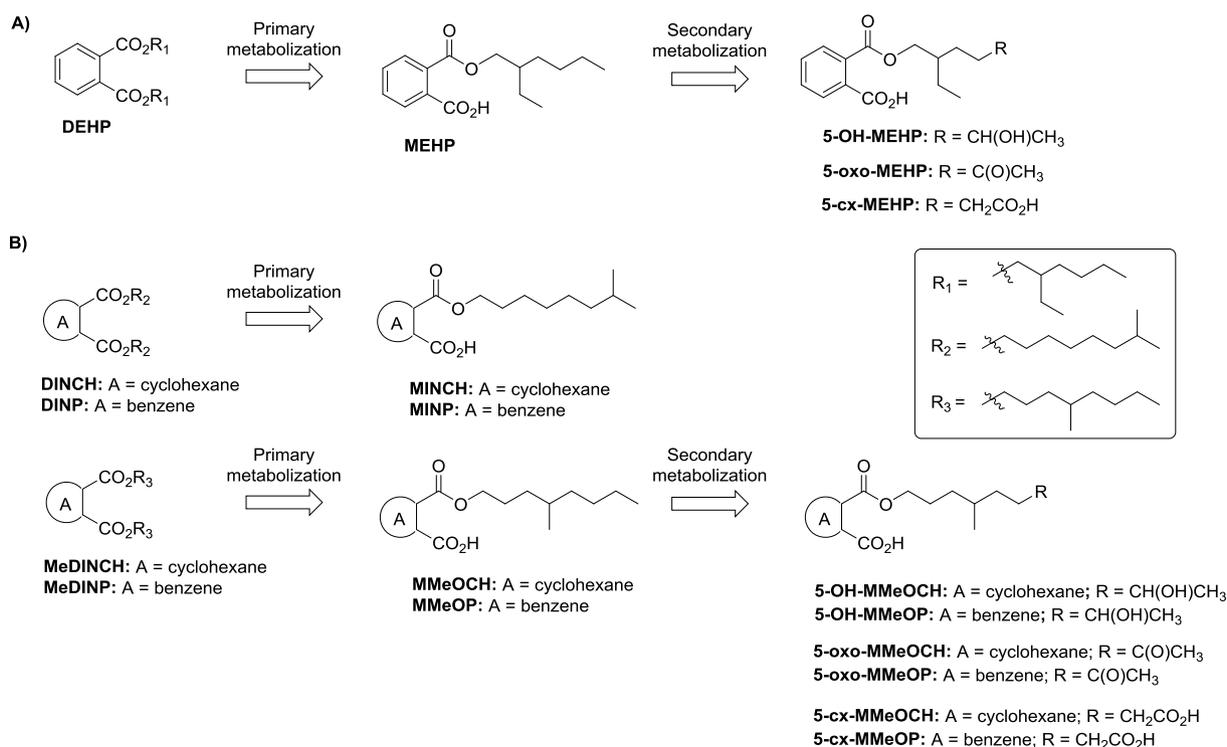

Fig. 1. Primary and secondary metabolites of **DEHP**, **DINCH**, **DINP**, **MeDINCH** and **MeDINP**.

MMeOP, the primary metabolite of **DINP**, is not cytotoxic at these concentrations (Eljezi et al., 2017). Taking into account that DINP and DINCH are metabolized in the same way as DEHP, secondary metabolites are also found in the urine (7-oxo-MMeOCH, 7-OH-MMeOCH, 7-cx-MMeOCH, 7-oxo-MMeOP, 7-OH-MMeOP and 7-cx-MmeOP) in a glucuroconjugated form or not. According to the data in the literature, it appears that 26% of cx-MINCH are excreted in its unconjugated form in urine (Koch et al., 2013). These results are similar to the previous work concerning DEHP and DINP (Silva et al., 2013). In continuation of our work on alternative plasticizers to DEHP, we therefore undertook the total synthesis of the secondary metabolites of DINP and DINCH in order to be able to carry out a first cytotoxicity study on the NCTC clone 929 in accordance with the EN ISO 10-993-5 standards documents.

2. Materials and methods

2.1. Chemicals, biochemicals and reagents

Unless otherwise mentioned, all manipulations were performed under an argon atmosphere. All reagents were purchased from the following commercial suppliers: Sigma-Aldrich, Acros Organics, Carlo Erba, TCI Europa, and Alpha Aesar. Anhydrous DMF and anhydrous triethylamine were purchased from Acros Organics. THF was distilled over benzophenone and sodium. Dichloromethane was distilled over calcium hydride. Nuclear magnetic resonance (NMR) spectra were acquired on a Bruker AC-200 or AC-400 instrument operating at 200 MHz and 50 MHz or 400 MHz and 100 MHz for 1H NMR and 13C NMR, respectively. All 1H NMR and 13C NMR spectral peaks are reported in δ units, parts per million (ppm), and the coupling constants are indicated in hertz (Hz). The following abbreviations are used for spin multiplicity: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet, and br = broad. The chromatographic system consisted of an HTC PAL autosampler, and a Transcend TLX-1 HTLC System (ThermoFisher Scientific, San Jose, United States). The HRMS analysis was performed using a Thermo Exactive benchtop Orbitrap® instrument. TLC was performed on pre-coated silica gel sheets (POLYGRAM® 60F254 plates) and visualized under UV light (254 nm). Revelators used were KMnO4 (1.5 g KMnO$_4$, 10 g K$_2$CO$_3$, and 1.25 mL 10% NaOH in 200 mL water) and ninhydrin (1.5 g ninhydrin in 100 mL of n-butanol with 3 mL AcOH). Column chromatography was performed using a silica gel normal phase (35–70 μm). A HPLC Prominence-i LC-2030 CSCHIMA-DZU with Kinetex 5 μm EVO C18 (Phenomenex) column with pore diameter (A) 82–102, particle size (μm) 4.5–4.8, and column dimensions of 50 × 4.6 mm was used to purify secondary metabolites. Uncorrected melting points (Mp) were recorded on an Electrothermal IA9300 apparatus. Infrared spectra (IR) were recorded on a Bruker FT Vector 22. The 5-oxo-MEHP, 5-OH-MEHP and 5-cx-MEHP compounds were synthesized using the procedure described by Nüti et al. (Nuti et al., 2005).

Dimethylsulfoxide DMSO (ref: D8418 CAS: 67-68-5), Mouse fibroblasts L929 (ref: 85011425 lot: 10L019), MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, ref: M2128) were also purchased from Sigma Aldrich. Dulbecco's modified Eagle Medium (DMEM) (fetal calf serum (SVF), phosphate saline buffer (PBS), penicillin (P), streptomycin (S), and actinomycin D) were purchased from Life technologies SAS, France. For more details, see supporting information.

2.2. Methods for the synthesis of the secondary metabolites 7-oxo-MMeOCH, 7-OH-MMeOCH, 7-cx-MMeOCH, 7-oxo-MMeOP, 7-OH-MMeOP and 7-cx-MMeOP

Metabolites **7-oxo-MMeOCH**, **7-OH-MMeOCH**, **7-cx-MMeOCH**, **7-oxo-MMeOP**, **7-OH-MMeOP** and **7-cx-MMeOP** were synthesized from the appropriate anhydrides (hexahydroisobenzofuran-1,3-dione or phtalic anhydride) and 7-(benzyloxy)-4-methylheptan-1-ol previously

synthesized and characterized (^{1}H, ^{13}C NMR and HRMS) by our lab (Eljezi et al., 2017). The benzyl group at ω-position was then removed under hydrogen atmosphere and the resulting alcohol was oxidized into an aldehyde compound which was converted into vinyl compounds **6** or **18**. From the appropriate vinyl compound (**6** or **18**), **7-oxo-MMeOCH** or **7-oxo-MMeOP** could be obtained by oxidation in a mixture of PdCl$_2$ and parabenzoquinone. These were directly converted into **7-OH-MMeOCH** or **7-OH-MMeOP** by a regioselective reduction in the presence of NaBH$_4$. Concerning **7-cx-MMeOCH** or **7-cx-MMeOP**, they were obtained from vinyl derivatives **6** or **18** by hydroboration followed by an oxidation in the presence of Jones reagent. The purity of all synthesized metabolites was over 95% (HPLC/MS). All experimental details are described in the supporting material.

2.3. Biocompatibility assays

For the solubilization of the plasticizers, cell cultures, viability assays for the synthesized metabolites and statistical analysis, we used the same procedures previously described by us (Eljezi et al., 2017). For more details, see supporting information.

3. Results

3.1. Synthesis of MeDINCH and MeDINP secondary metabolites

The three metabolites of the **MeDINCH** plasticizer, i.e. **7-oxo-MMeOCH**, **7-OH-MMeOCH** and **7-cx-MMeOCH,** were synthesized from monoprotected alcohol **1** (Eljezi et al., 2017) and hexahydroisobenzofuran-1,3-dione (Scheme 1). Acid compound **2** was esterified, followed by a catalytic hydrogenation to produce compound **4**. The hydroxy group at ω-position of the alkyl chain was oxidized into an aldehyde. Compound **5** reacted with isopropyltriphenylphosphonium iodide (Wittig reaction) to obtain vinyl derivative **6**. According to the procedure previously described (Nuti et al., 2005), this vinyl function produced a ketone function at position C-7 of the alkyl chain (compound **7**) which was converted into **7-oxo-MMeOCH** after a soft saponification. Compound **7** was also reduced in the presence of NaBH$_4$ followed by a treatment with lithium hydroxide monohydrate to give **7-OH-MMeOCH**. Finally, the third secondary metabolite, **7-cx-MMeOCH**, was obtained from compound **6** after a hydroboration of the vinyl function, followed by an oxidation of the primary alcohol function with Jones reagent and a soft saponification. All of these syntheses are depicted in Scheme 1.

The secondary metabolites **7-oxo-MMeOP**, **7-OH-MMeOP** and **7-cx-MMeOP** were synthesized using a similar strategy developed for secondary metabolites of **MeDINCH** (Scheme 2). Briefly, vinylic precursor **18** was obtained by condensation of the monoprotected alcohol **1**, and anhydride phthalic, followed by an esterification, deprotection of the ω-hydroxy group at C-7 position, then its oxidation and a Wittig reaction. Finally, this vinyl function enabled **7-oxo-MMeOP** to be obtained in two steps (Wacker oxidation and a soft saponification), then **7-OH-MMeOP** by a reduction in presence of NaBH$_4$. Compound **7-cx-MMeOP** was obtained after a hydroboration, followed by an oxidation with Jones reagent.

3.2. Viability of cells when exposed to the secondary metabolites of plasticizers

Fig. 2 shows the results of viability tests, at day 7 (D7), performed on L929 cells cultured in the presence of the three concentrations of plasticizers after 7 days of contact. Compounds **5-cx-MEHP**, **5-OH-MEHP**, **7-cx-MMeOCH**, **7-OH- MMeOCH**, **7-cx-MMeOP**, **7-OH-MMeOP**, **7-oxo-MMeOP** were not toxic to the cells at concentrations of 0.01, 0.05, and 0.1 mg/mL compared with the control containing DMSO at the same concentrations. A decrease in cell viability was observed for **5-oxo-MEHP** and **7-oxo-MMeOCH** from the concentration of
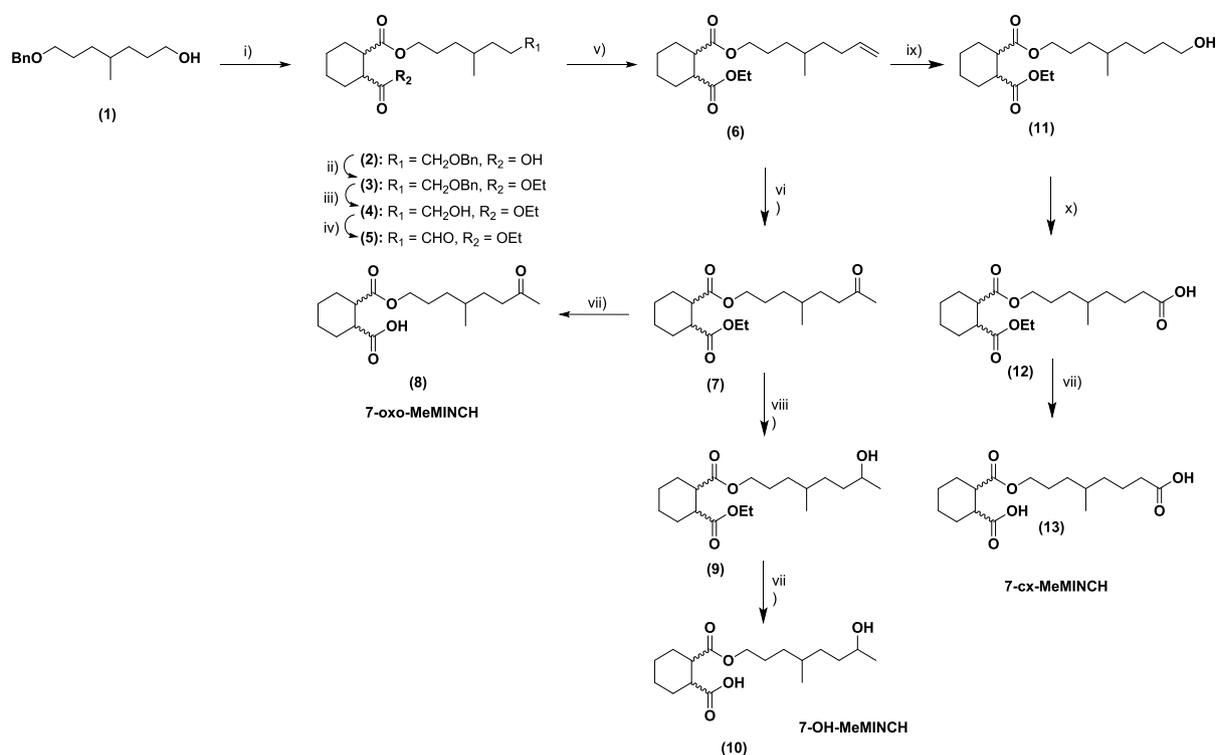
aConditions and reagents: i) 1,2-cyclohexane dicarboxylic anhydride, pyridine, 125°C; ii) EtOH, H$_2$SO$_4$, Δ; iii) H$_2$, Pd/C (1 bar); iv) PCC, dichloromethane, rt; v) isopropyltriphenylphosphonium iodide, n-BuLi, THF, -78°C to rt; vi) PdCl2, p-benzoquinone, DMF/H$_2$0 (7/1; v/v), rt; vii) LiOH (1M), THF/methanol (3/1; v/v), rt; viii) NaBH$_4$, ethanol, rt; ix) B$_2$H$_6$ (1M), THF, rt; x) Jones reagent, rt;

Scheme 1. Synthesis of secondary metabolites **7-oxo-MMeOCH**, **7-OH-MMeOCH** and **7-cx-MMeOCH**.
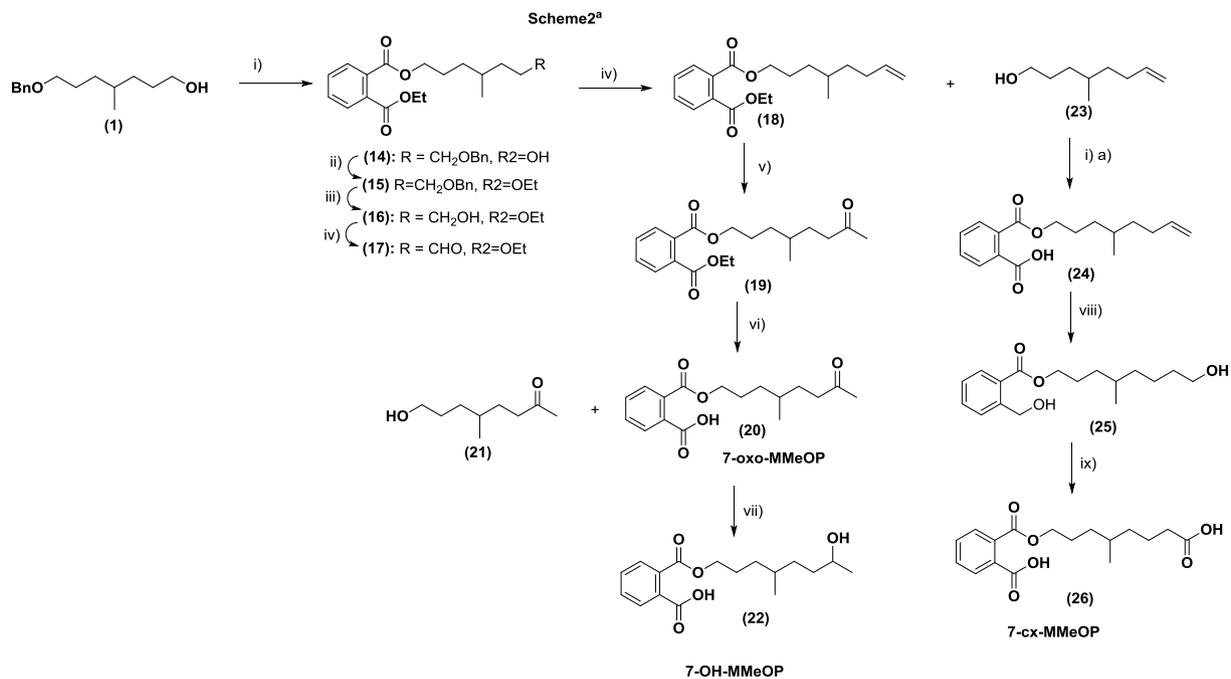
aConditions and reagents: i) a) phthalic anhydride, pyridine, 125°C; b) EtOH, H$_2$SO$_4$, Δ; ii) H$_2$, Pd/C (1 bar); iii) PCC, dichloromethane, rt; iv) isopropyltriphenylphosphonium iodide, n-BuLi, THF, -78°C to rt; v) PdCl2, p-benzoquinone, DMF/H$_2$0 (7/1; v/v), rt; vi) LiOH (1M), THF/methanol (3/1; v/v), rt; vii) NaBH$_4$, ethanol, rt; viii) B$_2$H$_6$ (1M), THF, rt; ix) Jones reagent, rt;

Scheme 2. Synthesis of secondary metabolites **7-oxo-MMeOP**, **7-OH-MMeOP** and **7-cx-MMeOP**.
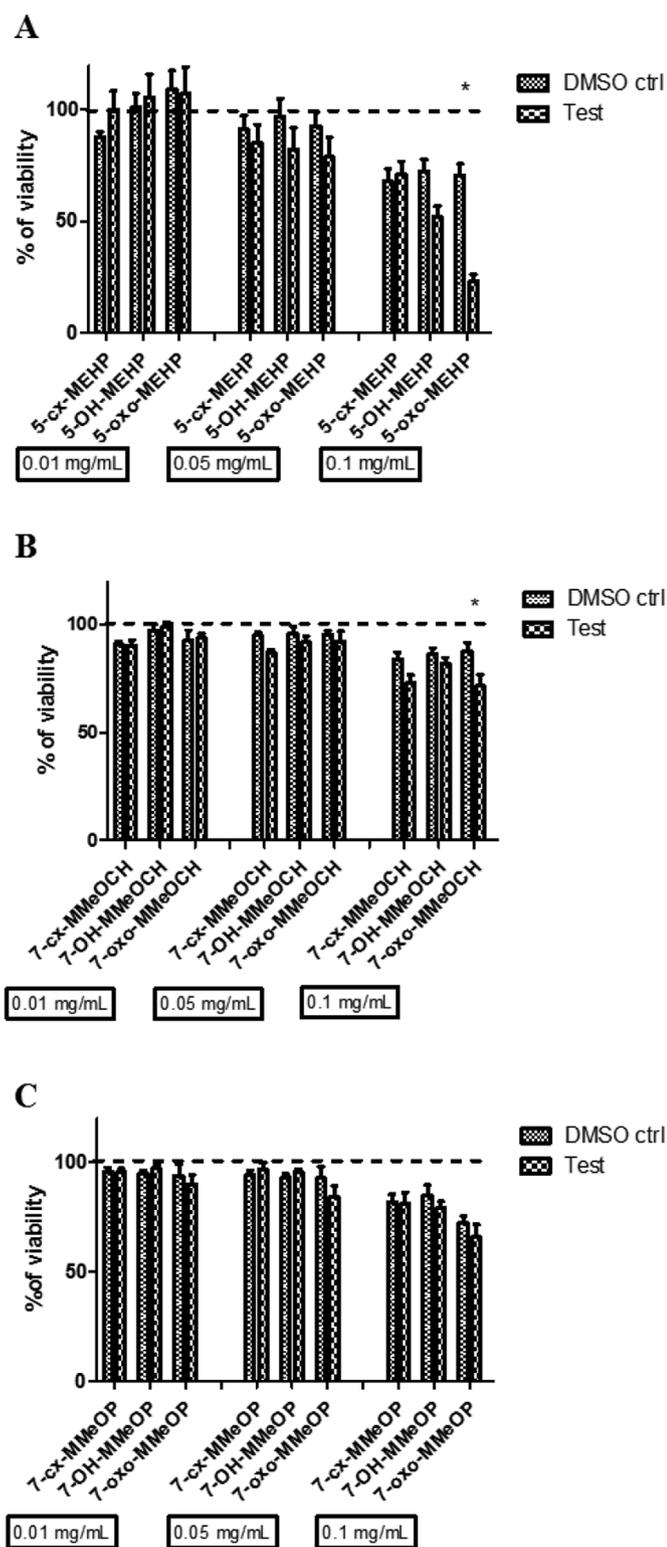
A

B

C

Fig. 2. Cytotoxicity tests of secondary metabolites at 0.01 mg/mL, 0.05 mg/mL and 0.1 mg/mL, on day 7. Outcomes of the cell viability analysis using a MTT assay. L929 cells were seeded at 10^4 cells/well and cultured for 7 days in three conditions: control (complete medium), control DMSO (complete medium supplemented with 0.1%, 0.5% and 1% DMSO) and test (working solutions of the plasticizer in complete medium at 0.01 mg/mL, 0.05 mg/mL and 0.1 mg/mL). MTT assays and quantification of the viability percentage were carried out every day. *p < 0.05 DMSO control vs Test.

0.1 mg/mL. A significant decrease in viability of over 50% was observed for **5-oxo-MEHP**.

4. Discussion

For several years, our research program has focused on **DEHP** and some of the corresponding alternatives in medical devices. **DINCH** and **DINP** are two plasticizers commonly used as alternatives to **DEHP** in PVC medical devices. DINCH is used in parenteral and enteral nutrition tubings as well as in hemodialysis lines (Strømmen et al., 2016). DINP is added to some PVC infusions tubings or blood transfusion sets (Frederiksen et al., 2014; Bernard et al., 2015; Bourdeaux et al., 2016). Like DEHP, these plasticizers are also known to migrate from medical devices and to enter into contact with the patient's body where they are metabolized into primary and secondary forms (Bernard et al., 2015). Based on data obtained from ICU studies carried out in recent years (Frederiksen et al., 2014; Huygh et al., 2015; Strømmen et al., 2016), it seems that these metabolites are less present in the urine than those of DEHP. Although these metabolites are mainly eliminated in their conjugated form in the urine, about 26% of the acid form is in its free form (5cx-MINCH) (Koch et al., 2013). Similar data have been published for DEHP and DINP (Silva et al., 2013). The cytotoxicity of their respective primary metabolites (**MINCH**, **MeMINCH**, **MINP** and **MeMINP**) has also been evaluated (Eljezi et al., 2017) but the cytotoxicity of the secondary metabolites of **DINCH** and **DINP** have not currently been studied. We thus synthesized these secondary metabolites (**7-oxo-MMeOCH, 7-OH-MMeOCH, 7-cx-MMeOCH, 7-oxo-MMeOP, 7-OH-MMeOP** and **7-cx-MMeOP**) in sufficient quantities to complete preliminary cytotoxicity test. Based on the urinary concentrations found for these secondary metabolites (conjugates or not), the concentrations of the secondary metabolites of **DEHP** are much higher than those of **DINP** and **DINCH** (0.3–5 µg/mL for ΣDEHPm versus 0.008–0.1 µg/mL for ΣDINPm and ΣDINCHm) (Frederiksen et al., 2014; Huygh et al., 2015; Strømmen et al., 2016). We therefore evaluated the cytotoxicity of the secondary metabolites of **DINP** and **DINCH** over a larger concentration range (0.01–1 mg/mL), which is a very pessimistic scenario, and compared these results with those obtained for the secondary metabolites of **DEHP**. We chose this concentration range in order to take into account the fact that DINCH and DINP could be increasingly used in the composition of medical devices, thus exposure to them is expected to increase in the coming years. It must also be taken into consideration that patients in intensive care units are exposed to several medical devices that may contain these alternative plasticizers (Huygh et al., 2015; Strømmen et al., 2016). Metabolites **7-OH-MMeOP**, **7-OH-MMeOCH**, **7-cx-MMeOP**, **7-cx-MMeOCH** do not cause any cytotoxicity up to concentrations of 0.1 mg/mL, just like the corresponding secondary metabolites of **DEHP**, i.e. **7-OH-MEHP** and **5-cx-MEHP**. This is also true for **7-oxo-MMeOP**, the third secondary metabolite of **DINP**. However, **7-oxo-MMeOCH**, like **5-oxo-MEHP**, has been shown to be cytotoxic at 0.1 mg/mL. No cytototoxicity was observed at a concentration below 0.05 mg/mL. The concentrations tested in our study were chosen on the basis of a complete transformation of the plasticizers released after direct contact between a MD and the patient's blood (SCENHIR, 2016, Eckert et al., 2015, 2016). However, the secondary metabolites, derived from the hepatic metabolism of the primary metabolite of the plasticizer (itself derived from an enzymatic hydrolysis of the plasticizer) are present at much lower concentrations in body fluids. Human biomonitoring studies (Schütze et al., 2014; Koch et al., 2007a, 2007b) performed after the introduction of **DINCH** and **DINP** on to the market show that the concentrations of their secondary metabolites in urinary samples are in the µg/L range (example 0.71 µg/L for **7-OH-MMeOCH**, 0.33 µg/L for **7-oxo-MMeOCH**, 0.61 µg/L for **7-cx-MMeOCH**, 14.9 µg/L for **7-OH -MMeOP**, 8.9 µg/L for **7-oxo-MMeOP** and 16.4 µg/L for **7-cx-MMeOP**), which is about 100–1000 times lower than the concentrations tested. The **DEHP** secondary metabolites found in the urine of humans exposed for two decades are also in the order of

μg/L (21.0 μg/L for **5-OH-MEHP**, 16.7 μg/L for **5-oxo-MEHP**, 26,9 μg/L for **5-cx-MEHP**) (Wittassek et al., 2007). Therefore, we can consider that these secondary metabolites of **DINCH** and **DINP** do not present a risk of cytotoxicity at the doses found in the body. This information, combined with the toxicity data obtained in animals, is reassuring with regard to the use of MDs plasticized with **DINCH** or **DINP** for situations where there is no direct contact with the patient's biological fluids (infusers, extension lines, nutrition tubings). In the case of a direct contact between the plasticized PVC in medical device and the patient's blood (extracorporeal circulation in cardiac surgery, dialysis), the risk could be different. In our previous study, we showed the cytotoxicity of primary metabolites of **DINP** and **DINCH** at 0.05 mg/mL (Eljezi et al., 2017). During an extracorporeal circulation, the plasticizer released from the MD (extracorporeal circulation line) is hydrolyzed into its glucuroconjugated primary metabolite. However, studies on plasticized DINCH blood bags have shown that this does not affect plasma quality and the amount of DINCH released is less pronounced than that of DEHP (Lagerberg et al., 2015). These results seem to be in agreement with those obtained in rats after i.v. injection or infusion of DINCH for which no apparent systemic effect had been observed up to 300 mg/kg bw/day (David et al., 2015).

It should be noted that these preliminary results attempt to show that the secondary metabolites of **DINCH** and **DINP** do not exhibit cytotoxicity according to EN ISO 10-993-5 standards documents. We will consider these different aspects when our biomonitoring study on alternative plasticizers and their corresponding metabolites are complete within the framework of ARMED® and ARMED-NEO projects. We will then select the different compounds for complementary studies.

Declaration of interest

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2019.03.005.

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