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Methylimidazolium ionic liquids - A new class of forever chemicals with endocrine disrupting potential

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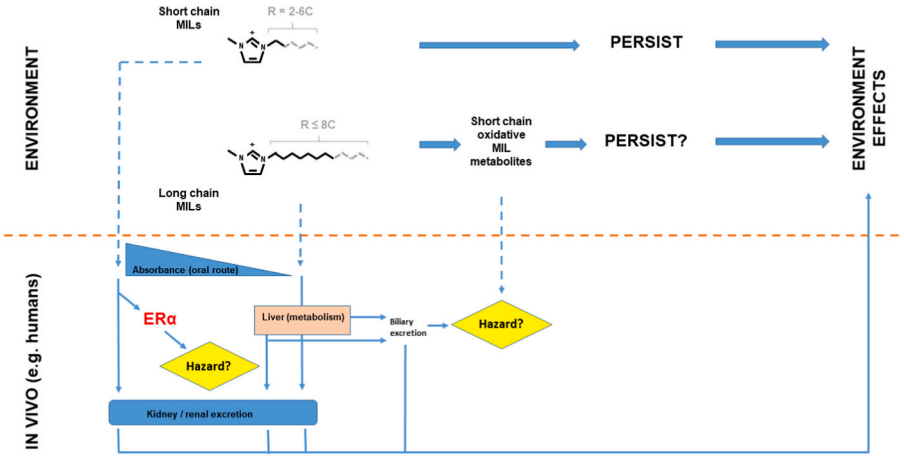
HIGHLIGHTS

- Short chain MILs underwent negligible metabolism and mineralisation in river water.
- Short chain MILs were not metabolised by human liver.
- Short chain MILs activated the human estrogen receptor alpha.
- Short chain MILs were estrogenic in vivo in rats.
- Longer chain MILs were metabolised and partially mineralised.

GRAPHICAL ABSTRACT



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ABSTRACT

A class of chemical with a potentially important perceived future contribution to the net zero carbon goal (as "green" solvents) is the methylimidazolium ionic liquids (MILs). These solvents are used in industrial processes such as biofuel production yet little is known about their environmental stability or toxicity in man although one MIL – 1-octyl-3-methylimidazolium (M8OI) – has been shown to activate the human estrogen receptor alpha

Abbreviations: AhR, aryl hydrocarbon receptor; BMI, 1-butyl-3-methylimidazolium (also referred to as C4mim); BPA, bisphenol A; BPC, bisphenol C; DBD, DNA binding domain; DMI, 1-decyl-3-methylimidazolium (also referred to as C10mim); E2, 17β-Estradiol; EMI, 1-ethyl-3-methylimidazolium (also refered to as C2mim); ERα, estrogen receptor alpha; HMI, 1-hexyl-3-methylimidazolium (also referred to as C6mim); LBD, ligand binding domain; M8OI, 1-octyl-3-methylimidazolium (also referred to as C8mim); MI, methyimidazolium; MIL, methylimidazolium ionic liquid; PFAS, perfluoroalkyl-and polyfluoroalkyl substances; TC, total carbon; TIC, total inorganic carbon; TOC, total organic carbon.

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Xenoestrogen
Oxidation
Mineralisation
Persistence

(ERα). The stabilities of the chloride unsubstituted methylimidazolium (MI) and MILs possessing increasing alkyl chain lengths (2C, 1-ethyl-3-methylimidazolium (EMI); 4C, 1-butyl-3-methylimidazolium (BMI); 6C; 1-hexyl-3-methylimidazolium (HMI), 8C, M8OI; 10C, 1-decyl-3-methylimidazolium (DMI)) were examined in river water and a human liver model system. The MILs were also screened for their abilities to activate the human ERα in vitro and induce uterine growth in pre-pubertal rats in vivo. Short chain MILs (EMI, BMI and HMI) underwent negligible metabolism and mineralisation in river water; were not metabolised in a model of human liver metabolism; activated the human ERα in vitro and were estrogenic in vivo in rats. A structure-based computational approach predicted short chain MIL binding to both the estrogen binding site and an additional site on the human estrogen receptor alpha. Longer chain MILs (M8OI and DMI) were metabolised in river water and partially mineralised. Based on structure-activity considerations, some of these environmentally-derived metabolites may however, remain a hazard to the population. MILs therefore have the potential to become forever chemicals with adverse effects to both man, other animals and the environment in general.

Funding

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1. Introduction

Ionic liquids define a structurally-diverse range of chemical salts that display an attractive range of physical properties. Prominent of these are their normally liquid natures and low volatilities at ambient temperatures (Welton, 2018). These specific aspects, combined with a limitless array of potential cation and anion moieties, provide invaluable solvent options for use in a variety of industrial processes. Their low volatility also means they are considered as *environmentally-friendly* or *green* solvents (Leitch et al., 2020). Methylimidazolium ionic liquids (MILs) are a class of ionic liquids characterised by a positively charged N-alkyl chain substituted methylimidazolium moiety (refer to Fig. 5a for structures). The methylimidazolium cations are combined with an anion such as chloride (but a variety of other anions are commonly used) to form a salt that is normally a liquid at ambient temperatures (Leitch et al., 2020). However, recent work detected the 8C variant cation, 1-octyl-3-methylimidazolium (M8OI), in soil around a landfill waste site (Probert et al., 2018; Leitch et al., 2020). A subsequent limited pilot screening study identified low levels of M8OI in 6 out of 30 human serum samples (Leitch et al., 2021). More recently, 300 human serum samples were screened for a variety of MILs, resulting in detection of 1-hexyl-3-methylimidazolium (HMI) in 19% of samples and M8OI in 8.3% of samples, with concentrations ranging from 0.02 to 111.70 μg/L and 0.09–16.99 μg/L, respectively, (Li et al., 2024).

One outcome from the examination of soils around the landfill contaminated with M8OI was that there was also high levels of unidentified activators of the human estrogen receptor alpha (hERα) and aryl hydrocarbon receptor (hAhR) at many of the sample sites (Meyer et al., 2017; Leitch et al., 2020; Probert et al., 2018). A subsequent study showed that M8OI is an activator of the hERα using a transactivation reporter gene assay developed in house (Leitch et al., 2018). However, there are no data on whether any of the related MILs are capable of activating the hERα and hAhR.

Given these observations, it was hypothesised that MILs are environmentally- and metabolically-persistent and capable of interacting with receptors frequently contacted by xenobiotics. To test this hypothesis, the chloride salts of the 2C (1-ethyl-3-methylimidazolium, EMI: CAS# 65039-09-0), 4C (1-butyl-3-methylimidazolium, BMI: CAS# 79917-90-1), 6C (1-hexyl-3-methylimidazolium, HMI: CAS# 64697-40-1), 8C (M8OI: CAS# 64697-40-1) and 10C (1-decyl-3-methylimidazolium, DMI: CAS# 171058-18-7) MILs were examined for their stability in a fresh river water model and a human liver metabolic model. Their ability to activate the hERα and hAhR in vitro and the ERα in vivo (in a rat model) was then examined.

2. Materials and methods

2.1. Materials

The 1-alkyl-3-methylimidazolium ionic liquids (as the Cl− salt, >96% purity) were purchased from Sigma (Poole, UK). The monoxygenated (HO8IM) and carboxylic acid (COOH7IM) metabolites of M8OI were previously custom synthesised as described (Probert et al., 2018; Leitch et al., 2021). The proposed carboxylic acid metabolite for BMI, 3-(3-carboxypropyl)-1-methyl-imidazolium chloride salt, (BMI--COOH) was custom synthesised by Fountainbridge (Edinburgh, UK). Analytical data and purity for BMI-COOH are provided in the Supplementary data section (Supp. Fig. 1). The ERα-HeLa-9903 cell line was obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank through Tebu-bio. The HepG2-Lucia cell line was purchased from InvivoGen (France). HepaRG cells were obtained from INSERM Transfert SA (Biopark Paris, France via Biopredic). Cell culture, cell viability determination and hERα and hAhR activation potentials were determined as outlined in Supplementary Data.

2.2. Biodegradability in river water

Where feasible, the method followed was aligned to the OECD TG 306 (biodegradability in seawater) methodology (OECD, 1992). Approximately 2.5 L of Tyne river water was collected from a fast moving point of the river at Wylam (Northumberland, UK) in a clean glass bottle (Optima LC/MS bottle previously containing UHPLC-UV grade water). The collected water was kept in a cool box and transported to the laboratory at Newcastle. The contents were allowed to settle over 2 days in a cold room (4–8 °C) with exposure to filtered air, before the supernatant was decanted into another bottle to exclude large debris. 50 mL aliquots were then filtered through 100 μm sterile nylon mesh cell strainers (Fisher Scientific) to remove fine sediment and collected in 200 mL glass flasks (washed overnight in ethanol followed by 2 washes in ultra-pure H_2O prior to autoclaving and 5 rinses in ultra-pure H_2O. Each flask was supplied with 0.05 mLs of the following sterile nutrients: Nutrient stock A (1L contained 8.50 g KH_2PO_4, 21.75 g K_2HPO_4, 33.30 g $Na_2HPO_4.2H_2O$ and 0.5 g NH_4Cl); Nutrient stock B (1 L contained 27.50 g $CaCl_2$); Nutrient stock C (1 L contained 22.50 g $MgSO_4.7H_2O$) and Nutrient stock D (1 L contained 0.25 g $FeCl_3.6H_2O$).

MILs (final concentrations MI, 100 μM; EMI, 100 μM; BMI, 100 μM; HMI, 50 μM; M8OI, 20 μM or DMI, 10 μM) were added from 1000-fold concentrated sterile water stocks to flasks of river water and incubated in triplicate in a shaking incubator (100 rpm, 16 °C). Flasks were covered with tin foil containing small holes to permit the passage of air. Incubations were therefore subjected to variable degrees of evaporation. Two sets of positive control compounds in river water – 100 μM aniline and 1 mM sodium acetate – were also included in triplicate, as recommended by the OECD TG 306. EMI, M8OI, aniline and sodium acetate were also incubated in triplicate in sterile water (sterile water for

irrigation, [#UKF7114] Baxter, UK) at the same concentrations as those used in river water. River water and sterile water without any additions were included in triplicate as further controls.

1 mL aliquots were removed from flasks at various time points, centrifuged at 13,000 rpm (15 min at 4 °C) and the supernatant retained and stored at −20 °C prior to analysis. The final remaining volumes of all flasks were recorded at the end of the incubation period and submitted for carbon analysis. Sampling and evaporation was taken into account when calculating the extent of carbon oxidation. Occasionally, additional sterile water was needed to provide sufficient sample volume for carbon analysis (10 mL) and this was also taken into account.

2.3. Carbon measurements

Total river water sample carbon (TC) and total inorganic carbon (TIC) contents at 0 and 7 weeks were determined using a TOC Vario Select (Elementar) machine which incorporates magnesium perchlorate drying and chlorine removal prior to CO_2 determination using an infrared detector. Sodium carbonate was used as standard. For TIC measurements, samples were first acidified with phosphoric acid. Total organic carbon (TOC) was calculated by subtracting sample TIC levels from their corresponding TC levels.

2.4. hERα activation

Activation of the hERα was examined using the OECD test guideline (TG) 455-approved cell line, ERα-HeLa-9903 cells (OECD, 2021) and assays were performed essentially as recommended by the guideline. ERα-HeLa-9903 cells were routinely cultured as outlined in the Supplementary Data section. For screening assays, cells were sub-cultured into 24 well plates and expanded until approximately 40–60% confluent. The medium was renewed with the addition of chemicals added from a 1000-fold molar concentrated vehicle-solvated stock (in either DMSO or water). Separate vehicle controls were included in studies through addition of 0.1% (v/v) solvent. After 48 h, the medium was removed and luciferase activities were determined as previously outlined (Leitch et al., 2018).

2.5. Human AhR activation

Activation of the human AhR was examined in HepG2-Lucia cells as outlined in the Supplementary data section.

2.6. Molecular modelling

For molecular docking studies, high-resolution X-ray crystal structures of estrogen and androgen receptor ligand-binding domains were selected (PDB codes 2OCF and 2AM9, respectively). For GPER1, the AlphaFold model of full-length protein was used (Jumper et al., 2021). All receptors were considered in their monomeric states. For further details, see Supplementary Data section.

2.7. Uterine growth assay in vivo

The ability of MILs to affect the growth of the uterus was examined in vivo in rats essentially as previously conducted (Axon et al., 2012). The study was performed at the Faculty of Pharmacy, Cairo University, Egypt and was carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines. Ethical approval for the study was granted from both the Faculty of Pharmacy Ethics Committee (PT 3228) and the Animal Welfare and Ethical Review Body (#664) at the Faculty of Medical Sciences at Newcastle University. Reporting complies with the ARRIVE guidelines. Female Sprague Dawley rats (age 19–22 days) were weaned and randomly assigned to 6 separate treatment groups. Rats were weighed daily and administered 10 mL/kg bw MILs in 0.9% (w/v) saline vehicle (or 10 mL/kg bw saline vehicle as control) by intraperitoneal injection once per day for 6 days (for dose, see Table 1). 17β-Estradiol (E2) was administered daily at 10 ml/kg bw from a 50 μg E2/mL ethanol:olive oil (1:20, v/v) stock due to its solubility. To reduce animal use, a vehicle control was not included for this group as it is known to increase uterine wet weight in this assay (Axon et al., 2012). Twenty four hours after the final injection, rats were weighed, terminated by cervical dislocation and uteruses, livers and kidney (left) excised and weighed.

2.8. Statistics

Data are expressed as means ± standard deviation (SD). Comparisons between means were carried out using the Student's t-test for 2 group comparisons and one way analysis of variance (ANOVA) test for multiple group comparisons, followed by a Bonferroni-Holm comparison between groups test. For all statistical tests, the level of significance was fixed at $p < 0.05$ (two tailed).

2.9. Other methods

For additional methodological details, see Supplementary data section.

3. Results

3.1. An examination of the stability of MILs in river water

The MILs – EMI, BMI, HMI, M8OI and DMI – were separately added to river water as outlined in the methods section such that concentrations would be quantifiably detectable by carbon analysis (in addition to the relatively sensitive technique of LC-MS). Thus, the concentrations of MILs used decreased with increasing alkyl chain length, to limit toxicity to river water microbes. Incubation with methylimidazolium chloride (MI) was included since it is the chemical moiety present in all MILs that does not exist in nature. Positive control chemicals - sodium acetate and aniline – were also included in the study. Both positive controls are recommended for inclusion by the OECD TG 306 (OECD, 1992): acetate is a common metabolic metabolite; aniline is widely used industrial chemical. Both are expected to be readily oxidised.

Fig. 1a (upper panel) demonstrates that parent alkylated MILs were detectable based on LC migration and a screen for both parent and MI

Table 1
MIL exposure in rats results in a specific increase in relative uterus wet weight.

Dose group	Dose per day (Animals/ group)	Weight gain (g)	Uterus wet weight (g)	Relative wet weight (% of total body weight) mean (2 S. F.) ± SD (3 S.F.)		
				Uterus	Liver	Kidney
Control	saline vehicle (8)	23 ± 4.7	0.11 ± 0.028	0.14 ± 0.014	5.8 ± 1.16	0.56 ± 0.083
EMI	80 mg/kg bw (6)	27 ± 6.7	0.18 ± 0.135*	0.22 ± 0.162*	5.5 ± 0.53	0.45 ± 0.070$
BMI	50 mg/kg bw (9)	24 ± 4.2	0.12 ± 0.043	0.20 ± 0.085*	5.6 ± 0.42	0.54 ± 0.114
HMI	30 mg/kg bw (6)	29 ± 5.0	0.22 ± 0.159*	0.31 ± 0.162*	4.7 ± 2.15	0.50 ± 0.055
M8OI	10 mg/kg bw (6)	22 ± 3.7	0.11 ± 0.039	0.17 ± 0.045	5.6 ± 0.99	0.50 ± 0.067
E2	0.5 mg/kg bw (5)	21 ± 6.7	0.29 ± 0.085*	0.44 ± 0.142*	5.6 ± 0.98	0.56 ± 0.064

Data are the mean and SD of the number of animals per group. $p < 0.05$ significantly greater*/lower$ from the control using the Student's T test.

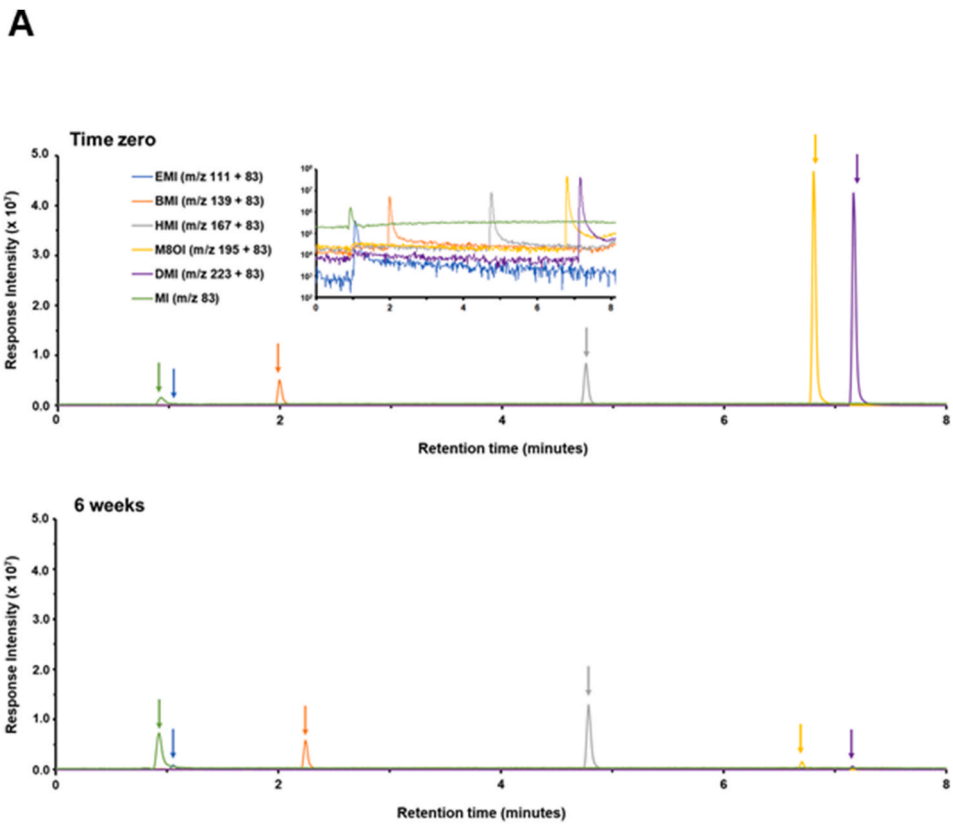

Fig. 1. Parent short chain MILs are subject to negligible degradation in river water. A, detection of parent MILs in river water samples at time 0 (upper panel) and after 6 weeks separate incubation with MILs (lower panel) by LC-MS. Note, determinations of each MIL are presented on the same chromatogram to facilitate comparison and demonstrate the method is capable of differentiating between MILs with different alkyl chain lengths, results typical of 3 separate experiments. **B**, time course for the presence of acetate or the indicated parent MILs, data are the mean and SD of 3 separate experiments.

positive ions. MI detection was therefore necessarily restricted to a single ion (MI^+). The increase in response intensity with increasing alkyl chain length (at time zero, despite decreasing concentration) is due to increasing stability of parent MIL ion with increasing chain length.

After 7 weeks, only the longer alkyl chain parent MILs – M8OI and DMI – had markedly fallen in concentration (Fig. 1a, lower panel). A quantification of parent MILs with time (Fig. 1b) indicates MI and the short chain MILs (EMI, BMI and HMI) were not depleted, rather there were variable increases in their concentrations with time, associated with variable evaporation (due to requirement that incubations be exposed to air). This contrasts with acetate, which was oxidised within 1 week in river water. In contrast to short chain MILs, parent M8OI and DMI concentrations fell to low levels by around week 3–4 in river water (Fig. 1b). Two sterile water groups containing EMI or M8OI were also included in the study and in both cases, there was no fall in concentration (rather, an increase in concentration due to evaporation of solvent water). The absence of any M8OI loss in sterile water suggests that chemical instability was likely the cause of acetate loss in sterile water after 5 weeks (Fig. 1b) and that MIL changes in river water were associated with microbial metabolism.

A screen for MIL metabolites was undertaken based on the prediction that oxidation would occur on the terminal alkyl carbon as observed for M8OI when incubated with human hepatocytes (Probert et al., 2018; Leitch et al., 2021, 2024). These data and the present data identify a methylimidazolium moiety fragment (*m/z* 83), not a fragment commensurate with oxidation on the methyl group present in MILs. Further, oxidative metabolites of M8OI have identical retention times to authentic HO8IM and COOH7IM metabolites (Probert et al., 2018; Leitch et al., 2021). Supp. Fig. 2a indicates appearance of these metabolites for M8OI and DMI in river water, but an absence of any equivalent metabolites in river water containing MI, EMI, BMI or HMI. A time course analysis for the appearance of predicted oxidative metabolites of M8OI and DMI demonstrated there was an accumulation and subsequent loss (Supp. Fig. 2b). The appearance of several chromatographically-distinct metabolites with the same mass fragment signature indicates - in contrast to human hepatocytes (Probert et al., 2018; Leitch et al., 2021) (see also Fig. 5) – that oxidation on the alkyl chain at positions other than the terminal carbon occurred (Supp Figs. 2c and 2d; see also Supp. Figs. 3a–f and Supp. Figs. 4a–j for potential structures). Interestingly, metabolism of DMI resulted in metabolites with truncated alkyl chain lengths that did not appear when the short chain parent MILs were incubated with river water. For example, metabolite i – likely a 4C carboxylic acid metabolite (Supp. Fig. 4f) – was not observed in river water incubated with BMI (Fig. 1).

Fig. 2a demonstrates that control river water contained both inorganic and organic carbon and that there was a statistically significant loss of total organic carbon (TOC) over the 7 week incubation period, presumably associated with microbial organic carbon oxidation. There was a statistically significant decrease in TOC in both positive controls and in river water containing EMI, M8OI and DMI (Fig. 2a). However, when mean background TOC oxidation was subtracted, only river water containing the positive control compounds saw a statistically significant TOC oxidation (Fig. 2b). The concentrations of M8OI and DMI were necessarily low to avoid toxicity to microbes. However, these concentrations were also near the quantification limit for carbon determination, particularly for DMI. Given that the carbon associated with M8OI was calculated to be 2.9 mg/L, the mean M8OI-derived carbon oxidation can be estimated to be around 60%. It is likely therefore, that a similar

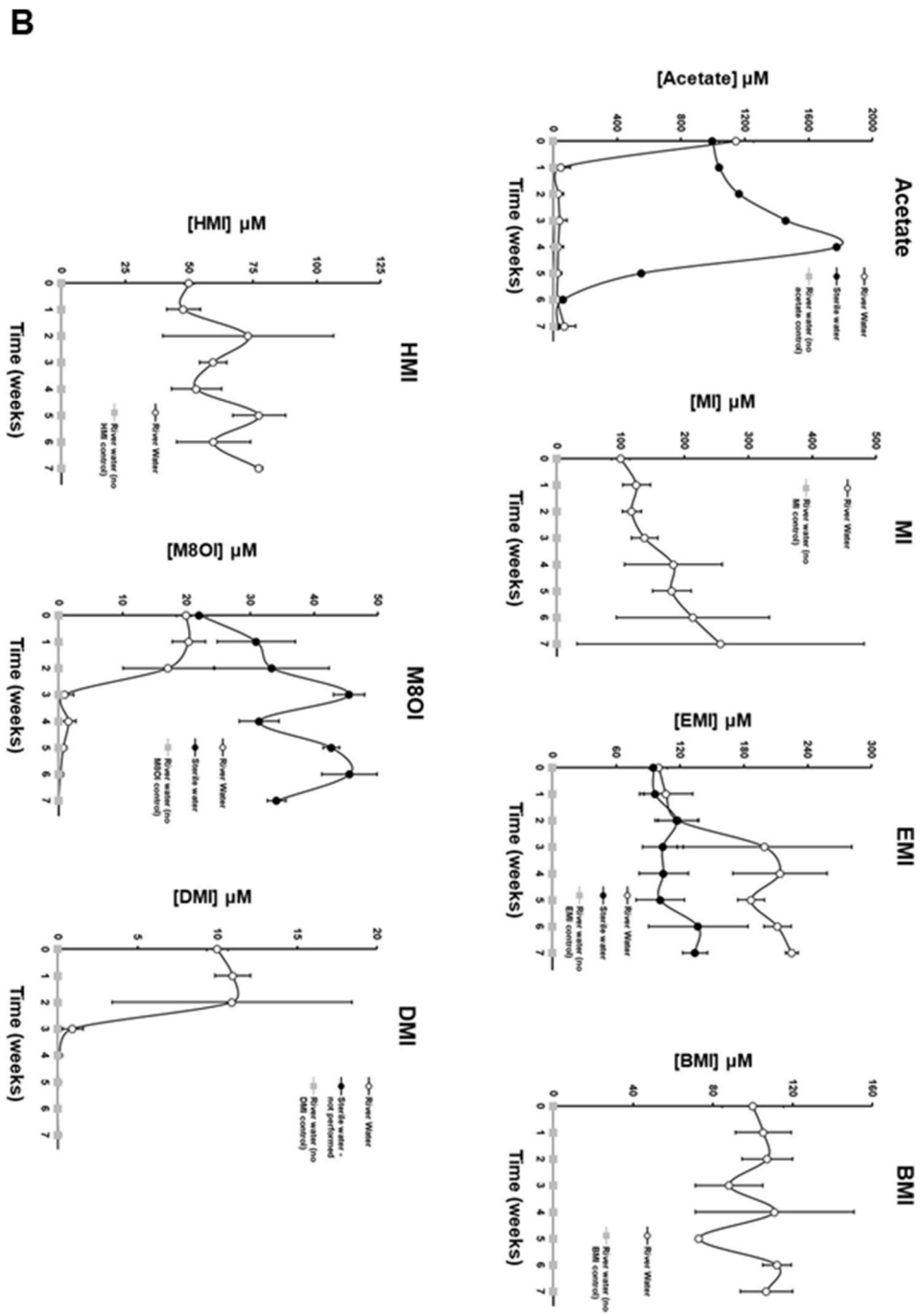

Fig. 1. (*continued*).

degree of oxidation was seen with DMI. Sterile water controls with EMI or M8OI saw no reductions in TOC (Fig. 2a/b).

These data suggest that short chain MILs are oxidised to a negligible extent in river water. In contrast, longer chain MILs are oxidised via microbial action and to some extent mineralised over a 7 week period.

3.2. Short chain MILs are activators of the hERα and activation is inhibited by ERα antagonists

Prior to testing MILs for their ability to activate the hERα, the toxicities of both MILs (Supp. Fig. 5a) and other relevant compounds (Supp. Fig. 5b) in ERα-HeLa-9903 were determined to ensure that only non-toxic concentrations (>80% MTT reduction capacity relative to vehicle controls) were examined. Fig. 3 demonstrates that EMI, BMI and HMI were activators of the hERα, with approximate half maximal activation for each MIL in the 10^{-7}-10^{-6} M range. Previous studies identified M8OI as a hERα activator in a transient transfection assay at concentrations above 25 µM (i.e. $>10^{-5}$ M) (Leitch et al., 2018). However, this concentration was toxic to HeLa 9903 cells (Supp. Fig. 5a) and therefore M8OI was not seen to be positive using ERα-HeLa-9903 cells (Fig. 3). Supp. Fig. 6a demonstrates that there was no metabolism of M8OI by ERα-HeLa-9903 cells and therefore the lack of hERα activation was not due to metabolism of M8OI to inactive metabolites (HO8IM and COOH7IM do not activate the hERα in MCF-7 cells, in contrast to M8OI (Leitch et al., 2018)).

DMI also failed to activate the hERα (Fig. 3). Supp. Fig. 6b demonstrates that DMI did not inhibit luciferase activity (induced via E2 pretreatment) when added to ERα-HeLa-9903 cell lysates in contrast to

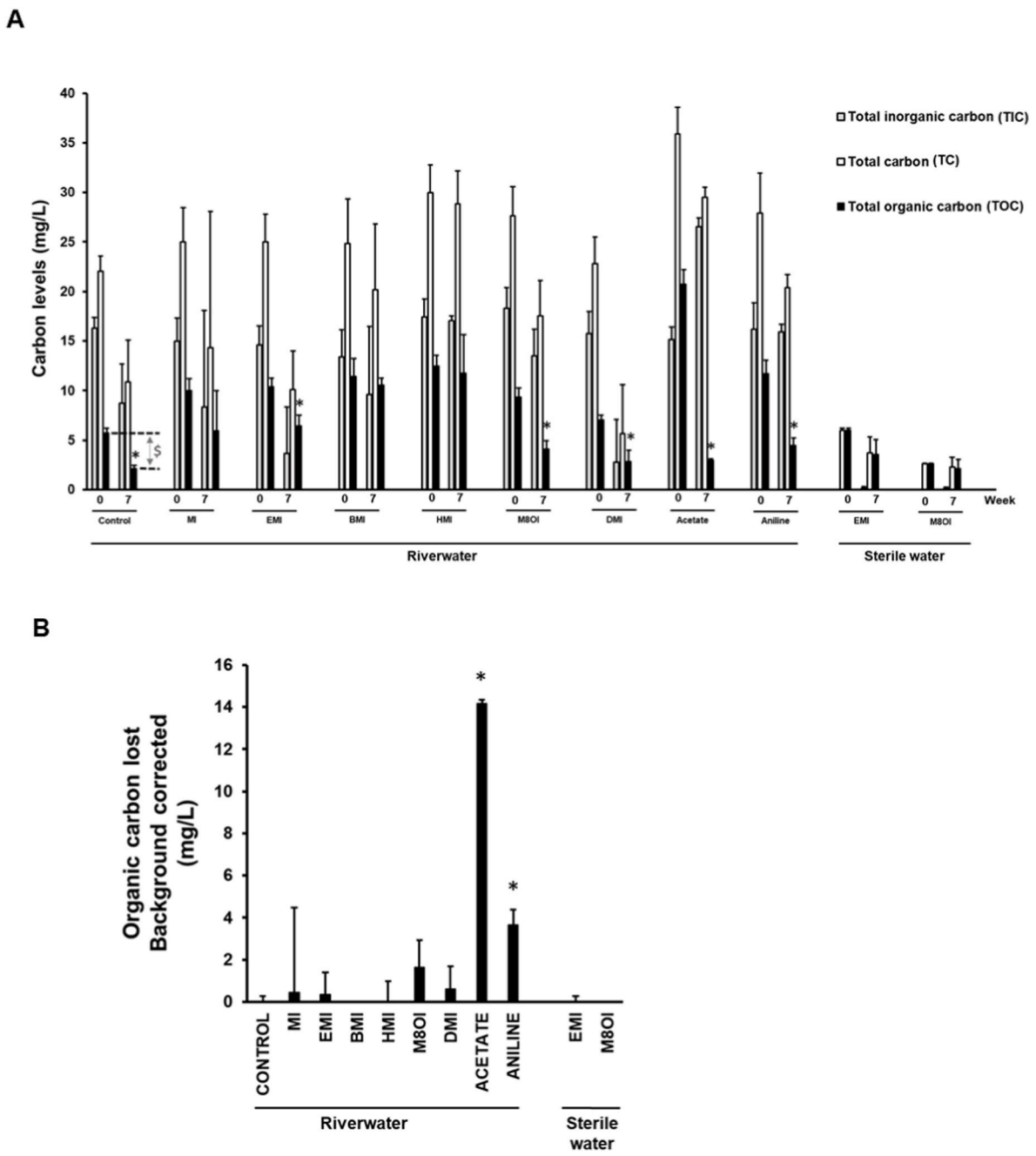

Fig. 2. Short chain MILs are subject to negligible mineralisation in river water. A, Carbon measurement performed on water samples after 7 weeks of incubation. **B**, Estimation of organic carbon lost in river water samples after 7 weeks incubation, after subtraction of control background organic carbon loss. All data are the mean and SD of 3 separate experiments. *$p < 0.05$ from the control using the Student's T test.

established luciferase inhibitors – resveratrol and β-naphthoflavone (BNF) (Braeuning, 2015). Non-toxic concentrations of DMI also did not further activate nor inhibit E2-dependent activation of the hERα (Supp. Fig. 6c). Therefore, DMI likely does not interact with the hERα, either as an agonist or as an antagonist. Activation of the hERα by E2 (Supp. Fig. 7a), EMI (Supp. Fig. 7b), BMI (Supp. Fig. 7c) and HMI (Supp Fig. 7d) are all similarly antagonised by the ERα antagonist fulvestrant (Kojetin et al., 2008) and tissue-selective ERα antagonist tamoxifen (Wakeling et al., 1991). Of note, tamoxifen similarly acted as a type I anti-estrogen or partial agonist/antagonist with MILs.

For an examination of hAhR activation, the HepG2-Lucia cell line was used. None of the MILs activated (or antagonised) the hAhR (Supp. Fig. 8).

3.3. Structure-based activity predictions

The potential interactions between MILs (EMI, BMI, HMI and M8OI) and the hERα were further examined using a structure-based computational approach. Using X-ray crystallographic data, AlphaFold2 modelling and other experimental data (Huang et al., 2018), a model of the hERα LBD-DBD dimer was constructed. Two plausible models satisfied the site-directed mutagenesis and small-angle X-ray scattering restraints: one, where the DBD-LBD interface comprises each monomer separately and one where the domains are swapped (Fig. 4a).

As previously reported, M8OI was shown to be an activator of the hERα in MCF7 cells (Leitch et al., 2018) and molecular docking calculations predict that M8OI binds to hERα at the orthosteric site (Fig. 4b). The complex is stabilised primarily by hydrophobic interactions

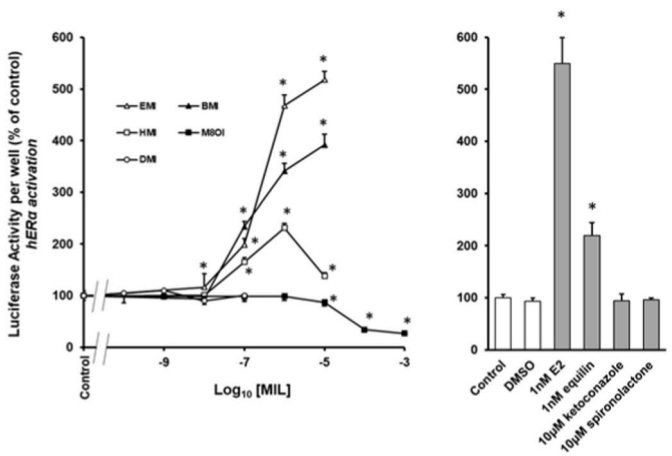

Fig. 3. Short chain MILs activate the hERα in ERα-HeLa-9903. ERα-HeLa-9903 were treated with the indicated chemicals for 48 h prior to determination of luciferase activities as outlined in methods section. Left panel: effect of the indicated MIL on luciferase activity; right panel: typical example of vehicle, OECD TG 455 positive (E2 and equilin) and negative controls (ketoconazole, spironolactone) routinely implemented within batch screens. Data are the mean and SD of 6 separate determinations from the same experiment, typical of at least 3 separate experiments. *$p < 0.05$ from control using ANOVA/Bonferroni-Holm comparison between groups.

between the hydrophobic n-octyl chain and a cluster of hydrophobic residues including M343, L384, L387, W383, M388, L391, F404, M421, L428, L540 and L525. BMI and HMI adopt the same binding mode and molecular docking calculations. This interaction is similar to that seen with bisphenol A (BPA), with no intermolecular H-bonds stabilising ligand-protein binding. By comparing ERα binary complexes with BPA (an ERα agonist (Brzozowski et al., 1997)) and bisphenol C (BPC, an ERα antagonist (Shiau et al., 1998)), BMI, HMI and M8OI are predicted to act as hERα agonists (Fig. 4c).

Solvent-mapping of the models identified 2 "drugg-able" binding sites: an orthosteric (E2) binding site in each LBD domain and a smaller allosteric binding site at each LBD-DBD interface (Fig. 4d). EMI is predicted to ostensibly bind to the allosteric site as depicted in Fig. 4d. Top-ranking binding poses predict interactions with residues in the DBD (Y184, W200) and LBD (I326, W393, M396, E397). Indeed, for MILs with a short carbon chain ($n < 3$), it was feasible that binding could occur at both an allosteric and the orthosteric site. EMI would likely not displace E2 based on predicted affinities at the orthosteric site, as its calculated binding affinity is in mid-μM range, whereas the affinity of E2 was calculated to be in the high-pM to low-nM range, which agrees very well with its determined K_d of 0.15 nM (Harmon and Kimmel, 1981). Therefore, EMI is more likely to act as a hERα activator (via the allosteric site) and/or as an allosteric positive modulator of orthosteric binding by activators of the hERα (such as E2).

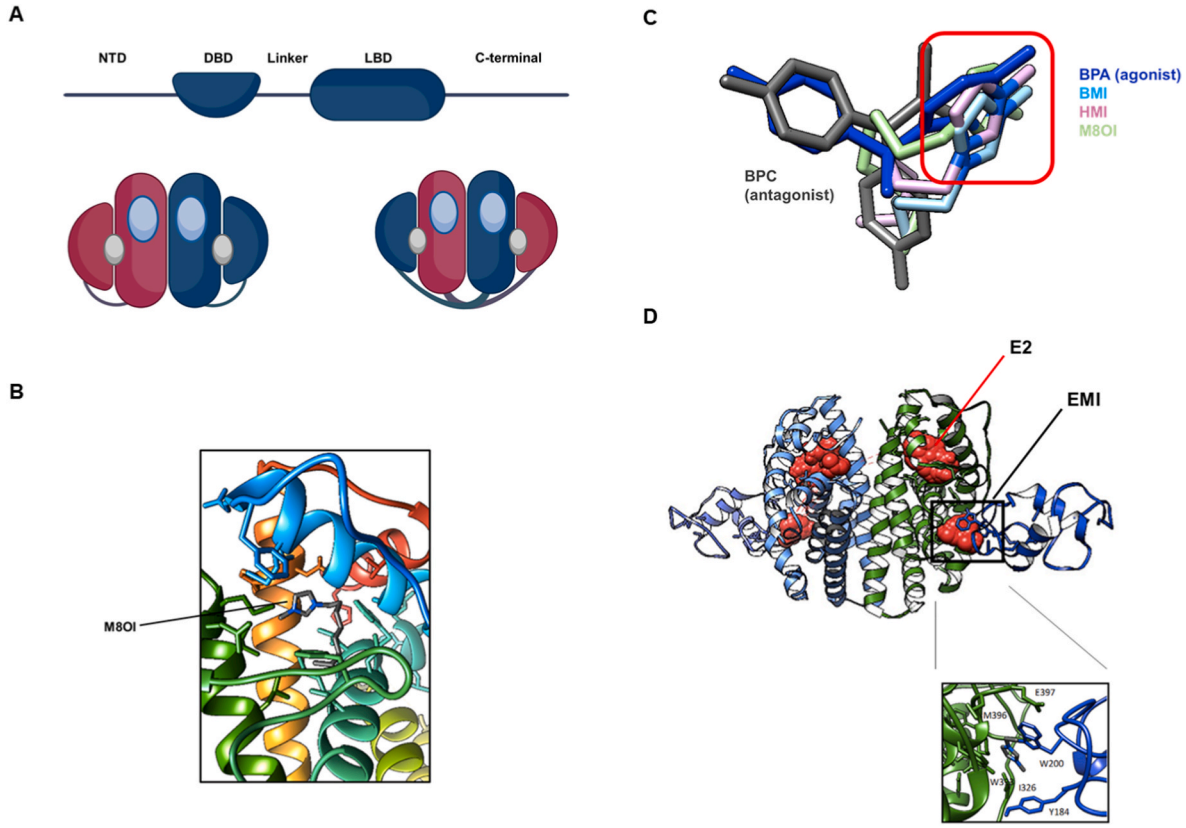

Fig. 4. Short chain MIL binding to the hERα. A, schematic illustration of a generic nuclear receptor such as ERα. Top panel, NTD - N-terminal domain (not included in the model, DBD, linker and LBD - modelled, C-terminal domain - not included in the model. Bottom panels illustrate 2 possible dimer arrangements. In both cases, the orthosteric sites (blue ovals) at the LBDs and interfacial LBD-DBD allosteric sites (grey ovals) remain unaffected by the dimer topology. **B**, M8OI bound to the orthosteric binding site in the hERα. Non-hydrogen atoms of M8OI, coloured by element, and ER side chains involved in interactions are shown. **C**, predicted binding modes BMI (marine blue), HMI (purple) and M8OI (green) overlayed with the binding mode of BPA (PDB code: 3UU7, blue – a hERα agonist) and BPC (PDB code: 3UUC, grey - a hERα antagonist). **D**, Detailed view of EMI bound at the allosteric binding site marked at panel C. The EMI molecule is rendered as sticks and coloured by element (carbon – grey; nitrogen – blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

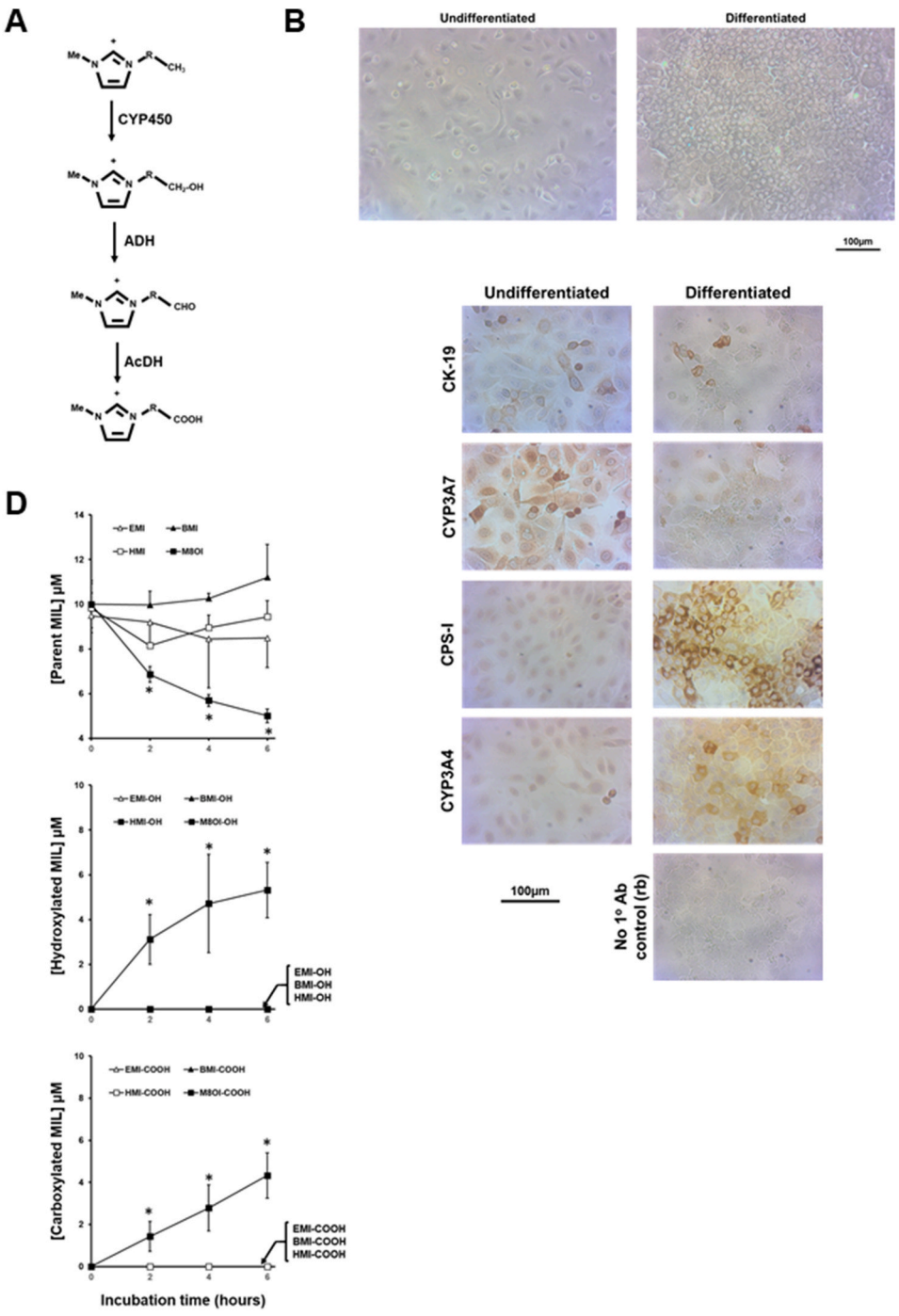

Fig. 5. Metabolism of MILs by human hepatocyte-like (differentiated) HepaRG cells. A, Generic metabolic pathway for MILs. Note, R = - (CH_2) - for EMI; - $(CH_2)_3$ - for BMI; - $(CH_2)_5$ - for HMI; - $(CH_2)_7$ - for M8OI and - $(CH_2)_9$ - for DMI and their metabolites. **B**, Light micrographs of typical morphology of undifferentiated HepaRG cells and differentiated HepaRG cells. **C**, Typical LC-MS/MS detection of MILs (EMI and M8OI) and potential carboxylate metabolite at time zero and after 6 h of incubation. See Supp. Fig. 7 for equivalent data with BMI and HMI. **D**, timecourse for the disappearance of MILs from medium and appearance of hydroxylated and carboxylate MIL metabolites. Hydroxylated and carboxylated M8OI metabolites were quantitated using authentic standards. A carboxylate BMI was also custom-synthesised to validate detection of proposed metabolites (Supp. Fig. 1). Data are the mean and SD of 3 separate experiments. *$p < 0.05$ from control using ANOVA/Bonferroni-Holm comparison between groups.

3.4. Short chain MILs have endocrine disrupting potential in vivo

Although the in vitro methodologies applied above identify biological targets, their toxicokinetics dictate tissue concentrations in vivo, which heavily influences likelihood of target protein interaction and biological or toxicological effect(s). Table 1 indicates that EMI, HMI and E2 (a positive control) treatment all lead to statistically significant increases in uterus wet weight in rats in pre-pubescent rats showing similar weight gains to controls (i.e. not showing overt signs of general toxicity). When expressed relative to total body weights, all small chain MILs showed a statistically significant increase in uterus weight, in addition to E2. In contrast, there was no change in relative wet liver and

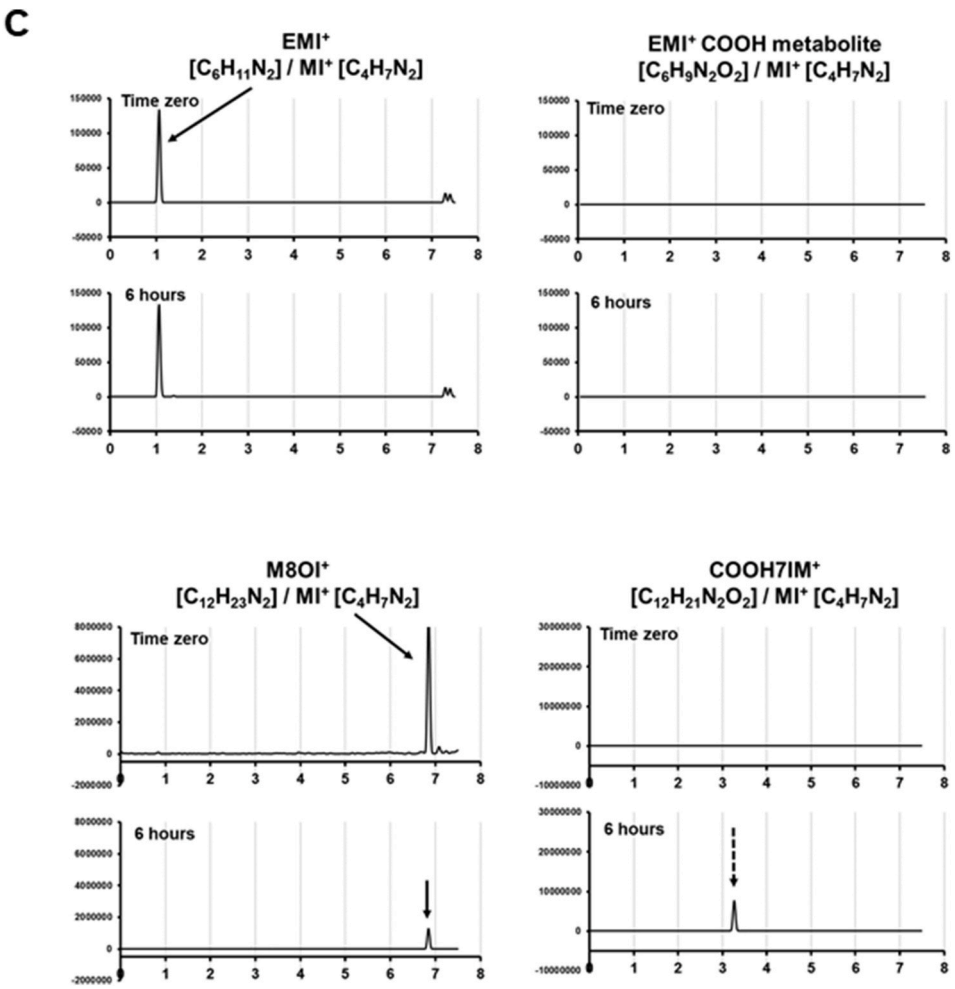

Fig. 5. (*continued*).

kidney wet weights, suggesting a target organ effect of small chain MILs and E2 on uterus growth. EMI-treated rats did show a significant decrease in relative kidney weight, which is either incidental or indicative of renal toxicity. Exposure to M8OI did not lead to any change in relative uterus wet weight. These data indicate that the small chain MILs are capable of activating the rat ERα in vivo.

3.5. Short chain MILs are resistant to degradation by human hepatocytes

To determine whether smaller chain MILs are metabolised similarly in man, their metabolism was examined using a similar strategy to that applied to M8OI. This involved screening for the presence of both parent or predicted metabolite ions in combination with the methylimidazolium fragment ion present in all of the MILs (Fig. 5a). Fig. 5b demonstrates a conversion of the human HepaRG cell into hepatocyte-like cells on the basis of morphological changes and widespread expression of a number of liver-specific proteins, including the major drug metabolising cytochrome P450 CYP3A4.

Fig. 5c and d confirms previous data that M8OI is readily removed from the medium along with a time-dependent increase in the hydroxylated and carboxylic acid metabolites over a 6 h period. In contrast, EMI did not significantly disappear from the medium and there was no appearance of any hydroxylated or carboxylated metabolites (Fig. 5c and d). Similarly to EMI (Fig. 5c and c), BMI and HMI did not significantly disappear from the medium and there was no appearance of any hydroxylated or carboxylated metabolites (Supp. Fig. 9).

4. Discussion

Using an LC-MS analytical approach previously applied to M8OI (Probert et al., 2018; Leitch et al., 2021) a range have MILs have been examined in both river water and cell culture medium for the first time. This LC-MS procedure is an inherently sensitive technique able to characterise chemicals using both chromatographic retention time as well as mass fragment patterns. It is evident that the technique is less able to detect MILs as the alkyl chain becomes shorter due to decreased parent MIL ion stabilities. This did not impact on our investigations since – due to their lower toxicity - higher concentrations of short chain MILs could be used in each component of the study. However, given their solubility in aqueous solvents (making it challenging to extract them from biological and environmental samples and concentrate using volatile extractions), this feature of short chain MILs could impact on their being detectable in the environmental. For future studies, a formal assessment of MIL detection (limit of detection; limit of quantification) in different matrices would be informative with regard to limits of MIL detection. This would assist in any future screening study, both in exposure studies and in environment screens. To our knowledge, MILs are not currently included on the list of chemicals routinely screened by environmental agencies.

MILs are relatively simple man-made chemicals that do not exist naturally (i.e. the alkylated methylimidazolium entity is not, to our knowledge, synthesised by living organisms or present in the environment due to natural geological processes that occur on Earth). They are stable chemicals under normal temperatures and pressures, relatively

non-volatile and readily water-soluble (Welton, 2018; Leitch et al., 2020). Given that biota will have only encountered MILs in the last few decades, efficient metabolic pathways to mineralisation are unlikely to have specifically evolved for their degradation. Accordingly, as is the case for some other classes of man-made chemicals (e.g. perfluoroalkyl-and polyfluoroalkyl substances (De Silva et al., 2021; Daghorn et al., 2023 see https://www.lemonde.fr/en/les-decodeurs/article/2023/02/23/forever-pollution-explore-the-map-of-europe-s-pfas-contamination_6016905_8.html), there is the potential for an environmental accumulation of MILs in the future and for widespread adverse effects to occur.

There is limited data on MIL stability in the environment. These comprise an examination of BMI stability in river water based on biochemical oxygen measurements. The authors reported there was no increase in oxygen demand over 28 days, suggesting an absence of BMI oxidative metabolism (Wells and Coombe, 2006). There has been no examination of MIL stability in seawater. In aerobic sewage sludge experiments, short chain MILs appear to be resistant to degradation. For example, less than 10% of EMI was oxidised (based on dissolved organic carbon measurements) after 28 days when incubated with activated sewage sludge (ECHA, 2022); there was no apparent oxidation of BMI (using Br^- salt) after 43 days (Docherty et al., 2007) whereas around 60% oxidation was reported for M8OI based on increased biochemical oxygen demand in a mixture of soil and waste sewage sludge using the OECD test method 301F (Markiewicz et al., 2015). In all these cases however, parent MILs and their metabolites were not directly determined and therefore it is not possible to definitively confirm oxidation of parent and/or exclude that there was an accumulation of metabolites.

A novel observation from the studies reported herein, is that longer chain MILs were converted to short chain carboxylic acid metabolites that were, intriguingly, not generated from similar length short chain parent MILs. This was only observed in the environment, and not in metabolic studies with liver cells. Future biochemical studies could examine why this is the case and identify the enzymes involved.

This study indicates for the first time that short chain MILs are activators of the human ERα in vitro. These studies were conducted following an OECD TG approach and provide strong initial evidence that short chain MILs may be a hazard with the respect to endocrine disruption via the ERα. Based on modelling, MILs were predicted to bind to the orthosteric site in a similar fashion to the xenoestrogen bisphenol A (BPA). Work by others has shown that the crystal structures of ERα in complex with xenoestrogens bisphenol A (BPA) and bisphenol C (BPC) have different binding poses (Delfosse et al., 2012, 2014). BPA acts as an agonist and induces an agonist-bound conformation in which H12 covers the ligand-binding pocket (Brzozowski et al., 1997). In contrast, BPC triggers a repositioning of H12 into the ERα surface groove and adopts a similar LBD conformation induced by the antagonist 4 hydroxytamoxifen (Shiau et al., 1998). However, EMI may bind additionally at another site in the ERα. The conserved DNA-binding (DBD) and C-terminal ligand-binding (LBD) domains in ERα are joined by an intrinsically disordered hinge region. In this respect, functionally relevant inter-domain interactions between the DBD and LBD have been identified in several nuclear receptors, including the human ERα that allosterically regulates receptor function (Huang et al., 2018). Small-angle X-ray scattering (SAXS), hydroxyl radical protein footprinting and molecular modelling have identified specific sites on each domain interface involved in DBD–LBD interactions along with site-directed mutagenesis have demonstrated the functional role of the DBD–LBD interface (Huang et al., 2018). However, the authors did not follow up with any “druggability” assessment of this interface. Using molecular modelling and in silico solvent mapping (Jumper et al., 2021), we identified a set of unique “drugg-able” binding sites at this interface, and by subsequent molecular docking, found that these pockets may bind short-chained (<3C alkyl chain) but not long-chained MILs.

However, the risk for endocrine disruption in the human population will be dependent on the level of exposure and the toxicokinetics of each MIL, aspects which have not been examined to any significant extent. Therefore, the extent of the risk that short chain MILs might contribute to endocrine disruption in the human population is unknown. Future studies should examine in greater detail the mechanism and extent of absorption, first pass metabolism, systemic availability and excretion of MILs. Data in this study suggest that short chain MILs are not metabolised by the human liver and therefore would not be cleared by metabolism. However, to fully consider the risks to the general population, in the absence of ethical studies in humans - orally-exposed animal models may be needed. An animal model could provide toxicokinetic data and, after taking into account probable differences and/or uncertainties between the model species and humans, provide a dose estimate for internal exposure in man. In addition, such studies could examine effect by - and on -gastrointestinal microbiota since there may be metabolism by the microbiota given that both BMI and M8OI have been shown to cause changes in microbial populations (Young et al., 2020). Tissue concentrations (i.e. uterine and other organs) could also be determined.

A limited animal study was performed to examine the potential for ERα activation in vivo. The route of exposure chosen was designed to maximise systemic MIL levels in combination with an appropriate dose to reduce chances of general toxic effects. This approach therefore does not reflect relevant general human population exposure, which is most likely via the oral route. A further drawback of the study was the choice of a single dose level for each MIL. All these options were chosen in order to reduce the number of animals used in compliance with a 3Rs approach. However, the data suggest there was activation of the ERα in rats in vivo in response to short chain MILs. Future studies could therefore examine whether there is activation of the ERα in vivo in response to an oral route of exposure to (a selected) short chain MILs. Inclusion of a comprehensive dose range will likely enable the identification of a no effect level, since such effects would be expected to have a threshold.

5. Conclusion

In conclusion, this study shows that short chain MILs (EMI, BMI and HMI) resisted any metabolic alterations in both river water and a human liver model. Longer chain MILs (M8OI and DMI) were subject to oxidative metabolism. In both systems and for all short chain MILs, there was no compelling evidence for mineralisation to any significant extent. Using an in vitro screen, we show that short chain MILs activate the hERα - but not the hAhR – and in vivo have estrogenic effects in orally-exposed rats.

This study therefore identified for the first time that short chain MILS are environmentally persistent activators of the hERα in vitro and have potential endocrine disrupting properties in vivo. MILs are therefore an emerging risk as a new type of forever chemical with the potential for endocrine disruption.

CRediT authorship contribution statement

Tarek M. Abdelghany: Writing – review & editing, Investigation. **Shireen Hedya:** Investigation. **Alex Charlton:** Investigation. **Lanyu Fan:** Investigation. **Narges Fazili:** Investigation. **Ben Air:** Investigation. **Alistair C. Leitch:** Investigation. **Martin Cooke:** Writing – review & editing, Supervision. **Agnieszka K. Bronowska:** Writing – review & editing, Supervision, Investigation. **Matthew C. Wright:** Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.142827.

References

Axon, A., May, F.E., Gaughan, L.E., Williams, F.M., Blain, P.G., Wright, M.C., 2012. Tartrazine and sunset yellow are xenoestrogens in a new screening assay to identify modulators of human oestrogen receptor transcriptional activity. Toxicology 298 (1–3), 40–51. https://doi.org/10.1016/j.tox.2012.04.014.

Braeuning, A., 2015. Firefly luciferase inhibition: a widely neglected problem. Arch. Toxicol. 89 (1), 141–142. https://doi.org/10.1007/s00204-014-1423-3.

Brzozowski, A.M., Pike, A.C., Dauter, Z., Hubbard, R.E., Bonn, T., Engström, O., Ohman, L., Greene, G.L., Gustafsson, J.A., Carlquist, M., 1997. Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389 (6652), 753–758. https://doi.org/10.1038/39645.

Daghorn, G., Aubert, R., Horel, S., Martinon, L., Steffen, 2023. Forever pollution': Explore the map of Europe's PFAS contamination. LeMonde. https://www.lemonde.fr/en/les-decodeurs/article/2023/02/23/forever-pollution-explore-the-map-of-europe-s-pfas-contamination_6016905_8.html.

Delfosse, V., Grimaldi, M., Pons, J.L., Boulahtouf, A., le Maire, A., Cavailles, V., Labesse, G., Bourguet, W., Balaguer, P., 2012. Structural and mechanistic insights into bisphenols action provide guidelines for risk assessment and discovery of bisphenol A substitutes. Proc. Natl. Acad. Sci. U. S. A. 109 (37), 14930–14935. https://doi.org/10.1073/pnas.1203574109.

Delfosse, V., Grimaldi, M., Cavaillès, V., Balaguer, P., Bourguet, W., 2014. Structural and functional profiling of environmental ligands for estrogen receptors. Environ. Health Perspect. 122 (12), 1306–1313. https://doi.org/10.1289/ehp.1408453.

De Silva, A.O., Armitage, J.M., Bruton, T.A., Dassuncao, C., Heiger-Bernays, W., Hu, X.C., Kärrman, A., Kelly, B., Ng, C., Robuck, A., Sun, M., Webster, T.F., Sunderland, E.M., 2021. PFAS exposure pathways for humans and wildlife: a synthesis of current knowledge and key gaps in understanding. Environ. Toxicol. Chem. 40 (3), 631–657. https://doi.org/10.1002/etc.4935.

Docherty, K.M., Dixon, J.K., Kulpa Jr., C.F., 2007. Biodegradability of imidazolium and pyridinium ionic liquids by an activated sludge microbial community. Biodegradation 18 (4), 481–493. https://doi.org/10.1007/s10532-006-9081-7.

ECHA, 2022. https://echa.europa.eu/registration-dossier/-/registered-dossier/16488/5/3/2.

Harmon, J.R., Kimmel, G.L., 1981. Estrogen receptor characterization following selective sedimentation separation of estrogen-binding components in immature rat uterine cytosol. J. Recept. Res. 2 (5–6), 555–574. https://doi.org/10.3109/10799981809038885.

Huang, W., Peng, Y., Kiselar, J., et al., 2018. Multidomain architecture of estrogen receptor reveals interfacial cross-talk between its DNA-binding and ligand-binding domains. Nat. Commun. 9, 3520. https://doi.org/10.1038/s41467-018-06034-2.

Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S.A.A., Ballard, A.J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A.W., Kavukcuoglu, K., Kohli, P., Hassabis, D., 2021. Highly accurate protein structure prediction with AlphaFold. Nature 596, 583–589.

Kojetin, D.J., Burris, T.P., Jensen, E.V., Khan, S.A., 2008. Implications of the binding of tamoxifen to the coactivator recognition site of the estrogen receptor. Endocr. Relat. Cancer 15 (4), 851–870. https://doi.org/10.1677/ERC-07-0281.

Leitch, A.C., Lakey, A.F., Hotham, W.E., Agius, L., Kass, G.E.N., Blain, P.G., Wright, M.C., 2018. The ionic liquid 1-octyl-3-methylimidazolium (M8OI) is an activator of the human estrogen receptor alpha. Biochem. Biophys. Res. Commun. 503 (3), 2167–2172. https://doi.org/10.1016/j.bbrc.2018.08.008.

Leitch, A.C., Abdelghany, T.M., Probert, P.M., Dunn, M.P., Meyer, S.K., Palmer, J.M., Cooke, M.P., Blake, L.I., Morse, K., Rosenmai, A.K., Oskarsson, A., Bates, L., Figueiredo, R.S., Ibrahim, I., Wilson, C., Abdelkader, N.F., Jones, D.E., Blain, P.G., Wright, M.C., 2020. The toxicity of the methylimidazolium ionic liquids, with a focus on M8OI and hepatic effects. Food Chem. Toxicol. 136, 111069 https://doi.org/10.1016/j.fct.2019.111069.

Leitch, A.C., Ibrahim, I., Abdelghany, T.M., Charlton, A., Roper, C., Vidler, D., Palmer, J.M., Wilson, C., Jones, D.E., Blain, P.G., Wright, M.C., 2021. The methylimidazolium ionic liquid M8OI is detectable in human sera and is subject to biliary excretion in perfused human liver. Toxicology 459, 152854. https://doi.org/10.1016/j.tox.2021.152854.

Leitch, A.C., Abdelghany, T.M., Charlton, A., Cooke, M., Wright, M.C., 2024. The Ionic Liquid 1-Octyl-3-Methylimidazolium (M8OI) Is Mono-Oxygenated by CYP3A4 and CYP3A5 in Adult Human Liver. J. Xenobiot. 2024, 14(3), 907-922. https://doi.org/10.3390/jox14030050.

Li, M., Wu, Z., Yu, Q., Fang, M., Liu, X., Cao, W., Wen, S., Li, J., Wu, Y., Liu, X., 2024. High-sensitivity liquid chromatography-tandem mass spectrometry quantitative for alkyl imidazolium ionic liquids in human serum: advancing biomonitoring of human exposure concerns. Talanta 276, 126257.

Markiewicz, M., Jungnickel, C., Cho, C.W., Stolte, S., 2015. Mobility and biodegradability of an imidazolium based ionic liquid in soil and soil amended with waste sewage sludge. Environ Sci Process Impacts 17 (8), 1462–1469. https://doi.org/10.1039/c5em00209e.

Meyer, S.K., Probert, P.M., Lakey, A.K., Leitch, A.C., Blake, L.I., Jowsey, P.A., Cooke, M.P., Blain, P.G., Wright, M.C., 2017. Environmental xenoestrogens super-activate a variant murine ER beta in cholangiocytes. Toxicol. Sci. 156 (1), 54–71. https://doi.org/10.1093/toxsci/kfw234.

OECD, 1992. Test No. 306: biodegradability in seawater. OECD Guidelines for the Testing of Chemicals, Section 3. OECD Publishing, Paris. https://doi.org/10.1787/9789264070486-en.

OECD, 2021. Test No. 455: performance-based test guideline for stably transfected transactivation in vitro assays to detect estrogen receptor agonists and antagonists. OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264265295-en.

Probert, P.M., Leitch, A.C., Dunn, M.P., Meyer, S.K., Palmer, J.M., Abdelghany, T.M., Lakey, A.F., Cooke, M.P., Talbot, H., Wills, C., McFarlane, W., Blake, L.I., Rosenmai, A.K., Oskarsson, A., Figueiredo, R., Wilson, C., Kass, G.E., Jones, D.E., Blain, P.G., Wright, M.C., 2018. Identification of a xenobiotic as a potential environmental trigger in primary biliary cholangitis. J. Hepatol. 69 (5), 1123–1135. https://doi.org/10.1016/j.jhep.2018.06.027.

Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., Greene, G.L., 1998. The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95 (7), 927–937. https://doi.org/10.1016/s0092-8674(00)81717-1.

Wakeling, A.E., Dukes, M., Bowler, J., 1991. A potent specific pure antiestrogen with clinical potential. Cancer Res. 51 (15), 3867–3873.

Wells, A.S., Coombe, V.T., 2006. On the freshwater ecotoxicity and biodegradation properties of some common ionic liquids. Org. Process Res. Dev. 10 (4), 794–798. https://doi.org/10.1021/op060048i.

Welton, T., 2018. Ionic liquids: a brief history. Biophys Rev 10 (3), 691–706. https://doi.org/10.1007/s12551-018-0419-2.

Young, G.R., Abdelghany, T.M., Leitch, A.C., Dunn, M.P., Blain, P.G., Lanyon, C., Wright, M.C., 2020. Changes in the gut microbiota of mice orally exposed to methylimidazolium ionic liquids. PLoS One 15 (3), e0229745. https://doi.org/10.1371/journal.pone.0229745.