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Evaluation of fluorinated biphenyl ether pro-drug scaffolds employing the chemical-microbial approach

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ABSTRACT

Incorporation of fluorine in a drug can dramatically affect its metabolism and methods to assess the effect of fluorine substitution on drug metabolism are required for effective drug design. Employing a previously developed chemical-microbial method the metabolism of a series of fluorinated biphenyl ethers was determined. The substrates were synthesized via Ullmann-type condensation reactions between bromotoluene and fluorophenol. The ethers were incubated with the fungus *Cunninghamella elegans*, which oxidises xenobiotics in an analogous fashion to mammals, generating a number of hydroxylated biphenyl ethers and acids. The propensity of the fluorinated ring to be hydroxylated depended upon the position of the fluorine atom, and the oxidation of the methyl group was observed when it was *meta* to the oxygen. The experiments demonstrate the applicability of the method to rapidly determine the effect of fluorine substitution on CYP-catalysed biotransformation of pro-drug molecules.

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Fluorine is an important element in drug development and design owing to its physicochemical properties, in particular Van der Waal's radius (1.35 Å), electronegativity (4 on the Pauling scale) and strength of the carbon–fluorine bond (115 kcal/mol); consequently, approximately 25% of drugs currently available are fluorinated.[1] One of the main reasons for incorporating fluorine into a drug is to improve its metabolic stability, particularly by preventing oxidative attack by cytochrome P450 enzymes, thereby prolonging its activity in vivo.[2,3] There are a number of approaches to identifying sites of metabolic attack, including in vivo, in vitro (such as microsomes, hepatocytes and recombinant enzymes), in silico and computational methods.[4] Our contribution to this important field concerns the development of a simple and effective method that employs the fungus *Cunninghamella elegans*, which is a model of mammalian oxidative drug metabolism,[5] in conjunction with synthetic fluorine chemistry, to evaluate the most readily metabolised site of a drug or drug candidate. This approach enables the design of a more metabolically stable fluorinated derivative.[6,7] The method has the advantages of being cost-effective, easy-to-use and allows for ready scalability facilitating the generation of isolable amounts of metabolites. In addition to offering a complementary approach to drug development, the method can be used to produce sufficient quantities of mammalian metabolites for toxicity testing,[8] which is important given the MIST (Metabolites In Safety Testing) guidelines defined by the FDA.[9] In this Letter we expand the range of drug-related systems to which this methodology can be applied by preparing a range of mono-fluorinated biphenyl ethers and assessing their biotransformation in *C. elegans* since biphenyl ether structural sub-units are found in several non-steroidal anti-inflammatory drugs (NSAIDs) such as nimesulide and fenoprofen (Fig. 1). Here we assess the fungal metabolism of methyl biphenyl ethers as models for this class of structural subunit.

Fluorinated 2- and 3-methylphenoxybenzene model compounds were synthesised by Ullmann-type condensation reactions between 2- and 3-bromotoluene with appropriate fluorophenol derivatives (Scheme 1), using an adapted literature procedure involving CuCl and 2,2,6,6-tetramethyl-3,5-heptanedione (TMHD) as catalysts and cesium carbonate as base in DMF reaction media.[10,11]

C. elegans was cultivated in 50 mL Sabouraud dextrose broth in 250 ml Erlenmeyer flasks for 72 h,[12] after which biphenyl ether (0.1 mg/mL) was added. Control experiments were conducted in which either no biphenyl ether was added to fungal cultures, or substrate was added to uninoculated flasks. Metabolites were extracted from culture supernatants in ethyl acetate and after removal of the solvent the residue was derivatised with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) prior to analysis by GC–MS. New peaks were observed in extracts from all flasks in which the biphenyl ethers were incubated with fungus. It is clear from the data shown in Table 1 that the new metabolites

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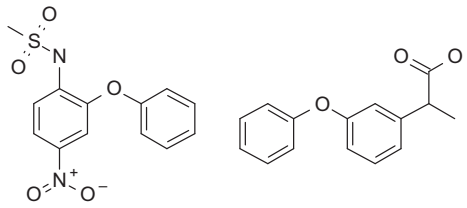
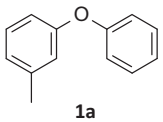
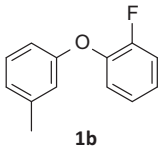
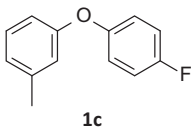
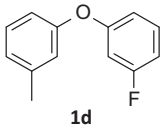
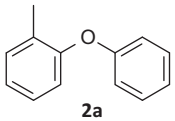
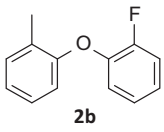
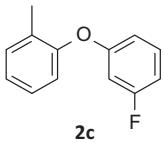
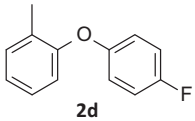


Figure 1. Examples of drugs with a biphenyl ether subunit.

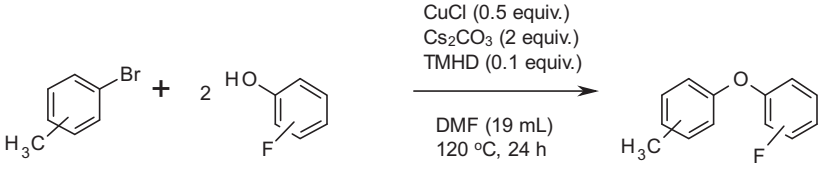
had common molecular ions (M+) which corresponded to various combinations of ring hydroxylation and methyl group oxidation to carboxyl (Scheme 2). For example, the non-fluorinated biphenyl ether **1a** was biotransformed to two products with molecular ions *m*/*z* 374 and 462; these masses correspond to those expected for silylated mono- and di-hydroxylated phenoxybenzoic acids. Biotransformation of the fluorinated biphenyl ethers **1b–d** revealed the production of monohydroxylated fluorophenoxybenzoic acids (*m*/*z* 392), dihydroxylated fluorophenoxybenzoic acids (*m*/*z* 480), fluorophenoxybenzoic acids (*m*/*z* 304), monohydroxylated fluorobiphenyl ether (*m*/*z* 290) and dihydroxylated fluorobiphenyl ether (*m*/*z* 378). In contrast, the methyl group of the biphenyl ethers **2a–d** was not oxidised as the masses of the biotransformation products corresponded to mono- or di-hydroxylated biphenyl ethers only. One possible explanation for this is that the proximity of the electronegative ether oxygen to the methyl group inhibits oxidation through inductive deactivation. For substrates **1c** and **2a–d** the presence of metabolites with similar mass spectra and different retention times indicated isomers of mono- and di-hydroxylated biphenyl ethers and phenoxybenzoic acids. For example, **1c** is biotransformed to three monohydroxylated fluorophenoxy benzoic acids (*m*/*z* 392).

Time course analysis of the biotransformation of **1a** demonstrated that the mono-hydroxylated product was present after 2 h and after 16 h the dihydroxylated product was detected. The sequential observation of mono- then di-hydroxylated products is consistent with previous observations concerning the biotransformation of biphenyl carboxylic acids.[6] Extracts from 16 h incubations with **1a** were analysed by HPLC and revealed the absence of starting material and the presence of the monohydroxylated product, which reflected the GC–MS analysis (Table 1). The mass recovery of the biotransformation product was much less than expected (<4% of the mass of starting material) indicating substantial material loss and subsequent experiments with control flasks (no fungus) revealed that much of the substrate was lost through evaporation and work-up. Nevertheless, through multiple biotransformation experiments it was possible to isolate sufficient monohydroxylated metabolite for ^{1}H NMR analysis.[13] Compared with the starting compound the methyl group resonance (δ 2.5 ppm) disappeared, confirming oxidation to carboxylic acid derivative. The appearance of a diagnostic AX system centred at 6.92 ppm confirmed the structure of 3-(4-hydroxyphenoxy)benzoic acid. The production of this metabolite is as expected based on our previous studies with biphenyl-4-carboxylic acid and 4-fluorobiphenyl,[6] in which hydroxylation was observed most prominently at the 4′ position.

Table 1
Summary of metabolites detected by GC–MS from biphenyl ether biotransformation

Biphenyl ethers			Metabolites		
	RT (min)	M+(*m*/*z*)	RT (min)	M+(*m*/*z*)	Yield[a] (%)
 **1a**	5.2	184	12.8 14.2	374 462	93 7
 **1b**	5.2	202	8.6 12.6 13.9 14.2	290 392 480 480	<0.5 99 <0.5 <0.5
 **1c**	5.3	202	9.5 10.5 11.0 12.1 12.5 13.1	304 378 392 392 392 480	7 7 14 30 25 18
 **1d**	4.9	202	12.5 13.8	392 480	99 1
 **2a**	4.7	184	7.8 8.3 8.6 10.5 11.2 11.7	272 272 272 360 360 360	41 15 18 13 9 8
 **2b**	4.9	202	8.1 8.5 11.3	290 290 378	78 1 22
 **2c**	4.6	202	7.6 8.1 10.3 10.9 11.5	290 290 378 378 378	15 38 1 37 10
 **2d**	4.7	202	7.8 8.3 10.3	290 290 378	51 35 14

[a] The yields were calculated from the areas of the GC–MS peaks. Incubation time for compounds **1a–d** was 72 h and for compounds **2a–d** was 24 h. No starting material remained in any of the flasks.



Scheme 1. Synthesis of fluorinated methyl-phenoxybenzenes via Ullmann-type condensation.

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