**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 synthesied 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.

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 paper 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.



**Figure 1.** Examples of drugs with a biphenyl ether 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].



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

*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

**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 72h and for compounds 2a-d was 24 h. No starting material remained in any of the flasks.

incubated with fungus. It is clear from the data shown in Table 1 that the new metabolites 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).



**Scheme 2.** Fungal biotransformation of fluorinated methyl-phenoxy-benzenes to hydroxylated ethers and acids. The masses given are those of the trimethylsilyl derivatives.

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 1H 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.



B

A

**Figure 2.** F-19 NMR spectra of compound **1b** before (A) and after (B) incubation with *C. elegans*. The insets show the coupling patterns of the most prominent resonances.

Compound **1b** was almost entirely converted to a monohydroxylated fluorophenoxybenzoic acid (*m/z* 392) according to GC-MS analysis of the extracted metabolites, and this was confirmed by HPLC. Whilst HPLC analysis of the extracts from all of the fluorinated biphenyl ether experiments revealed the same poor mass recovery as from **1a**, the most abundant metabolite of any experiment with the fluorinated substrates was the monohydroxylated fluorophenoxybenzoic acid formed from **1b** (up to 44 % of the starting material). F-19 NMR analysis confirmed the disappearance of the starting material (δ -131 ppm) and the appearance of a major (δ -127 ppm) and minor (δ -130 ppm) metabolite (Figure 2). The major metabolite is most likely to be the monohydroxylated fluorophenoxybenzoic acid, based on HPLC and GC-MS analyses. Furthermore, the change in the splitting pattern of this metabolite compared with the starting material indicates that the hydroxylation occurred on the fluorinated ring. Following the work of Peelen et al. [14] it is possible to predict sites of hydroxylation on fluoroaromatic derivatives, as *ortho*-, *meta*- and *para*- monohydroxylations result in characteristic chemical shifts of -23, +1.3 and -11 ppm respectively, compared to the non-hydroxylated starting compound. Thus, the main metabolite from **1b** is hydroxylated *meta*- to fluorine and from our previous studies on biotransformation of biphenyl systems it is most likely that the hydroxyl is *para* to the ether oxygen. This was confirmed by H-1 NMR analysis of the product (Supplemental Information), which revealed a doublet of doublets at δ 6.69 ppm (3JH-F = 11.5 Hz, 4JH-H = 3 Hz) that became a doublet upon F-19 decoupling (J=3Hz), demonstrating the absence of a second larger coupling (ca. 11 Hz) to the proton *ortho* to fluorine. The splitting of the minor metabolite’s resonance is very similar to the starting material, thus is most likely that this is 3-(2-fluorophenoxy) benzoic acid.

Metabolite derived from **1d** was purified by HPLC and the F-19 NMR spectrum showed a ddd with coupling constants of 11.3, 9.9 and 1.6 Hz, which indicated two 3JHF couplings and one 4JHF coupling, consistent with hydroxylation *ortho* to the phenoxy group. It appears that the fluorine atom at C-3 sufficiently deactivates the adjacent C-4 position, which is analogous with the slower metabolism observed with similar fluorinated biphenyl systems. However, in contrast to biphenyl derivatives, the presence of the ether oxygen can more effectively stabilise intermediate radical cations formed at the *ortho* position, resulting in hydroxylation at this site.

Experiments to investigate microsomal biotransformation of the biphenyl ethers were also conducted, using a previously described protocol [15]. However, neither substrate nor metabolites were detected after 2 h incubation. The incubation temperature employed (37 °C) was higher than that used for the fungal experiments, thus the absence of substrate strongly suggests that the rapid evaporation of the starting material was the reason for the lack of detectable metabolites. The comparison of xenobiotic metabolism in *Cunninghamella* spp. and microsomes has been reported in other studies, and it is generally accepted that the metabolites formed are comparable [16]. The present study also highlights an advantage of using the fungus rather than the microsomes if the substrates are volatile.

Our previous experiments also demonstrated that when the site of cytochrome P450-catalysed hydroxylation is blocked by fluorine, no biotransformation occurs with either 4’-fluoro-biphenyl-4-carboxylic acid [6], or 4’fluoro-flurbiprofen [7]. Therefore, it was surprising that numerous hydroxylated metabolites were detected from the fluorinated biphenyl ethers **1c** and **2d**, both of which are fluorinated *para* to the phenoxy. However, analysis by HPLC revealed that the main metabolite formed from **1c** was the fluorophenoxybenzoic acid (~ 17 % of starting material, compared to ~ 3 % of the hydroxylated metabolites), which is in contrast to the main product from **1b**, which was predominantly mono-hydroxy fluorophenoxybenzoic acid (up to 44 % of starting material). Therefore, the site of fluorination does affect the degree of CYP-catalysed hydroxylation of fluorobiphenyl ethers, consistent with our previous observations. However, in contrast to the absence of CYP-catalysed hydroxylation of 4’-fluoro-biphenyl-1-carboxylic acid observed previously, 4’fluorobiphenyl ether is biotransformed to a minor degree probably reflecting the ability of the ether oxygen to more effectively stabilise radical cations formed during oxidation. This indicates the requirement to evaluate metabolism of new fluorinated drug systems and highlights the applicability of the fungal method in the drug discovery process.

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