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The potential of ^{19}F NMR spectroscopy for rapid screening of cell cultures for models of mammalian drug metabolism

Olivia Corcoran,*[a] John C. Lindon,[a] Richard Hall,[b] Ismail M. Ismail[c] and Jeremy K. Nicholson[a]

[a] *Biological Chemistry, Division of Biomedical Sciences, Imperial College of Science, Technology and Medicine, Sir Alexander Fleming Building, South Kensington, London, UK SW7 2AZ. E-mail: olivia.corcoran@bruker-biospin.com*
[b] *Biotransformation and Natural Product Chemistry, Chemical and Analytical Technologies, GlaxoWellcome Medicines Research Centre, Gunnells Wood Road, Stevenage, Hertfordshire, UK SG1 2NY*
[c] *International Development, Biomet Division, GlaxoWellcome, Park Road, Ware, Hertfordshire, UK SG12 ODP*

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The use of microbial cultures as a complementary model for mammalian drug metabolism has been well established previously. Here is a preliminary investigation into the potential of ^{19}F NMR spectroscopy as a rapid screening tool to quantify the biotransformations of fluorine-containing model drugs. Biotransformations of three model drugs in 48 taxonomically diverse organisms were measured by acquiring ^{19}F NMR spectra at 376 MHz. The presence of fluorine in the molecules allowed rapid, simultaneous detection of over 20 biotransformation products without sample pre-treatment, chromatography, mass spectrometric techniques or the use of radiolabelled substrates. The detection limit at 376 MHz using 5 mm NMR tubes was *ca.* 0.3 μg ml^{-1} using a typical analysis time of 20 min per sample. With the recent advent of flow injection NMR technology, analysis time of 5 min could be achieved with less sample. This approach may be used to develop fast small-scale microbial screens for the biosynthesis of metabolite standards and production of novel drug analogues, whilst also having a role in reducing animal experiments needed to identify animal and human metabolites of fluorinated xenobiotics.

Introduction

One of the major analytical challenges in the drug discovery process is the identification and quantitation of drug metabolites in mammalian biofluids. In particular, with the design of increasingly potent drugs *via* combinatorial chemistry, levels of any drug metabolites are much lower than previously. Because of this many animals may need to be dosed, with subsequent pooling of samples, in order to provide sufficient metabolite material for structural elucidation. Otherwise, it is usually necessary to synthesise reasonable quantities of putative metabolites as standards for confirmation of structure. Both of these approaches are time-consuming and expensive and, therefore, are not usually employed in the early stages of drug discovery. The use of micro-organisms, particularly those with cytochrome P450 biotransformation enzymes or those genetically modified to contain human P450s, is now well established as a complementary model for mammalian metabolism of drugs.[1–4] This so-called 'microbial models of mammalian metabolism' approach provides an attractive, low-cost alternative to chemical synthesis for the production of important metabolites. Indeed, human metabolites are now commonly produced by microbial fermentations at the preparative scale, employing relatively mild conditions compared with chemical synthesis and allowing reactions to be performed on sensitive molecules with minimal degradation.[5,6] Furthermore, fermentation conditions can be manipulated to alter the yield and nature of the products formed.[1,3–6] These products can then be used as analytical standards to assist in the identification of mammalian metabolites and also to facilitate assay validation. Moreover, novel drug analogues thus produced can also be tested for bioactivity or toxicological activity and also aid the construction of structure–activity relationships at an earlier stage in the drug discovery process.

Currently, however, rapid screening of large numbers of micro-organisms for models of mammalian drug metabolism can be limited by conventional analytical technologies which need time-consuming sample pre-treatment. Optimising fermentations for a specific biotransformation route, for instance using 96- or 384-well plate technology, relies on a rapid and sensitive assay that is selective only for substrate-related products. This can be achieved using ^{14}C-labeled substrate but requires TLC or HPLC to separate the resulting structural analogues.[1,3–6] Moreover, radiolabel-containing drug candidates are generally not available in the early drug discovery process. Chromatographic methods can be limited by co-elution of metabolites that are close analogues of the substrate. Similarly, preparative HPLC is limited by large volumes of organic solvents and requires off-line UV/MS and NMR spectroscopic studies to identify the metabolites.[6–8] Commonly, these methods require lengthy method development, with the risk of degrading reactive analytes.

^{19}F NMR spectroscopy is an attractive technique for monitoring metabolic fate in drug metabolism and agrochem-

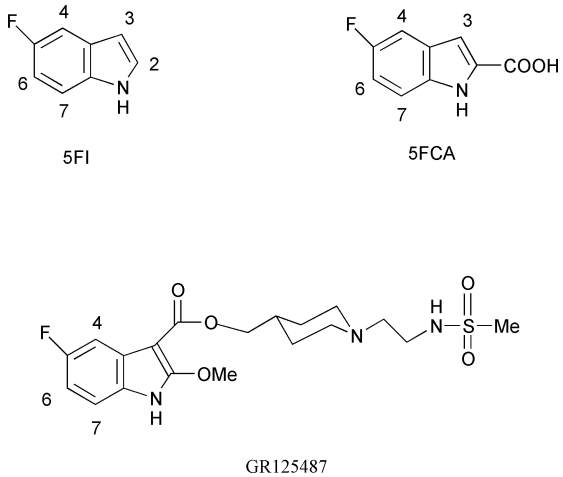

Fig. 1 Structures and numbering system for 5-fluoroindole (5FI), 5-fluoroindole-2-carboxylic acid (5FCA) and GR125487.

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ical studies,[9,10] as it requires minimal sample preparation, is relatively sensitive (detection limit, *ca.* < 50 ng ml^{-1} at 9.4 T), non-destructive and exquisitely selective for substrate-related material. Indeed the strategic use of fluorine in drug design programmes as a tool for directing metabolism on aromatic molecules, is possibly reflected in the escalating number of drugs containing one or more fluorine atoms. Here we have applied ^{19}F NMR spectroscopy to screening the microbial biotransformations of three model fluorine-containing drugs typical of those in a drug discovery strategy, for which there are no published biotransformation data.

Experimental

Chemicals

5-fluoroindole (5FI) and 5-fluoroindole-2-carboxylic acid (5FCA) were obtained from SigmaAldrich, Dorset, UK. GR125487 (1-{2[(methylsulfonyl)amino]ethyl}piperidin-4-yl) methyl 5-fluoro-2-methoxy-1H-indole-3-carboxylate) was supplied by GlaxoWellcome R&D, Stevenage, UK. Structures are shown in Fig. 1.

Microbiological experiments

48 micro-organisms were screened including: *Actinoplanes sp.* (ATCC 53771), *Amycolatopsis orientalis* (NRRL 2452), *Streptomyces mashuensis* (ISP 5221), *Streptomyces platensis ss. malvinus* (NRRL 3761), *Streptomyces rimosus* (NRRL 2234 and 2455), *Streptomyces aureofaciens* (ATCC 10762), *Streptomyces rimosus* (NRRL 2234), *Streptomyces punipalus* (NRRL 3529), *Streptomyces griseus* (ATCC 13273), *Rhizopus arrhizus* (ATCC 11145), *Mucor parasiticus* (ATCC 6476). Two strains namely *Actinomyces sp.* (3986E) and *Streptomyces fradiae* (1218C) were obtained from the GlaxoWellcome Culture Collection. The remainder of the strains used were 'in-house' isolates identified to genus level as *Streptomyces sp*, again obtained from the GW Corporate Culture Collection. Micro-organisms had previously been cultured on agar plates and transferred to polypropylene beads (Microbank™, Prolab Incorporated, Ontario, Canada) and stored at −80 °C until required. Bacteria and fungi were cultured as described

Table 1 Summary of the quantitative results for turnover of three model fluorodrugs in a library of 48 microbial species.

Micro-organism identifier	Taxonomic information[b]	Turnover[c]		
		5FI	5FCA	GR125487
SB1[a]	Bacterial culture medium (no micro-organism present)	−	−	−
B35	*	+++	−	−
B44	*	−	−	−
B51	*	+++	−	−
S1	*	+++	−	−
S4	*Actinomyces sp.* (3986E)	+++	+++	+++
S5	*Amycolatopsis orientalis* (NRRL 2452)	+++	+++	−
S7	*	+++	−	−
S10	*	+++	−	−
S11	*Streptomyces fradiae* (1218C)	+++	+++	+++
S13	*Streptomyces mashuensis* (ISP 5221)	+++	−	+++
S14	*Streptomyces platensis ss. malvinus* (NRRL 3761)	+++	−	−
S15	*Streptomyces rimosus* (NRRL 2455)	+	+++	−
S16	*	+++	−	−
S17	*Actinoplanes sp.* (ATCC 53771)	+++	+++	+++
S19	*Streptomyces aureofaciens* (ATCC 10762)	+++	+++	+++
S20	*Streptomyces sp.* (3992E)	+++	−	−
S21	*	−	−	−
S22	*Streptomyces rimosus* (NRRL 2234)	+++	+++	−
S23	*	+	−	−
S24	*Streptomyces punipalus* (NRRL 3529)	+++	−	−
S25	*Streptomyces griseus* (ATCC 13273)	+++	+++	−
S26	*	−	−	−
S28	*Streptomyces sp.* (4789E)	+++	−	−
FB1[a]	Fungal culture medium (no micro-organism present)	−	−	−
F1	*	−	−	−
F7	*	−	−	−
F14	*	−	−	−
F19	*	−	−	−
F24	*	−	−	−
F29	*Rhizopus arrhizus* (ATCC 11145)	−	+	−
F31	*Mucor parasiticus* (ATCC 6476)	+	+	−
F39	*	−	−	−
F41	*	−	−	−
F42	*	−	−	−
F44	*	−	−	−
F50	*	−	−	−
F51	*	−	−	−
F57	*	−	−	−
F62	*	−	−	−
F63	*	−	−	−
F73	*	−	−	−
F74	*	−	−	−

[a] SB1 and FB1 denote incubations of the nutrient media with parent drug,*i.e.* no micro-organism present.[b] Where no external culture number is provided, GW 'in-house' isolates are indicated.[c] Turnover is indicated as follows: (−) = undetectable, (+) = < 10% and (+++) = > 10% turnover of fluorinated substrate.

previously.[6] On day 1, single beads containing the individual micro-organisms were innoculated aseptically into 2 ml of the appropriate medium in 24-well plates. Individual plates contained a control and a positive well for each of 12 micro-organisms. Control wells contained cultures that were not fed substrate, in order to provide pre-dose and post-dose samples for each micro-organism. Four plates were thus required for each substrate, resulting in a total of 288 samples for ^{19}F NMR spectroscopic analysis. On day 4 the substrates, dissolved in dimethyl sulfoxide (5 mg ml^{-1} stock solutions), were added to each positive well to give a final concentration of 250 μg ml^{-1}, equivalent to *ca.* 1 mM substrate concentration. All cultures were then reincubated for a further 7 days. Experiments were terminated on day 10, by adding an equal volume (2 ml) of acetonitrile–methanol (4:1) to each well and mixing the contents with a Gilson 2 ml pipette. The contents were transferred to 2 ml Eppendorf™ tubes and centrifuged at 10000 rpm for 5 min to produce a supernatant which was stored at −20 °C until analysis by ^{19}F NMR spectroscopy.

^{19}F NMR spectroscopy

For ^{19}F NMR analysis, 500 μl aliquots of supernatants were added to 100 μl D_2O in 5 mm NMR tubes. Proton-decoupled fluorine NMR spectra (^{19}F{^{1}H} NMR) were obtained using composite pulse ^{1}H decoupling on a Bruker AMX-400 NMR spectrometer operating at 9.4 T (376.50 MHz ^{19}F observation frequency). The spectrometer was equipped with a dedicated 5mm ^{19}F-^{1}H probe and an automatic sample changer. Spectra were measured using 45° pulses with a 20000 Hz spectral width and each spectrum comprised 512 free induction decays (FIDs) collected into 32K data points with an acquisition time of 0.72 s and a relaxation delay of 2.5 s between pulses to minimise differential relaxation effects for different ^{19}F signals. The temperature was maintained at 303 K. An exponential apodization function was applied to the FID corresponding to a line broadening of 3 Hz. ^{19}F NMR chemical shifts were then referenced internally to the substrates (confirmed by spiking experiments) which were in turn externally referenced to trifluoroethanol at δ −77.02 (at which $CFCl_3$ = δ 0.00). The quantitative turnover of substrate by each micro-organism was then expressed in terms of a percentage of the total area under the observed fluorine signals in each spectrum.

Results and discussion

Although ^{19}F NMR only applies to screening fluorinated molecules, many pharmaceutical drug design optimisation schemes use –F and –CF_3 substituents as common variants, and so this specific spectroscopic technique can often be used to advantage for screening of complex microbial matrices. Previously, investigations of microbial models of mammalian drug metabolism have used radiolabelled substrate or clinical drugs for which metabolite standards are available, in order to identify organisms capable of a wide range of biotransformations.[3,5,7] However, at the drug discovery stage it is not possible to justify radiolabel synthesis and also metabolite standards are not readily available. Typical ^{1}H NMR spectra of whole cell cultures are biochemically complex due to the presence of resonances from residual nutrients and components endogenous to the microbial cell.[11] Although the endogenous biochemical complement could be investigated for evidence of drug safety issues, the quantitation of xenobiotic biotransformation is complicated. The use of ^{19}F NMR as a generic tool for the rapid quantitation of fluorinated biotransformation products was therefore investigated.

The results of the screen are summarised by quantitative turnover, determined by ^{19}F NMR, as described in Table 1. A wide diversity of products was observed according to the species used: some organisms produced several biotransformation products whereas others selectively produced only one or two major metabolites. Many of the fungal species produced no turnover. Of the three substrates, 5FI was extensively transformed to 10 products by almost all the bacterial species, whereas 5FCA was the most stable, with only 2 products in several species. GR125487 gave 5 products in most of the species. Only four micro-organisms were capable of efficiently biotransforming all three model drugs: *Actinomyces sp.* (3986E), *Streptomyces fradiae* (1218C), *Actinoplanes sp.* (ATCC 53771) and *Streptomyces aureofaciens* (ATCC 10762). The fungal species did not appear to biotransform the substrates under the conditions used here.

Typical ^{19}F{^{1}H}NMR spectra for extracts of the microbial cultures are shown in Fig. 2. The absence of resonances in the spectrum for *Streptomyces sp.* (3992E) prior to adding the 5FI substrate, indicates the lack of fluorine-containing molecules present in the microbial cellular matrix, Fig. 2a. After dosing 5FI to this species, the corresponding spectrum shows complete biotransformation to five metabolites M1–M5 (Fig. 2b) since no fluorine signal corresponding to the parent compound can be detected. The absence of substrate was confirmed by a spiking experiment. By contrast, a different pattern occurs on dosing 5FCA to *Streptomyces fradiae* (1218C), with 60% conversion to a single major metabolite M1 at δ −123.5, Fig. 2c. Finally, the spectrum for an extract of *Streptomyces aureofaciens* (ATCC 10762) after dosing GR125487 (Fig. 2d) shows conversion of the substrate to M1 (32%) and M2 (21%). In all three cases, samples from different species were then pooled to confirm common metabolites.

The limit of detection for fluorinated metabolites in ^{19}F NMR spectra acquired using 512 summed FIDs, was estimated as 10

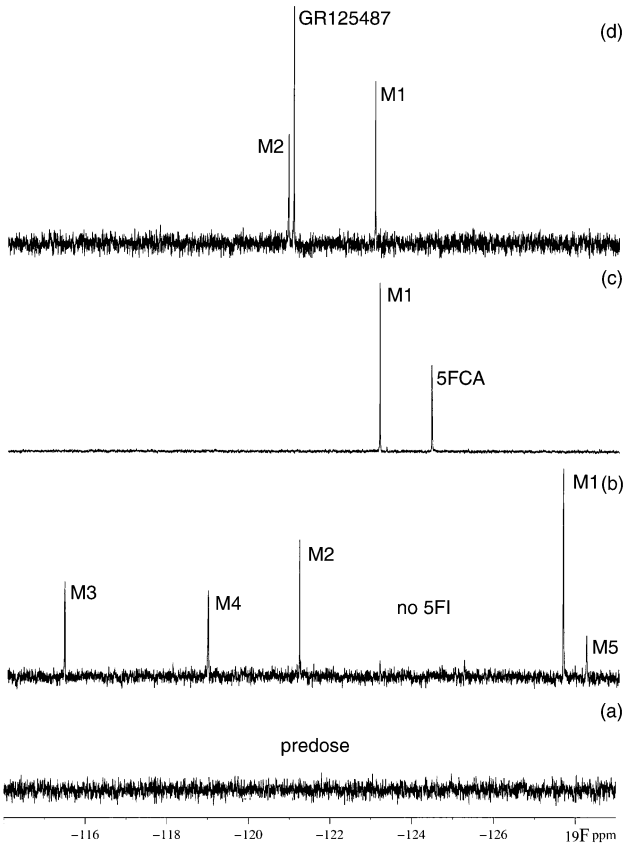

Fig. 2 Representative 376 MHz ^{19}F{^{1}H} NMR spectra of culture extracts obtained for the three model fluoroindole substrates, demonstrating a range of biotransformation products. (a) Spectrum from *Streptomyces sp.* (3992E) before administration of 5FI substrate. (b) Spectrum from *Streptomyces sp.* (3992E) extract after administration of 5FI. (c) Spectrum from *Streptomyces fradiae* (1218C) after administration of 5FCA. (d) Spectrum of *Streptomyces aureofaciens* (ATCC 10762) after administration of GR125487. *Metabolites are assigned as M1–MX in order of decreasing concentration.*

times the standard deviation of the peak-to-peak noise ratio, as this is a good estimate when the noise is assumed to be normally distributed. Therefore, the detection limit for parent drug material at 376 MHz by $^{19}F\{^{1}H\}$ NMR was estimated to be *ca.* 200 ng in 0.6 ml of extract. This is equivalent to a resonance representing *ca.* 0.6 % biotransformation of the parent drug. However, since this is at the level of the ^{13}C satellites of the parent resonance, this must be taken into account when assigning minor signals in the region of the parent signal. As the aim of this preliminary study was to identify micro-organisms capable of biotransforming $>10\%$ of substrate, the detection limits as defined here were sufficient. It is envisaged that in future studies using flow injection NMR technology, therefore, less substrate would be required and a larger set of organisms could be screened. This is particularly important with respect to the small quantities of material produced by combinatorial chemistry that must be made available for pharmacological and toxicological testing.

Metabolic defluorination was not observed for any of the substrates as evidenced by the lack of a characteristic ^{19}F NMR signal for the fluoride ion at *ca.* $\delta -120$. A further advantage of ^{19}F NMR over ^{1}H NMR is the wide chemical shift range that can result in detection of chemical modification up to 8 bonds from the fluorine atom.[12] Clearly, the higher dispersion of signals in the ^{19}F NMR spectra, coupled with the presence of one fluorine signal per metabolite greatly simplifies the quantitation of substrate turnover. For trifluoromethyl groups, commonly found in drugs, there is the added benefit of increased sensitivity. With regards to the microbial experiments, there is great scope for optimising future fermentations towards specific products using ^{19}F NMR as a generic screening tool.

In conclusion, it has been shown for the first time that high resolution ^{19}F NMR spectroscopy enables the simultaneous detection and quantitation of fluorinated substrates and their biotransformation products directly in complex culture supernatants. Novel and unexpected metabolites can be detected because no fractionation, extraction or other sample treatment procedures are necessary prior to the NMR analysis. The structural identification using HPLC-MS-NMR, of the many biotransformation products profiled here is underway. For fluorinated xenobiotics such as candidate drugs and agrochemicals, the combination of ^{19}F NMR with fully automated flow injection NMR technology[13] and directly coupled HPLC-MS-NMR[14] could be of value in the development of small-scale screens for investigating microbial models of mammalian drug metabolism.

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