Non-invasive *in Vivo* Assessment of 11 β -hydroxysteroid Dehydrogenase type 1 Activity by 19F-magnetic Resonance Spectroscopy

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Abstract

11 β -Hydroxysteroid dehydrogenase type 1 (11 β -HSD1) amplifies tissue glucocorticoid levels and is a pharmaceutical target in diabetes and cognitive decline. Clinical translation of inhibitors is hampered by lack of in vivo pharmacodynamic biomarkers. Our goal was to monitor substrates and products of 11 β -HSD1 non-invasively in liver via ¹⁹F magnetic resonance spectroscopy (¹⁹F-MRS). Interconversion of mono/poly-fluorinated substrate/product pairs was studied in Wistar rats (male, n=6) and healthy men (n=3) using 7 T and 3 T MRI scanners, respectively. Here we show that the limit of detection, as absolute fluorine content, was 0.625 μ mol in blood. Mono-fluorinated steroids, dexamethasone and 11-dehydrodexamethasone, were detected in phantoms but not in vivo in human liver following oral dosing. A non-steroidal polyfluorinated tracer, 2-(phenylsulfonyl)-1-(4-(trifluoromethyl)phenyl)ethanone and its metabolic product were detected in vivo in rat liver after oral administration of the keto-substrate, giving a readout of reductase activity. Administration of a selective 11 β -HSD1 inhibitor in vivo in rats altered total liver ¹⁹F-MRS signal. We conclude that there is insufficient sensitivity to measure mono-fluorinated tracers in vivo in man with current dosage regimens and clinical scanners. However use of a poly-fluorinated tracer allowed detection of hepatic reductase activity in rats and could be developed for translation to man.

Introduction

Excessive activity of glucocorticoids results in many adverse effects including obesity, hyperglycaemia and cognitive impairment. The hypothalamic-pituitary-adrenal axis regulates glucocorticoid levels in the circulation but an additional level of control exists within tissues through local metabolism. 11 β -Hydroxysteroid dehydrogenase type 1 (11 β -HSD1) catalyses the conversion of inactive 11-ketosteroids into active 11-hydroxyglucocorticoids (**Figure 1a**). This enzyme is expressed widely, including in liver, brain and adipose tissue ¹. In obesity, tissue-specific dysregulation of 11 β -HSD1 results in increased enzyme activity in adipose tissue, with elevated local glucocorticoid concentrations and hence metabolic dysfunction ². With ageing, higher hippocampal 11 β -HSD1 activity associates with more rapid cognitive decline in mice ³. Deletion or inhibition of 11 β -HSD1 in mice lowers tissue glucocorticoid levels and protects against weight gain, hyperglycaemia ^{4,5} and cognitive impairment ^{6,7}. In humans, a non-selective 11 β -HSD inhibitor, carbenoxolone, improved metabolic and cognitive indices ^{8,9}, and selective inhibitors have progressed to Phase II clinical trials ^{10,11}. However, efficacy has been inconsistent and most drug development programmes have stalled. One reason for variable responses in patients is that adequate enzyme inhibition may not have been achieved in key target tissues. Many chemical series of selective 11 β -HSD1 inhibitors exhibit species-specificity ¹² so the pharmacodynamics for clinical candidates cannot be reliably evaluated in pre-clinical models.

To date, whole-body 11 β -HSD1 inhibition has been measured in humans by urinary cortisol metabolite ratios ^{11,13} or during stable isotope labelled, tracer infusion ¹⁴. Inhibition in liver has been assessed indirectly by generation of plasma cortisol or prednisolone respectively after oral cortisone or prednisone

administration¹³. Enzyme activity in subcutaneous adipose tissue can be assessed *ex vivo* in biopsies² and *in vivo* using microdialysis⁹ and glucocorticoid regeneration has been quantified in hepatic and adipose tissue specifically by tracer infusion combined with selective venous sampling¹⁵ and *ex vivo* by mass spectrometry imaging¹⁶. However, none of these tools assess nascent enzyme activity *in vivo* at a tissue level, and those allowing serial tissue-specific measurements (d₄-cortisol infusion, microdialysis) are invasive and disturb the endogenous equilibrium. There is a need for pharmacodynamic tools to assess 11 β -HSD1 non-invasively *in vivo* in humans, including in target tissues such as liver and brain.

Magnetic resonance spectroscopy (MRS) identifies molecules according to the resonance of specific nuclei, which is dependent upon their molecular and chemical environment. In combination with magnetic resonance imaging, MRS can quantify specific compounds in designated regions of interest within organs *in vivo*. The most common nuclei detected are protons (¹H) and ¹H-MRS has been used to detect abundant endogenous molecules^{17,18}. The specificity of MRS is improved by measuring less common endogenous nuclei^{19,20} such as ³¹P or nuclei such as ¹⁹F, usually exogenous. ¹⁹F-MRS has been used successfully to study the distribution of fluorinated drugs²¹⁻²³ *in vivo* in humans particularly in the oncology and psychiatry fields. Interest has also been paid to the potential of ¹⁹F-MRS to monitor metabolism of fluorinated drugs due to the change in chemical shift of ¹⁹F²⁴⁻²⁸ wrought by the biotransformation. ¹⁹F-MRS has been reported recently for detection of fluorinated steroids *ex vivo* in urine extracts²⁹, and here we assessed the potential of ¹⁹F-MRS for non-invasive *in vivo* measurement of 11 β -HSD1 activity within liver, by differentiating fluorinated keto substrates and hydroxy products. Emphasis was placed on developing a protocol that minimised scanning time and tracer doses for pharmacodynamic use. The starting point was dexamethasone, a 9 α -fluorinated steroid, licensed for human use, which is regenerated from 11-dehydrodexamethasone by 11 β -HSD1 (**Figure 1b**)^{30,31}. To enhance sensitivity, potential poly-fluorinated substrates were screened, and one (**Figure 1c**) progressed to proof-of-principle pre-clinical studies *in vivo*.

Results

Evaluation of monofluorinated tracers

In vitro evaluation of ¹⁹F-MRS of monofluorinated 11-dehydrodexamethasone and dexamethasone

The anticipated ability to discriminate 11-dehydrodexamethasone (substrate) from dexamethasone (product) and the scanner sensitivity was confirmed *in vitro*. Conventional NMR spectroscopy (250 MHz ¹H spectrometer) showed different ¹⁹F resonance frequencies ($\Delta\delta_F$ 2.2 ppm) for the 9-fluorine atom according to whether the carbon in the 11-position oxidation state was determined by a ketone or hydroxy functionality (Supplementary Figure S1a).

Using a 7 T small animal MR scanner, 11-dehydrodexamethasone and dexamethasone were easily distinguished in organic solution ($\Delta\delta_F > 2$ ppm), closely matching the separation measured on the NMR spectrometer. Scanning conditions were optimised across a range of concentrations of 11-dehydrodexamethasone in chloroform. The shortest measured Repetition time (T_R) that did not affect signal intensity was 0.5 s and linearity of the concentration/signal intensity relationship was maintained between 50 and 1000 μM with scanning times of at least 100 s (with signal-to-noise (S/N) above 5 at the lowest point), (Supporting Figure S2). The limit of detection as moles of fluorine (LOD_F) was 0.250 μmol (98 μg of 11-dehydrodexamethasone) in chloroform. ^{19}F -MRS signal intensities and indices of linearity of response of dexamethasone were the same as for 11-dehydrodexamethasone (Figure 2a). Signal line width was between 68 and 72 Hz across all concentrations. Increasing scanning times did not change signal areas but did improve sensitivity by substantial gains in S/N, so an interval of at least 400 s per scan was selected as a suitable duration for further *in vivo* pharmacodynamic measurements.

Whole blood was used as a biological matrix to mimic *in vivo* scanning with the 7 T pre-clinical animal scanner. Optimal measured T_R was 1.0 s. 11-Dehydrodexamethasone and dexamethasone were scanned together at a 2:1 ratio. Both steroids were easily detected with peak maxima ≈ 2.2 ppm apart but signals were broader (Figure 2b, dexamethasone 245 Hz, 11-dehydrodexamethasone 189 Hz) than in chloroform, which caused some peak overlapping. However, individual peak areas could still be calculated reliably using jMRUI processing (measured ratio 1.9:1). The LOD_F measured in blood was 0.625 μmol (250 μg of 11-dehydrodexamethasone).

***In vivo* ^{19}F -MRS of 11-dehydrodexamethasone/dexamethasone in humans**

Next the detection of dexamethasone and 11-dehydrodexamethasone signals was evaluated on the a 3T clinical human scanner, requiring T_R of 1.5 s with 640 repetitions (16 minute scan time) and providing a S/N of 2.5 and 5.2 with 2 and 10 mg of dexamethasone respectively. This indicated that LOD_F on the 3 T scanner were hindered by at least one order of magnitude compared to measurements on at 7 T.

Discrimination of dexamethasone and 11-dehydrodexamethasone ^{19}F -MRS signal was also poorer on the 3 T clinical scanner (Figure 2c), compared to the data obtained with the 7 T preclinical scanner.

Three healthy men were subsequently studied after oral administration of increasing doses of dexamethasone. The 10 minute scans used fewer repetitions than the *in vitro* validation experiments (400 vs 640), aiming to achieve a more detailed pharmacokinetic profile of dexamethasone. A clear ^{19}F -MRS signal was detected for dexamethasone in the phantom used to validate the scanning set-up (Figure 2d (i)) but ^{19}F -MRS signals for dexamethasone or 11-dehydrodexamethasone were not detected *in vivo* (Figure 2d (ii)). The raw Free Induction Decay (FID) data for the 6 spectra were then combined (equivalent to 1 h long acquisition spectra) to compensate for the decreased number of acquisitions in each individual spectrum, but ^{19}F -MRS signals for dexamethasone or 11-dehydrodexamethasone were still not

detected. Quantitation of dexamethasone in plasma *ex-vivo* indicated a peak in circulating concentrations of around 170 µg/L. 11-Dehydrodexamethasone was detected with peak concentrations of around 12 µg/L (≈ 0.43 and 0.03 µmol/L, respectively). It was concluded that fluorinated tracers held potential but that *in vivo* sensitivity in human using a 3 T clinical scanner with standard product MRS acquisition sequences is insufficient to detect mono-fluorinated compounds, such as the dexamethasone and 11-dehydrodexamethasone pair, within appropriate dose range. The use of polyfluorinated tracers was then investigated.

Identification of polyfluorinated substrates of 11 β -HSD1

A library of potential preclinical tracers were studied which had at least one carbonyl group that could potentially be reduced by 11 β -HSD1 and contained 3 to 12 magnetically equivalent fluorine atoms (Figure 3). The library was screened for inhibition of cortisone metabolism by human and rat 11 β -HSD1. Compounds c1a, c3a, c4a, c6a and c10a had IC₅₀s in the low µM range for human 11 β -HSD1. Of these, only c1a and c4a showed a competitive interaction with rat 11 β -HSD1 (Figure 3), indicating differential inter-species tracer suitability. Compound c1a was selected for progression to proof-of-principle ¹⁹F-MRS studies in rat, since it had the lowest IC₅₀ and was a known substrate for 11 β -HSD1 ³². Synthesis of c1a stock and its reduced (hydroxy) from c1b was carried in house (Supplementary Material).

In vitro evaluation of ¹⁹F-MRS of polyfluorinated compounds c1a and c1b

Using the 7 T preclinical scanner, compound c1a in methanol was scanned across similar a concentration (31-1000 µM) range previously validated for the monofluorinated steroidal tracer and in similar rapid conditions (400s per spectra, TR= 0.5s) with monofluorinated dexamethasone/11-dehydrodexamethasone. ¹⁹F-MRS signal intensity was consistently three-fold higher for c1a than for dexamethasone or 11-dehydrodexamethasone at similar concentrations (Figure 4a). LOD_F with S/N >5 in these conditions for c1a was 0.470 µmoles (50 µg of c1a). Linearity and LOD of the c1b signal vs concentration/total fluorine content in the sample was also matching that of c1a at the studied concentration range (Figure 4a). Chloroform was also used as diluting solvent for in-vitro experiments with identical results to methanol (not shown)

Switching to blood as matrix and maintaining the scan time at 400 s, the measured LOD was approximately four-fold higher than when in solvent. For example, for c1b LOD_F was 1.87 µmoles of equivalent fluorine (0.625 µmoles or 0.2 mg of c1b) with a S/N of 6.5. When c1a and its reduced metabolite c1b were scanned in rat blood *ex vivo*, the ¹⁹F-MRS signals observed were substantially broader than in organic solvents and the overlap between keto and hydroxy signals for c1a/c1b was

extensive (Figure 4b). The separation between signals closely matched the 0.6 ppm ¹⁹F-NMR signal difference (Supporting Figure S1b) between c1a and c1b in CDCl₃.

jMRUI AMARES processing was used for signal modelling and the accuracy of this procedure to measure changes in concentration at levels near the limit of detection (0.1 to 0.4 mg) was validated by comparing observed with expected peak area ratio from blood samples containing c1a and c1b (Table 1).

Table 1

Validation of spectral modelling of overlapping ¹⁹F-MRS peaks for c1a and c1b using jMRUI software

c1a : c1b mg : mg	¹⁹ F-MRS signal area		¹⁹ F-MRS signal ratio c1a:c1b	
	c1a	c1b	Observed	Expected
0.4 : 0	4033	—	n. a.	n.a
0.4 : 0.1	4261	1158	3.68	4
0.4 : 0.2	3628	2177	1.66	2
0.4 : 0.4	4125	4160	0.99	1

In vivo ¹⁹F-MRS of polyfluorinated compounds c1a and c1b in rat and 11β-HSD1 activity detection

Conversion of c1a into cb1 could be detected following ex vivo hepatic perfusion of c1a (figure 5a). The total ¹⁹F-MRS signal measured from the excised liver remained constant over the spectra acquisition time. A change on the c1a:c1b ratio measurable over time. jMRUI peak fitting suggested greater broadening of the F-MRS peaks in whole liver (Figure 5b) than previously seen in blood.

A stand-alone, first in-vivo, validation experiment by ¹⁹F-MRS, involving oral administration of c1a to a rat was then carried with 1600 repetitions (800 s) per scan to test sensitivity under oral tracer dosage, with encouraging results (Supporting figure S3). This was followed by replicate experiments in 6 animals (3 controls and 3 with prior dosing of an 11β-HSD1 inhibitor candidate). In all 6 animals the in vivo ¹⁹F-MRS spectra of the liver showed the two signals of c1a and c1b separated by ≈1 ppm, (Figure 6a, representative). Of note the signals were much weaker than those of isofluorane anaesthetic.

For the group dosed with the inhibitor candidate (Merck544), the ¹⁹F-MRS signal of the substrate (c1a) was lower than for the control group (p<0.04) (Figure 6b). Total ¹⁹F-MRS signal (c1a+c1b) on the inhibitor group was also lower (p<0.05), although there were no in vivo measurable or detectable inhibition effects that could be seen from c1b ¹⁹F-MRS signals.

Discussion

These data provide novel evidence that ^{19}F -MRS discriminates keto and hydroxy compounds and may be used to develop tracers to quantify individual reactions in drug metabolism, such as those related to the activity of dehydrogenase/reductase enzymes such as $11\beta\text{-HSD1}$, or indeed other steroid hydroxysteroid dehydrogenases. This provides valuable insight over and above measurements of drug and metabolite concentrations in tissues *in vivo*²⁴⁻²⁸.

Mono-fluorinated tracers were evaluated initially, using dexamethasone and 11-dehydrodexamethasone, which can be distinguished readily in standard ^{19}F NMR. Dexamethasone is licensed for human use and hence offered a rapid route to translation. As anticipated, signals in general were broader in MRS experimental settings than in NMR due matrix effects and lack of sample spinning. Nonetheless in biological matrices, blood and perfused liver examined *ex vivo*, the ^{19}F -MRS signals for dexamethasone and 11-dehydrodexamethasone could be distinguished albeit being even broader than in solvent alone. Peak broadening can be attributed to a number of factors, including association of the tracer with larger molecules in the matrix or interaction with iron, affecting $T2^*$ relaxation³⁸. This raised the limit of detection (approximately four-fold higher in blood than in organic solutions) due to a detrimental effect on signal/noise. These data also confirmed that ^{19}F -MRS signal was linearly related to the total fluorine content within the scanned volume. Importantly the individual intensity of ^{19}F -MRS signal in biological matrix (blood and liver) has the same proportionality vs concentration for by the keto and hydroxy tracer forms interconverted by catalytic action of $11\beta\text{-HSD1}$, consistent with the ^{19}F -MRS signal being proportional to the number of magnetically equivalent fluorine nuclei present in the tracer.

While detection of mono-fluorinated steroids was feasible using a 7 T preclinical scanner, on a 3 T clinical scanner, however, adequate sensitivity for detection could not be achieved *in vivo* in humans even after administration of a single high dose of dexamethasone. This is due in part to the sensitivity limits of ^{19}F -MRS and potentially could be overcome by polyfluorinated tracers should they be licensed for human use. However it is worth also noting that here, using licensed doses of dexamethasone, the circulating concentrations of dexamethasone achieved were substantially lower than the concentrations of other fluorine-containing drugs detected successfully by MRS. Indeed, although doses of dexamethasone used here were as high as might reasonably be used in healthy volunteers, they do not approach the doses of other (often poly-fluorinated) drugs used for previous ^{19}F -MRS studies (>500 mg^{22,25,28}). The limited scope to further increase dosing of monofluorinated steroids was unlikely to yield the sensitivity gains required. The detection of (and resolution between) ^{19}F -MRS signals from structurally very similar tracer and metabolite, achieved using the readily available and suitable for human fluorinated molecule dexamethasone and on a clinical scanner set up, was still a promising finding particularly in view of the low LOD measured using relatively short acquisition times.

Therefore, to address future potential, the use of poly-fluorinated non-steroidal tracers in conjunction with a more powerful 7 T pre-clinical scanner was investigated. For comparative purposes, we introduced the

use of LOD_F (limit of detection adjusted for total fluorine content, expressed in moles of fluorine) rather than the LOD (limit of detection as tracer concentration) usually reported in the literature²⁰ to account for both the number of equivalent nuclei per molecule and the total amount of fluorine within the coil volume of detection. Using feasible scanning times (<20 minutes), an LOD_F of 0.250 to 0.450 μmoles of fluorine for molecules in the library was consistently achieved in organic solutions. This is an order of magnitude improvement compared with the LOD_F of ~5 μmoles measured with the 3 T scanner and highlighted the increased challenge of sensitivity on experiments at 3 T field that is the more common field strength for clinical scanners.

The improved sensitivity gained with the polyfluorinated tracer, used on a 7 T scanner, allowed the ¹⁹F-MRS signal to be readily detected in rat liver *in vivo*, using moderate weight matched doses and achieving circulating levels much lower than the isoflurane used as anaesthetic. However, the fact that the fluorine nuclei were more distant in the polyfluorinated molecule from the keto/hydroxy group than in dexamethasone meant that the ¹⁹F-MRS signals were less shifted by the magnetic environment change and were less readily discriminated between enzyme substrate and product. Accordingly, spectral modelling analysis was used to measure ¹⁹F-MRS peaks from the substrate and product of 11β-HSD1. In blood, the modelling procedure for signals of similar magnitude was accurate at near LOD, with an observed signal variation between scans of ≈7%. The procedure was similarly useful in monitoring activity in excised liver at mid-high tracer content. However, increased broadening of peaks and greater difference in signal intensities may render it less accurate for *in vivo* data acquisition when using very low tracer dosages or when looking for small differences; Thus precision would need assessed in workflows designed to measure pharmacodynamics for the 11βHSD1 target for which enzyme inhibition of only 50% (as that of Merck 544) may be sufficient to elicit the desired efficacy⁶. Nonetheless we did detect both tracer and metabolite in all animals dosed with tracer **c1a**, proving that ¹⁹F-MRS can be used to measure signals from closely related tracer/metabolite pairs originated by oxidoreductase activity.

A single dose of the 11β-HSD1 inhibitor Merck544, (an established inhibitor of the rat enzyme *in vitro*¹²) was administered which altered the ¹⁹F-MRS signals *in vivo* in rats, by unexpectedly reducing the overall abundance of **c1a** in the liver, whereas a difference in the amount of metabolite, **c1b**, formed was not observed. This paradox could be a result of a drug-drug interaction affecting the bioavailability or clearance of **c1a** and provides insight into the pharmacodynamics of this specific drug. Demonstration of the ability to detect tracer and metabolite in the specific tissue of interest by ¹⁹F-MRS highlights the value of having *in vivo* measurements when extrapolating candidate inhibitors from the *in vitro* setting and the potential of the approach. There are significant advantages of knowing tissue specific pharmacodynamics *in vivo* versus solely whole organism exposure studies by autoradiography.

This work provides a basis for future development of *in vivo* tracers, in particular defining the likely LOD_F and substrate/product peak separation required, but was limited in the number of potential fluorinated enzyme substrates available. The number of human subjects tested was small but sufficient to establish that increases in sensitivity were required for translation to humans. Preparation of polyfluorinated

derivatives of keto-steroids (e.g. based on cortisone or 11-dehydrocorticosterone) may offer a potential route ahead, but these syntheses are challenging. To be useful, and for the signal to be additive, the synthesis must yield steroids containing multiple magnetically equivalent fluorine atoms. Non-steroidal compounds are more likely to provide a suitable skeleton for polyfluorinated tracers. Indeed some further members of the library investigated here have more than 3 magnetically equivalent fluorines, and some had greater separation of ^{19}F -MRS signal between keto and hydroxy forms (unpublished data) but these were not substrates for 11 β -HSD1. Thus a more extensive screen for such compounds, or modification of the lead compounds identified here, may provide suitable tools in future.

Scanning with phased-array coils that cover a larger sample volume in the organs of larger animals may result in enhanced sensitivity compared with rodents. As higher field strength MR is introduced into medical and large animal practice, even tri-fluorinated compounds may become suitable for tracer use³⁹. Indeed commercial MRI systems of up to 21 T are available and 9.4 T magnets are now increasingly used for preclinical scanning⁴⁰. However unless sensitivity can be enhanced to the point of measuring mono-fluorinated steroids, a hurdle remains in the expense of toxicology studies required for use in humans that may limit the utility of ^{19}F -MRS tracer studies to the pre-clinical arena.

^{19}F MRS offers an approach to monitor metabolism of steroids by 11 β -HSD1. Single reaction oxidoreductase metabolic steps could thus be monitored in a tissue specific manner, but the approach is currently limited by sensitivity. Advances in MRS technology, such as increased magnetic field strength, or the availability of polyfluorinated enzyme substrates may open doors for *in vivo* pharmacokinetic/pharmacodynamic studies and investigation of pathology and improves understanding during *in vitro* to *in vivo* translation.

Methods

Fluorinated Tracers and Chemicals

11-Dehydrodexamethasone was from Steraloids (Newport, Rhode Island, USA) and dexamethasone for pre-clinical studies from Alfa-Aesar (Heysham, UK). Dexamethasone tablets for clinical use were from Essential Generics (Egham, UK). 2-(Phenylsulfonyl)-1-(4-(trifluoromethyl)phenyl)ethanone (Figure 1c, compound c1a) was originally provided by Wyeth (now Pfizer, Sandwich, UK); subsequent batches were synthesised in-house as previously described³² (Supplementary information). All other non-steroidal polyfluorinated keto compounds (Figure 2) were from Apollo Scientific Ltd (Stockport, UK), with the exception of methyl 6-(trifluoromethyl)-nicotinoyl acetate (c6a) and 4,4'-bis(trifluoromethyl)benzophenone (c8a) which were from Marshalton Research Laboratories (King, North Carolina, USA). Corresponding hydroxyl metabolites were synthesised in house by reduction with sodium borohydride (Supporting information). The selective 11 β -HSD1 inhibitor Merck544 (3-adamantan-1yl-6,7,8,9-tetrahydro-5H-[1,2,4]triazolo[4,3-a]azepine)³³ was from Enamine Ltd (Kiev, Ukraine). All other chemicals were from Sigma-Aldrich (Poole, UK) and used without further purification

unless otherwise stated. Solvents (HPLC grade where appropriate) were from Sigma-Aldrich, Fisher Scientific (Loughborough, UK) or Rathburn (Walkerburn, UK).

Library of Polyfluorinated substrates for 11 β -HSD1

Putative polyfluorinated non-steroidal keto tracers (Figure 3, compounds c1a to c12a) were selected by consideration of the published literature^{34,35} and structural similarity with the library lead, compound c1a³² and screened for inhibition of cortisone reductase activity. Tracers c1a to c12a (or vehicle) were added to HEK293 cells stably transfected with either human or rat *Hsd11b1* and inhibition measured in duplicate using a scintillation proximity assay as described³⁶. Percentage inhibition was determined relative to vehicle control and IC₅₀ determined using a four parameter logistic equation.

Structural characterisation of steroidal and polyfluorinated tracers

NMR spectra (¹H, ¹³C and ¹⁹F) were acquired using an ARX250 Bruker BioSpin NMR spectrometer (Billerica, USA) and referenced to a solvent peak (CDCl₃ or d₄-methanol). (Supplementary Figure S1).

Measurement of steroidal and polyfluorinated tracer ex vivo by ¹⁹F-MRS at 7 T (pre-clinical scanner) in phantoms

A 7 T pre-clinical scanner (Agilent Technologies, Yarnton, UK) fitted with a 400 mT/m gradient set and with a 30 mm diameter round surface coil for resonance frequency (RF) transmission and signal reception was used. The scanner was tuned to the RF of ¹⁹F (280 MHz). Fluorinated keto tracers (including 11-dehydrodexamethasone and c1a) and their hydroxy metabolites were scanned in glass vials (sample volumes 1 to 20 mL) in organic solvent (methanol or chloroform) and whole blood (20:1 in 0.5 M ethylenediaminetetraacetic acid). Care was taken so all the sample volume was contained within the sphere of detection. To determine the linearity of the relationship between signal intensity and concentration and the limit of detection, samples were prepared by serial dilution in chloroform (5 mL) or whole blood, the latter spiked with tracer stock solutions (8-10 mg/mL prepared in chloroform, chloroform/dimethylsulphoxide (DMSO) 9:1 or DMSO). A range of concentrations of 11-dehydrodexamethasone in 5 mL was scanned using 50 to 3200 repetitions per spectra (25, 100, 400 and 1600 s scan time). Scans were carried out at room temperature without stirring or spinning. When using blood as matrix, samples were occasionally shaken between scans. Simple pulse-acquire scans were acquired with T_R of 0.5 s and with 50 to 3200 repetitions per spectrum, as indicated in Results.

Dexamethasone measurement in phantoms by ^{19}F -MRS at 3 T (clinical scanner)

A 3 T Siemens Verio scanner (Siemens Healthineers, Erlangen, Germany) with circularly polarized ^{19}F -tuned transmit-receive flexible surface coil (180 mm x 244 mm, Rapid Biomedical, GmbH, Rimpfing, Germany) was used for scanning phantom solutions of dexamethasone and 11-dehydrodexamethasone prepared in methanol (20 mL in glass vials). Saline bottles were used to load the coil and the phantom was placed between the loading bottles and the coil. Spectra ranging from 64 to 640 averages were used to measure LOD_F and signal-to-noise versus tracer content using pulse-acquire scans. T_R of 0.5, 1 and 1.5 s were tested and the shortest one that did not affect signal to noise used.

Dexamethasone measurement in human liver by ^{19}F -MRS at 3 T (clinical scanner)

With local ethical approval (South East Scotland Research Ethics Committee 01) and written informed consent, three male healthy volunteers (ages 24, 26 and 32 years old) were recruited by advertisement. The study was performed in accordance with relevant guidelines/regulations, and in accordance with the Declaration of Helsinki. Inclusion criteria were age 16-60 years. Exclusion criteria were known hypersensitivity to dexamethasone or contraindications to MRI scanning; glucocorticoid therapy in the previous 3 months; diabetes mellitus; body mass index $>40 \text{ kg/m}^2$; alcohol intake >28 units/week; renal, thyroid or liver dysfunction on biochemical screening; history of dyspepsia or peptic ulcer disease; history of, or current treatment for, mental illness; pregnancy or lactation. Volunteers attended in the morning. A 20G IV cannula was inserted into the right antecubital fossa for serial venous sampling for *ex-vivo* measurement of dexamethasone and 11-dehydrodexamethasone (by liquid chromatography tandem mass spectrometry (LC-MS/MS)). Dexamethasone was administered orally (volunteer 1, 10 mg; volunteer 2, 12 mg; volunteer 3, 14 mg) with the aim of detecting a signal for dexamethasone in liver and determining the lowest dexamethasone dose which could be detected.

Volunteers were then scanned in the Verio 3 T scanner as for *in vitro* phantoms. The flexible surface coil was placed directly over the liver. A 90 degree excitation $100\mu\text{s}$ block pulse was used with a vector size of 128, and spectra obtained at isocentre with a second order semi-automated shim applied before acquisition. Pulse calibration was not possible and higher flip angles were tried empirically without any obvious increase in signal. No phase cycling was used, with an acquisition bandwidth of 10000 Hz. T_R of 1500 ms and echo time (T_E) of 0.15 ms were used with 400 repetitions (scan time 10 minutes) per spectrum. Anticipating a transient rise in hepatic dexamethasone concentrations within two hours post administration, six 10 minute scans were conducted at intervals from each participant between 30 and 100 minutes post dexamethasone administration. In two of the three subjects, a vial with dexamethasone solution in methanol was placed between the coil and patient liver to confirm coil operation and ^{19}F -MRS

signal detection prior to oral dexamethasone administration. The phantom was then removed, dexamethasone administered, and *in vivo* scanning of the liver commenced.

LC-MS/MS analysis of dexamethasone

Dexamethasone and 11-dehydrodexamethasone were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS). Stock solutions of analytes and internal standard were prepared (10 µg/mL in methanol) and stored at -20°C. Standards were prepared on the day of analysis by serial dilution of stock solutions. A standard curve was prepared representing a concentration range of 0-300 ng/mL for all analytes. 4,6α,21,21-Dexamethasone (d4-dexamethasone, internal standard, 15ng; C/D/N/ Isotopes, Quebec, Canada) was added to plasma (200 µL) and standard curve samples. Calibration curves and samples were processed by solid-phase extraction (Sep-Pak® C18, 200 mg cartridges (Waters, Wilmslow, UK), conditioning with 5 mL methanol, equilibration 5 mL water followed by sample loading, washing with 5 mL water and analyte elution with 2mL methanol). Eluates were dried under nitrogen (60 °C), resuspended in water (200 µL) and then extracted with ethyl acetate (2 mL). The supernatant was dried and dissolved in mobile phase. Injection volume was 10 µL. Analysis was performed on a Waters Acquity™ UPLC with autosampler (10 °C), coupled to an AB Sciex QTRAP® 5500 mass spectrometer (Warrington, UK), and operated with Analyst Software version 1.5.1. Separation was achieved on a SunFire™ C18 column (150x4.6 mm, 5 µm; Waters) at 20 °C, with a linear gradient from 60:40 to 45:55 (acetonitrile with 0.1% formic acid (FA): water with 0.1%FA) at a flow rate of 1.5 mL/min with a total run-time of 6 minutes. Ionisation was performed in positive electrospray mode with curtain gas 20 psi, collision gas medium, source temperature 500 °C and source gas 40 psi. Mass transitions of protonated ions monitored were (Declustering Potential, DP; Collision Energy, CE; Cell Exit Potential, CXP): dexamethasone *m/z* 393→373 (DP 71; CE 11; CXP 16); 11-dehydro dexamethasone *m/z* 391→253 (DP 71; CE 27; CXP 12); d4-dexamethasone *m/z* 397→377 (DP 51; CE 11; CXP 16). Intra-assay precision and accuracy respectively of the assay were 10.1% and 13.1% for dexamethasone and 7.7% and 0.3% 11-dehydrodexamethasone in the relevant concentration range (n=6 replicates), with a lower limit of quantitation of 0.25 ng/mL, measured against a 1/x weighted calibration line.

Measurement of polyfluorinated tracer in perfused via rat liver by ¹⁹F-MRS at 7 T (pre-clinical scanner)

In an initial *ex vivo* experiment, 32.8 mg c1a was dissolved in 4 mL DMSO:PEG₄₀₀ 1:1 and diluted in Krebs buffer (1 L) to a final concentration of 100 µM before perfusion into an excised rat liver (further details below) via the portal vein at 25-30 mL/min for 30 minutes. Subsequent scanning was carried out with 800 repetition (400 s) per spectrum (11 scans acquired) with the vial containing excised liver placed inside the coil.

Measurement of polyfluorinated tracers in rat liver in vivo by ^{19}F -MRS at 7 T (pre-clinical scanner)

Male Wistar rats (Harlan Olac, Bicester, UK), weighing between 415 and 515 g were studied under license from the UK Home Office and approved by the Animal Welfare and Ethical Review Board committee, University of Edinburgh. All procedures were performed under the UK Animals (Scientific Procedures) Act, 1986 and carried out in compliance with the ARRIVE guidelines. Rats were anesthetized with 4% isoflurane (Merial Animal Health Ltd., Harlow, UK), followed by 1.2-1.4% in oxygen enriched air for maintenance, and placed in an MRI compatible rat holder (Rapid Biomedical GmbH, Rimpar, Germany). To minimize the scanning time and maximize the signal-to-noise ratio of the ^{19}F spectra, no localization was applied. Positioning of the animal and coil were checked by the acquisition of fast gradient echo scout images (T_R 30 ms, T_E 2 ms, FOV 50 x 50 mm, matrix 128 x 128, and slice thickness 3 mm) in 3 orthogonal orientations. The homogeneity of the magnetic field was optimized using the ^1H signal before the coil was tuned to the resonance frequency of ^{19}F (280 MHz). Untriggered ^{19}F -MRS spectra using pulse-acquire sequence were acquired with following parameters: T_R 0.5 s; 800 signal averages (400 s) per spectra unless otherwise indicated; spectral width 20000 Hz centred near the tracer signal. After scanning was completed, rats were killed by cervical dislocation while still under anesthesia.

Preliminary validation used a range of concentrations (10-20 mg/kg of c1a and 20 mg/kg of c1b with up to 800 s (1600 repetitions) per spectrum. ^{19}F -MRS experiments to assess pharmacodynamics of tracer c1a and to test the effect of 11 β -HSD1 inhibition used c1a at 15 mg/kg (administered by gavage, dissolved in olive oil:PEG₄₀₀:DMSO 95:5:5 as vehicle at 5 mg/mL). After overnight fast, rats were gavaged with inhibitor (Merck544, 5 mg/mL in vehicle) at 30 mg/kg (n=3) or vehicle (n=3, weight matched as with inhibitor) then anesthetized and positioned prone on top of the surface coil and scout images obtained as above. The animals were removed from the scanner and dosed with tracer c1a. The tracer was administered 35-40 minutes after Merck544. The animals were repositioned into the holder (taking care to avoid including stomach or gut within the coil inner detection volume), scout images re-taken and the instrument switched to ^{19}F -MRS mode. Spectra (400 s per spectrum) were acquired sequentially from 25 ($\pm$ 2) minutes until 83 ($\pm$ 2) minutes post c1a gavage.

Data analysis

7 T-MRS signal processing

^{19}F -MRS spectra were processed using jMRUI ³⁷ AMARES algorithm. For phantom work, line broadening of 20 or 50 Hz was applied as it gave more reliable signal intensity results.. For animal work, 50 Hz line broadening was applied unless stated otherwise. The offset spike was removed; when present, the highest isofluorane peak was used as a reference for signal position and isofluorane peaks were filtered

out using the HLSVD algorithm before peak fitting and signal integration. If no isofluorane was present, a 0 ppm value was given to the centre of the spectrum. Overlapping tracer/metabolite ^{19}F -MRS peaks were resolved by using soft constraints on signal position and line width. Prior knowledge parameters were based on the values obtained from ^{19}F -MRS experiments when only one of the signals was present.

Statistical Analysis

^{19}F -MRS data are given as mean $\pm$ SEM and compared by repeated-measures ANOVA, with Fisher *post-hoc* tests as appropriate, with significance accepted at $p < 0.05$.

Declarations

Competing Interests Declaration

BRW is a holder of a relevant patent for use of 11β -hydroxysteroid dehydrogenase 1 inhibitors. All other authors do not have any no competing interests to declare.

Supplementary Information is available for this paper.

Correspondence and requests for materials should be addressed to Ruth Andrew. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author Contributions:

GNG: Project administration, Methodology, Investigation, Data curation, Formal analysis, Writing - original draft, review & editing; RU: Methodology, Investigation, Data curation, Formal analysis, Writing - review & editing. MJ: Methodology, Investigation, Data curation, Formal analysis, Writing - review & editing; SS: Methodology, Investigation, Formal analysis, Data curation, Writing - review & editing; OS: Methodology, Investigation, Formal analysis, Writing - review & editing; IM: Conceptualization, Funding acquisition, Methodology, Formal analysis, Writing - review & editing; BW: Conceptualization, Funding acquisition, Project administration, Formal analysis, Writing - review & editing. RA: Conceptualization, Funding acquisition, Project administration, Formal analysis Writing - review & editing.

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Figures

Figure 1

Figure 1

Metabolism of substrates by 11 β -hydroxysteroid dehydrogenases 11 β -Hydroxysteroid dehydrogenase (11 β -HSD) 1 reduces 11-keto steroids and non-steroidal tracers. a) 11 β -HSD1 converts inactive keto glucocorticoids cortisone or 11-dehydrocorticosterone into active hydroxy glucocorticoids, cortisol or

corticosterone, respectively. 11 β -HSD2 catalyses the reverse dehydrogenase reactions. b) 11 β -HSD1 and 11 β -HSD2 also interconvert synthetic fluorinated steroids, 11-dehydrodexamethasone and dexamethasone. c) The synthetic non-steroidal fluorinated compound c1a is also a substrate for 11 β -HSD1, being converted to c1b.

Figure 2

Figure 2

Evaluation of 11-dehydrodexamethasone as a tracer for 11 β -HSD1 activity by 19F-MRS a) Linearity of 19F-MRS signal vs concentration (50 μ M to 1000 μ M) ketosteroid 11-dehydrodexamethasone (11-dhdx; triangles) and hydroxysteroid dexamethasone (dex; rhombi). b) Broadening and overlap of peaks in whole blood biological matrix (5 mL) with 2.1 mg dex plus 4.2 mg 11-dhdx scanned on the Agilent 7 T pre-clinical MR scanner with 300 repetitions and total scan time of 5 minutes. The spectrum is centred on the 11-dhdx peak, and the dex peak is 2.2 ppm apart c) Discrimination of dex and 11-dhdx is attenuated on the clinical Verio 3 T scanner (10 mg each in 20 mL methanol). d) (i) In healthy men, dex was detected in a phantom containing 10 mg dex placed within the coil next to the patient. (ii) Dex was

not detected in the liver after oral administration of dexamethasone; a representative image is shown from a volunteer 10 minutes after a 12 mg dex dose.

FIGURE 3

Compound	IC50 Rat	IC50 Human	Compound	IC50 Rat	IC50 Human
c1a	0.34 μ M	0.13 μ M	c7a	>50 μ M	>50 μ M
c2a	>50 μ M	>50 μ M	c8a	>50 μ M	>50 μ M
c3a	>50 μ M	1.8 μ M	c9a	>50 μ M	>50 μ M
c4a	1.3 μ M	0.48 μ M	c10a	>50 μ M	1.9 μ M
c5a	>50 μ M	>50 μ M	c11a	>50 μ M	>50 μ M
c6a	>50 μ M	2.3 μ M	c12a	>50 μ M	>50 μ M

Figure 3

Screening putative polyfluorinated tracers for competition with cortisone for metabolism by 11 β -HSD1

Inhibition of cortisone reductase activity by potential fluorinated keto tracers (c1a-c12a) thought likely to

be substrates for 11 β -hydroxysteroid dehydrogenase (11 β -HSD 1) was evaluated in HEK293 stably transfected with human or rat Hsd11b1. IC50 values represent the mean of two experiments.

Figure 4

Figure 4

In vitro and ex vivo evaluation of compound c1a as a tracer for 11 β -HSD1 activity by 19F-MRS a) Increased 19F-MRS signal from trifluorinated c1a (circles) vs monofluorinated 11-dehydrodexamethasone (triangles), measured in chloroform solution on the 7 T pre-clinical scanner with TR of 0.5 s, 800 repetitions and 400 s acquisition time. The 19F-MRS signal was linearly correlated with tracer amount ($r^2 > 0.99$ for both c1a and 11-dehydrodexamethasone) and signal intensity increased linearly with numbers of fluorine atoms per molecule, as demonstrated by trifluorinated c1a giving a signal threefold higher than 11-dehydrodexamethasone. b) Overlap of 19F-MRS peaks (upper panel) for c1a and c1b in blood and their resolution (upper panel) by jMRUI software using predefined peak ppm values for c1a (1) and c1b (2).

Figure5

Figure 5

Ex-vivo (perfusion) and In-vivo conversion of keto compound c1a to hydroxy metabolite c1b detected by ^{19}F -MRS scan liver in rat. (a) Ex-vivo scan of perfused liver. Signal areas vs spectrum on the serial scans of the pilot experiment, each for 400 s, beginning ~20 minutes after the end of a 30 minutes perfusion with c1a 100 μM solution. c1b was formed and accumulated rapidly during the perfusion. Generation of c1b from c1a and continued to happen on the excised liver during scanning. (b) The ^{19}F -MRS spectra reveals further broadening of peaks compared with scanning in blood (representative spectrum 1 and signals deconvolution shown), with the overlap of wide signals from tracer and metabolite seen across all timepoints.

Figure 6

Figure 6

Effects of 11β -HSD1 inhibition on c1a and c1b in rat liver in vivo a) Sequential (front to back) in vivo ^{19}F -MRS spectra in rat liver after gavage of 15 mg/kg of c1a post administration of weight matched dose of vehicle. 800 repetitions (400 s) per spectrum were used to allow fine monitoring of signal change over time. ^{19}F -MRS signal from c1a and c1b were detected, with low in intensity compared to isofluorane peaks. b) Peak areas for c1a and c1b measured by ^{19}F -MRS after oral gavage of c1a in rats pre-treated with Merck544 (MK544) selective 11β -HSD1 inhibitor (c1a black squares, c1b black triangles) or vehicle (c1a white squares, c1b white triangles). Data are mean $\pm$ SEM from $n=3$ per group. The time shown is between c1a gavage and the start of the scan for each spectrum (aligned ± 2 minutes). Scan time per spectrum was 400 s (6 minutes 40 s). There were no statistically significant differences in amount of

metabolite formed or ratio of substrate to product. However, by repeated measures ANOVA ^{19}F -MRS signal of the substrate (c1a) on Merk544 group was lower than for the control group ($p < 0.04$).

Supplementary Files

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