MOL 107821

Title page

Probe-dependent negative allosteric modulators of the

long-chain free fatty acid receptor FFA4

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1

MOL 107821

Running Title Page

Running title: FFA4 Negative Allosteric Modulators

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Manuscript Information

Text pages 35

Tables 1

Figures 9

Words in Abstract 241

Words in Introduction 706

Words in Discussion 690

References 52

2

MOL 107821

Non-standard abbreviations

aLA, α linolenic acid; BRET, bioluminescence resonance energy transfer; DMEM, Dulbecco's modified Eagle's medium; eYFP, enhanced yellow fluorescent protein; FBS, foetal bovine serum; FFA1, Free fatty acid receptor 1; FFA4, Free fatty acid receptor 4; GPCR, G protein-coupled receptor; HBSS, Hanks' balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; NAM, negative allosteric modulator, PPARγ, peroxisome proliferator activator receptor γ,

Chemical names

AH-7614, 4-methyl-*N*-9*H*-xanthen-9-yl-benzenesulfonamide; GSK137647A, 4-methoxy-*N*-(2,4,6-trimethylphenyl)-benzenesulfonamide; Merck compound A, 3-[2-chloro-5-(trifluoromethoxy)phenyl]-3-azaspiro[5.5]undecane-9-acetic acid; TUG-770, (4-[2-[2-(cyanomethyl)phenyl]ethynyl]-2-fluorobenzenepropanoic acid); TUG-891, 4-[(4-fluoro-4'-methyl[1,1'-biphenyl]-2-yl)methoxy]-benzenepropanoic acid; TUG-1197, 2-(3-(pyridin-2-yloxy)phenyl)-2,3-dihydrobenzo[*d*]isothiazole 1,1-dioxide; TUG-1387, 4-methyl-*N*-(9*H*-xanthen-9-yl)benzamide, TUG-1506, 4-methyl-*N*-(9*H*-thioxanthen-9-yl)benzenesulfonamide.

MOL 107821

Abstract

High affinity and selective antagonists that are able to block the actions of both endogenous and synthetic agonists of G protein-coupled receptors are integral to analysis of receptor function and to support suggestions of therapeutic potential. Although there is great interest in the potential of free fatty acid receptor 4 (FFA4) as a novel therapeutic target for the treatment of type II diabetes, the broad distribution pattern of this receptor suggests it may play a range of roles beyond glucose homeostasis in different cells and tissues. To date a single molecule, AH-7614, has been described as an FFA4 antagonist, however it's mechanism of antagonism remains unknown. We synthesized AH-7614 and two chemical derivatives and demonstrate these to be negative allosteric modulators (NAMs) of FFA4. Although these NAMs did inhibit FFA4 signalling induced by a range of endogenous and synthetic agonists, clear agonist probe-dependence in the nature of allosteric modulation was apparent. Although AH-7614 did not antagonize the second long chain free fatty acid receptor, free fatty acid receptor 1, the simple chemical structure of AH-7614 containing features found in many anti-cancer drugs suggests that a novel close chemical analog of AH-7614 devoid of FFA4 activity, TUG-1387, will also provide a useful control compound for future studies assessing FFA4 function. Using TUG-1387 alongside AH-7614, we show that endogenous activation of FFA4 expressed by murine C3H10T1/2 mesenchymal stem cells is required for induced differentiation of these cells towards a more mature, adipocyte-like phenotype.

MOL 107821

Introduction

Free fatty acid receptor 4 (FFA4) (Davenport et al., 2013, Milligan et al., 2017), which is also
still frequently designated GPR120 (Hirasawa et al., 2005), was identified in 2005 as a G
protein-coupled receptor (GPCR) responsive to long-chain free fatty acids (Hirasawa et al.,
2005). Since then, not least because essential and other polyunsaturated ω-3 fatty acids with
health promoting properties are among the most potent fatty acid activators of this receptor
(Hirasawa et al., 2005, Christiansen et al., 2015), there has been strong interest in better
understanding the biological functions of FFA4 and in its potential as a therapeutic target
(Liu et al., 2015, Li et al., 2016). Because reported effects of FFA4 activation include
regulation of glucose homeostasis (Oh et al., 2014, Azevedo et al., 2016), the regulation of
release of incretins and satiety-regulating hormones (Hirasawa et al., 2005), modulation of
insulin sensitivity (Oh et al., 2010, Oh et al., 2014, Azevedo et al., 2016) and potential effects
on weight gain (Ichimura et al., 2012, Mo et al., 2013, Azevedo et al., 2016), a large focus of
studies on FFA4 has centred on the possibility that agonists of this receptor might be useful
anti-diabetic agents (Zhang and Leung, 2014, Cornall et al., 2014, Moran et al., 2016).
However, it is clear that the expression pattern of FFA4, which includes high levels in the
lung, in macrophage populations, and in various cancer cell types (Houthuijzen, 2016,
Milligan et al., 2017), indicates a broader set of roles and potential for therapeutic
interventions targeting this receptor (Milligan et al., 2016). This has resulted in screening for,
and the identification of, a number of series of FFA4 agonist ligands (Milligan et al., 2015,
Milligan et al., 2017), although chemical diversity within these series has, until recently (e.g.
Sparks et al., 2014, Azevedo et al., 2016), remained rather limited and based on carboxylate-
containing ligands that resemble synthetic fatty acids. FFA4 has been shown to be a potential
regulator of adipocyte development and differentiation (Gotoh et al., 2007), potentially via

5

increasing expression of the adipogenic transcription factor, peroxisome proliferator activator receptor γ (PPARγ) (Hasan et al., 2015, Song et al., 2016). Moreover, adipocyte differentiation from bone marrow mesenchymal stem cells has also been suggested to be promoted by FFA4 (Gao et al., 2015).

A major challenge in studying all aspects of both *in vivo* and *ex vivo* regulation of cell and tissue function by FFA4 is that the endogenous fatty acid ligands that are able to activate the receptor are present ubiquitously. Furthermore, being bound extensively by plasma proteins, the concentrations and disposition of 'free' fatty acids is often challenging to define (Ulven and Christiansen, 2015), as is the extent of receptor activation *in situ* that can be

attributed to such systemic, circulating fatty acids. As such, alongside various 'knock-down' and 'knock-out' strategies (Alvarez-Curto et al., 2016), well characterized and selective blockers of FFA4 function would be highly valuable tool compounds to further unravel biological roles of FFA4. To date, a single FFA4 'antagonist' has been described. Compound 39 (Sparks et al., 2014) (now designated AH-7614 by commercial vendors) was shown to block effects of both a fatty acid and a synthetic FFA4 agonist in FFA4-transfected U2OS human osteosarcoma cells and to antagonize both FFA4-mediated insulin secretion from mouse insulinoma MIN6 cells and the secretion of GLP-1 from the human intestinal cell line NCI-H716 (Sparks et al., 2014).

Herein we examine the antagonistic properties of AH-7614, and a small number of chemical derivatives, at FFA4. This demonstrated that AH-7614 is able to inhibit FFA4-mediated signals induced by a range of distinct fatty acids and synthetic FFA4 agonist ligands across all endpoints tested. Analysis of the mechanism of antagonism indicates that these compounds are negative allosteric modulators (NAMs) of FFA4 function, which display

'probe-dependence' in the nature of their inhibitory properties depending on the specific agonists they are inhibiting. This work also identified a close chemical derivative of AH-7614 that lacks antagonistic activity at FFA4, which given the current lack of knowledge about potential off-target effects of AH-7614, will be a useful tool in defining FFA4 specific biological effects. We have demonstrated the utility of this approach in assessing the role of FFA4 in adipogenesis of murine C3H10T1/2 mesenchymal stem cells.

Materials and Methods

Materials and compounds

Unless otherwise stated, all biochemicals and reagents were from Sigma-Aldrich Co. Tissue culture reagents and buffers were from Life Technologies Inc. TUG-891 [3-(4-((4-fluoro-4'-methyl-[1,1'-biphenyl]-2-yl)methoxy)phenyl)-propanoic acid] was synthesized as described previously (Shimpukade et al., 2012). Compound 34 (TUG-1197) (2-(3-(pyridin-2-yloxy)phenyl)-2,3-dihydrobenzo[d]isothiazole 1,1-dioxide) was synthesized as described by Azevedo et al., (2016). GSK137647A (4-methoxy-*N*-(2,4,6-trimethylphenyl)-benzenesulfonamide) was synthesized according to Sparks et al., (2014). TUG-770 (4-[2-[2-(cyanomethyl)phenyl]ethynyl]-2-fluorobenzenepropanoic acid) was synthesized as described previously (Christiansen et al., 2013), Cpd A (3-[2-chloro-5-(trifluoromethoxy)phenyl]-3-azaspiro[5.5]undecane-9-acetic acid) was synthesized essentially as described by Chelliah et al., (2014). AH-7614/compound 39 (4-methyl-*N*-9*H*-xanthen-9-yl-benzenesulfonamide) was synthesized as follows: Xanthone (100 mg, 0.5 mmol) was dissolved in methanol (10 mL),

MOL 107821

sodium borohydride (96 mg, 2.6 mmol) was added portion-wise over 20 minutes and the reaction stirred at room temperature for 24 hours. The reaction was partitioned between ethyl acetate and water. The two phases were separated and the aqueous phase was extracted twice with ethyl acetate. The organic phases were combined, washed with brine, dried over anhydrous sodium sulfate, filtered and concentrated. The crude product was used directly in the next step without further purification.. The crude was used directly in the next step without further purification. To the crude mixture was added p-toluenesulfonamide (95 mg, 0.56 mmol) followed by acetic acid (5 mL). The reaction was allowed to stir for 2 hours at room temperature. The reaction was partitioned between water and ethyl acetate. The two phases were separated and the aqueous phase was extracted twice with ethyl acetate. The organic phases were combined, washed with brine, dried over anhydrous sodium sulfate, filtered and concentrated. The crude product was purified by silica gel flash chromatography (ethyl acetate/petroleum ether 1:4) to give the desired compound as a white solid (136 mg, 77% over two steps): mp 194.1-195.5 ^{o}C; ^{1}H NMR (400 MHz, CDCl$_3$) 7.83 − 7.75 (m, 2H), 7.33 (d, J = 8.0 Hz, 2H), 7.30 − 7.23 (m, 2H), 7.15 (dd, J = 7.8, 1.4 Hz, 2H), 7.08 (dd, J = 8.3, 1.0 Hz, 2H), 6.99 (td, J = 7.7, 1.2 Hz, 2H), 5.77 (d, J = 8.6 Hz, 1H), 4.91 (d, J = 8.6 Hz, 1H), 2.47 (s, 3H); ^{13}C NMR (100 MHz, CDCl$_3$): 151.3, 143.6, 138.6, 129.8, 129.5, 127.2, 123.6, 120.4, 116.7, 49.2, 21.6; HRMS (ESI) m/z: calculated C$_{20}$H$_{17}$NaO$_3$S [M+Na^{+}]: 374.0821; found: 374.0804; HPLC 99.7 %. Data agrees with that of Liu et al. (2013). TUG-1387, 4-methyl-N-(9H-xanthen-9-yl)benzamide, was synthesized as follows: 4-Methylbenzamide (205 mg, 1.5 mmol) and 9H-xanthen-9-ol (150 mg, 0.76 mmol) were suspended in acetic acid (1.5 mL) and heated to 90°C. The compounds went in to solution after 1 min and a few min later a white solid started to precipitate. The reaction was heated overnight and cooled to room temperature, filtered and washed with acetic acid (0.5 mL),

8

then washed with cold ethyl acetate (3 mL). Subsequent drying gave the product as a white solid (180 mg, 75%): mp 229.8-231.6 °C; t_R = 12.88 min (HPLC purity: 98.7%); ^{1}H NMR (400 MHz, d_6-DMSO) δ 9.36 (d, J = 8.6 Hz, 1H), 7.82 (d, J = 8.2 Hz, 1H), 7.40 (d, J = 7.6 Hz, 1H), 7.37 – 7.31 (m, 1H), 7.26 (d, J = 8.0 Hz, 1H), 7.20 – 7.11 (m, 1H), 6.55 (d, J = 8.6 Hz, 1H), 2.34 (s, 1H); ^{13}C NMR (101 MHz, d_6-DMSO) δ 165.7, 150.6, 141.3, 131.1, 130.0, 128.9, 128.8, 127.5, 123.4, 121.9, 116.1, 43.0, 20.9; ESI-HRMS calcd for $C_{21}H_{17}NnaO_2$ [M+Na^{+}] 338.1151, found 338.1163. Data agrees with that of Patrick and Dolan (1973). TUG-1506, (4-methyl-N-(9H-thioxanthen-9-yl)benzenesulfonamide), was synthesized as follows: A 250 mL flask was charged with thioxanthone (2.0 g, 9.4 mmol), THF (30 mL) and methanol (10 mL). The solution was stirred at room temperature and NaBH$_4$ (1.07 g, 28.3 mmol) was added slowly. The initially yellow reaction mixture slowly turned orange. After 45 min the solution was diluted with water and extracted three times with ethyl acetate. The combined organic phases were washed with brine, dried over sodium sulfate and concentrated to give the crude alcohol as an orange solid (2.02 g) that was used immediately in the next step: ^{1}H NMR (400 MHz, d_6-DMSO) δ 7.70 (d, J = 7.6 Hz, 2H), 7.50 (dd, J = 7.7, 0.9 Hz, 2H), 7.36 (td, J = 7.5, 1.2 Hz, 2H), 7.28 (td, J = 7.5, 1.0 Hz, 2H), 6.40 (d, J = 5.8 Hz, 1H), 5.19 (d, J = 5.7 Hz, 1H); ^{13}C NMR (101 MHz, d_6-DMSO) δ 139.0, 130.8, 126.8, 126.6, 126.4, 125.3, 69.3. Data agrees with that of Cozzi and Zoli (2008). 9H-Thioxanthen-9-ol (2.02 g, 9.4 mmol) and p-toluenesulfonamide (2.22 g, 10.4 mmol) were dissolved in acetic acid (30 mL) and stirred at room temperature overnight. The resulting precipitate was isolated by filtration and washed first with cold acetic acid and then cold petroleum ether to afford the product as a white solid (2.2 g, 64%): mp 158.7-163.9 °C; t_R = 12.94 min (HPLC purity: 99.0%); R_f =0.40 (EtOAc:PE, 1:1); ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.76 (d, J = 8.4 Hz, 1H), 7.65 (d, J = 8.3 Hz, 2H), 7.50 – 7.46 (m, 2H), 7.37 – 7.28 (m, 4H), 7.28 – 7.19 (m, 4H), 5.23 (d, J = 8.4 Hz, 1H), 2.35 (s, 3H); ^{13}C NMR (101 MHz, d_6-DMSO) δ 142.4, 138.8, 134.9,

9

MOL 107821

132.2, 129.3, 127.3, 126.8, 126.7, 126.5, 126.3, 56.5, 20.9; ESI-HRMS calcd for

$C_{20}H_{17}NNaO_2S_2$ [M+Na$^+$] 390.0593, found 390.0585. Data agrees with that of Tamura et al.,

(1977).

Cell culture

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM)

supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C and 5% (v/v) CO_2. Flp-In^{TM}

T-REx^{TM} 293 cell lines, generated to inducibly express various tagged versions of human

FFA4 (hFFA4), human FFA1 (hFFA1) or murine FFA4 (mFFA4) following treatment with

doxycycline were maintained in DMEM supplemented with 10% (v/v) FBS, 100 U/mL

penicillin, 100 μg/mL streptomycin, 200μg/mL hygromycin B. Human brain astrocytoma

1321N1 cells stably transfected with hFFA1 were grown in DMEM supplemented with 10%

(v/v) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 400 μg/mL G418. To study

adipogenesis, murine C3H10T1/2 cells (Tang et al., 2004) were grown in DMEM containing

10% (v/v) FBS and 100 U/mL penicillin, 100 μg/mL streptomycin in a 37°C incubator with

5% (v/v) CO_2 and induced to differentiate in 10 cm dishes with IID (100 nM insulin, 500 μM

3-isobutyl-1-methylxanthine (IBMX) and 10 nM dexamethasone) for 5 days in the

presence/absence of either vehicle (0.1% (v/v) DMSO), 10μM AH-7614 or 10μM TUG-

1387.

Plasmids

Plasmids encoding either the human (short isoform) or mouse FFA4 receptors with enhanced

yellow fluorescent protein (eYFP) or the related fluorescent protein mVenus fused to their C

terminal and incorporating a N-terminal FLAG epitope tag in the pcDNA5 FRT/TO

10

expression vector were generated as previously described (Hudson et al., 2013, Butcher et al., 2014, Prihandoko et al., 2016, Alvarez-Curto et al., 2016).

β-Arrestin-2 interaction assay

β-Arrestin-2 recruitment to either human or mouse isoforms of FFA4 was measured using a bioluminescence resonance energy transfer (BRET)-based approach. HEK293T cells were co-transfected with eYFP-tagged forms of each receptor in a 4:1 ratio with a β-arrestin-2

Renilla luciferase (Rluc) plasmid using polyethylenimine. Cells were then transferred into white 96-well plates at 24 h post-transfection. At 48 h post-transfection, cells were washed with Hanks' balanced salt solution (HBSS) and then incubated in fresh HBSS prior to conducting the assay. Cells were pre-incubated for 15 mins with HBSS supplemented either with vehicle (1% (v/v) DMSO), AH-7614, TUG-1506, or TUG-1387. Cells were incubated with 2.5 µM of the Rluc substrate, coelenterazine h (Nanolight Tech, Pinetop, CA) at 37°C for 10 min and then stimulated with various FFA4 agonists for a further 5 min at 37°C. BRET resulting from receptor-β-arrestin-2 interaction was then determined by measuring the ratio of luminescence at 535 and 475 nm using a Pherastar FS fitted with the BRET1 optic module (BMG Labtech, Aylesbury, UK).

Ca^{2+} mobilization

Calcium assays were carried out on either Flp-In™ T-REx™ 293 cell lines, generated to inducibly express hFFA4 upon treatment with doxycycline, or 1321N1 cells stably expressing the hFFA1 receptor. One day prior to conducting the experiment, cells were seeded at 50000 cells/well (Flp-In™ REx™ 293) or 25000 cells/well (1321N1) in poly-D-lysine coated black clear-bottom 96-well microplates. Cells were allowed to adhere for 3-4 h before the addition

11

of 100 ng/mL doxycycline to induce receptor expression in the case of Flp-In[TM] T-REx[TM] 293 cell lines. The following day, cells were incubated in culture medium containing the calcium-sensitive dye Fura2-AM (3 μM) for 45 min. Cells were then washed three times and then allowed to equilibrate for 15 min in HBSS prior to conducting the assay. Fura2 fluorescent emission was measured at 510 nm following excitation at both 340 and 380 nm during the course of the experiment using a Flexstation plate reader (Molecular Devices, Brambleside, UK). Calcium responses were then measured as the difference between 340/380 ratios before and after the addition of the relevant compounds. For antagonism, the cells were pre-incubated for 15 minutes prior to agonist addition with HBSS supplemented with either vehicle (1% (v/v) DMSO), AH-7614 or TUG-1387 (10 μM) prior to the addition of either 50 μM aLA, 500 nM TUG-891 or 13 nM TUG-770.

High content imaging quantitative internalization assay

hFFA4-mVenus Flp-In[TM] T-REx[TM] 293 cells were plated 75000 cells per well in black with clear bottom 96-well plates. Cells were allowed to adhere for 3 to 6 hours before the addition of doxycycline (100 ng/mL) to induce receptor expression. After an overnight incubation, culture medium was replaced with serum-free DMEM containing the ligand to be assessed and incubated at 37°C for 30 min before fixation with 4% paraformaldehyde. After washing with phosphate-buffered saline, cell nuclei were stained for 30 minutes with Hoechst33342.

Plates were subsequently imaged using a Cellomics Arrayscan II high content plate imager (Thermo Fisher Scientific, Paisley, UK). Images were processed to identify internalized mVenus, which was then normalized to cell number based on nuclei identified by Hoechst33342 staining, to obtain a quantitative measure of hFFA4-mVenus internalization.

HTRF-based IP1 assay

Inositol monophosphate (IP1) assays (Cisbio Bioassays, Codolet, France) were performed according to the manufacturer's instructions. In brief, a suspension of 7500 cells/well was incubated with the stated concentrations of agonist for 1h in the presence of 10 mM lithium chloride (LiCl). IP1 accumulation was subsequently measured using a Pherastar FS plate reader (BMG Labtech, Aylesbury, UK).

RNA isolation and real-time qPCR

Total RNA was isolated from C3H10T1/2 cells using an RNEasy mini kit (Qiagen, Manchester, UK). Following RNA isolation, RT-PCR was performed using Superscript III (Life Technologies, Paisley, UK) and the resultant cDNA used as a template for quantitative PCR analysis using an ABI Prism 7300 sequence detector (Applied Biosystems, Paisley, UK). Cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. FFA4, PPARγ and Runx2 expression were then defined relative to cyclophilin using the $2^{-\Delta\Delta Ct}$ method.

Western blotting

Analysis of receptor phosphorylation was performed on mFFA4-eYFP cells that were pre-treated with either 10μM AH-7614 or TUG-1387 for 30 min after which time they were treated with 10μM TUG-891 for 5 min. Cell lysates were prepared and size fractionated on 4-12% SDS-PAGE gels and transferred to nitrocellulose membranes. Nitrocellulose membranes containing resolved receptor proteins were incubated in Tris buffered saline LI-COR blocking buffer and incubated subsequently with a phospho-specific antiserum produced in-house that is able to recognize phosphorylated Thr^{347} and Ser^{350} residues of the receptor

MOL 107821

(Prihandoko et al., 2016). Membranes were air-dried and scanned using an LI-COR Odyssey CLx Imager (Cambridge, UK).

Oil Red O staining

Untreated or differentiated C3H10T1/2 cells were fixed in 10% (v/v) formalin for 60 mins at room temperature. The cells were washed in 60% (v/v) isopropanol and then incubated for 10 mins with isopropanol supplemented with 0.6% (w/v) Oil Red O. The Oil Red O solution was then removed, cells washed x4 with dH_2O and then stored in phosphate-buffered saline

at 4^oC until visualization using an Invitrogen™ EVOS™ FL Auto Imaging System (Life Technologies, Paisley, UK). After removal of all liquid the Oil Red O stain was dissolved in isopropanol and the absorbance measured at 405nm using a Pherastar FS reader (BMG Labtech, Aylesbury, UK).

Statistical analysis

Data are presented as the mean ± S.E.M of a minimum of 3 independent experiments performed in triplicate, unless otherwise stated in the respective figure legend. Data analysis was carried out using GraphPad Prism software (package v5.02), with concentration-response data fitted to three parameter sigmoidal curves. For allosteric modulation experiments, data sets were globally fit to an operational model of allosterism described previously (Hudson et al., 2014). In order to fit these data the τ value for all allosteric modulators was constrained to reflect the lack of detectable agonism seen within these compounds, which allowed for curve fits yielding estimates of $\log\alpha$, $\log\beta$, $\log K_A$ and $\log K_B$. Statistical analyses were carried out using 1-way analysis of variance (1-way ANOVA) followed by Tukey's post-hoc test.

14

Results

We synthesized and assessed the functional activity of AH-7614 (**Figure 1**),

axanthene derivative of a diarylsulfonamide-based FFA4 agonist. This compound was

originally described as an antagonist of FFA4, able to block effects of both the

polyunsaturated, ω-6 fatty acid, linoleic acid and the synthetic FFA4 agonist GSK137647A

(**Figure 1**) (Sparks et al., 2014) and has since been used in a number of functional studies

(Quesada-López et al., 2016, Houthuijzen et al., 2017, Villegas-Comonfort et al., 2017) but

without analysis of its mode of action. Indeed, AH-7614 inhibited, in a potent and

concentration-dependent manner, (pIC_{50} versus EC_{80} concentrations of the agonists) the

ability of either the ω-3 fatty acid α linolenic acid (aLA) (pIC_{50} 7.51 ± 0.08, n = 3) or a

synthetic ortho-biphenyl-based agonist of FFA4, TUG-891 (**Figure 1**) (pIC_{50} 8.13 ± 0.08, n

= 3) (Shimpukade et al., 2012, Hudson et al., 2013, Butcher et al., 2014) to promote Ca^{2+}

mobilization in Flp-In^{TM} T-REx^{TM} 293 cells induced to express hFFA4-eYFP (**Figures 2A,

2B**). Similar results were obtained when measuring the ability of AH-7614 to inhibit agonist-

induced interactions of hFFA4 with β-arrestin-2 following co-expression of these two

proteins in HEK293T cells (**Figures 2C, 2D**). Importantly for subsequent studies, AH-7614

also functionally antagonized effects of both aLA and TUG-891 at the corresponding mouse

ortholog of the receptor (mFFA4) (**Figures 2E and 2F**). AH-7614 showed selectivity for

FFA4 as it was unable to antagonize activation of the other long-chain fatty acid receptor,

FFA1 (Milligan et al., 2015, Milligan et al., 2017) in studies in which the synthetic alkyne-

based FFA1 agonist TUG-770 (Christiansen et al., 2013) promoted elevation of intracellular

Ca^{2+} in 1321N1 cells stably transfected to express a human FFA1 construct (**Figure 2G**).

15

In similar EC_{80} inhibition experiments, AH-7614 was also effective in blocking TUG-891-mediated internalization of FFA4 from the cell surface (**Figure 3A**) ($pIC_{50} = 7.70 \pm 0.10$, n = 3). We therefore used this internalization assay, with end point 30 minutes after addition of agonist to facilitate achievement of ligand equilibrium, to probe the mechanism of AH-7614-mediated blockade of FFA4. Receptor internalization experiments were conducted in which the ability of defined concentrations of pre-added AH-7614 to block internalization in response to various concentrations of TUG-891 was assessed (**Figure 3B**). In these experiments it was clear that the primary inhibitory effect of AH-7614 was to produce a

decrease in the maximal response to TUG-891. Although this is consistent with outcomes reported by Sparks et al. (2014) using intracellular Ca^{2+} assays, the longer time course of the internalization studies largely eliminates potential issues of ligand hemi-equilibrium that can greatly influence outcomes and mechanistic interpretation in assays, such as those based on

Ca^{2+} mobilization, that employ much shorter end points (Charlton and Vauquelin, 2010). These findings indicated that AH-7614 is not a competitive antagonist of FFA4. Interestingly, even at the highest concentration of AH-7614 tested a residual response to TUG-891 remained. This response to TUG-891 was similar in the presence of 1 and 10 μM AH-7614 and, as such, the effect of AH-7614 appeared to saturate. Such an outcome is consistent with AH-7614 functioning as a NAM of FFA4.

As it is common for small chemical changes in allosteric GPCR ligands to significantly alter the details of their allosteric behavior (Wood et al., 2011, Hudson et al. 2014), we next synthesized derivatives of AH-7614 and assessed their activity at FFA4. Two key derivatives were considered. In the first the sulfonamide of AH-7614 was replaced with an amide (TUG-1387) whilst in the second the xanthine was replaced with a thioxanthene

16

(TUG-1506) (**Figure 4A**). These analogs were tested for their ability to antagonize EC_{80}

concentrations of TUG-891 (**Figure 4B**) in a β-arrestin-2 recruitment assay. The

thioxanthene-containing TUG-1506 retained the ability to inhibit TUG-891 ($pIC_{50} = 6.38 \pm$

0.09, n=3) but with lower potency than the xanthene containing AH-7614. In contrast, the

amide-containing TUG-1387 lacked all ability to inhibit TUG-891 mediated activation of

FFA4.

As AH-7614, and likely also TUG-1506, appeared to be NAMs of FFA4 function we

characterized and quantified the nature and extent of their NAM properties against synthetic

FFA4 agonists from several distinct chemotypes. A common feature of GPCR allosteric

modulators is that they may show 'probe dependence', producing different allosteric effects

depending on the orthosteric agonist being modulated (May et al., 2007). Therefore, we

conducted experiments where AH-7614 (**Figure 5A-D**) and TUG-1506 (**Figure 5E-H**) were

used to antagonize β-arrestin-2 recruitment to FFA4 induced by synthetic agonists from each

of 4 distinct chemotypes: TUG-891 (Shimpukade et al., 2012), TUG-1197 (Azevedo et al.,

2016), GSK137647A (Sparks et al., 2014), and Cpd A (Oh et al., 2014) (**Figure 1**). Data

from these experiments were then fit to an operational model of allosterism in order to

quantify the allosteric effect each NAM had on affinity of each orthosteric agonist (α), the

allosteric effect on efficacy of each orthosteric agonist (β), the affinity of the orthosteric

agonists (K_A) and the affinity of the modulator (K_B) (**Table 1**). AH-7614 reduced the

maximal response to each agonist tested, and also produced a modest reduction in agonist

potency (**Figure 5A-D**). This corresponded to logα (**Table 1, Figure 5I**) and logβ (**Table 1,**

Figure 5J) values that were both less than zero for each of the four agonists. However,

although the logα values were broadly similar for each agonist, the logβ for GSK137647A

17

was significantly lower (p<0.01) than the value obtained for either TUG-891 or Cpd A,

suggesting a subtle level of 'probe dependence' in AH-1764 modulation of FFA4 activity.

This can be noted in the concentration-response curves by the observation that although high

concentrations of AH-7614 eliminated measureable response to GSK137647A (**Figure 5C**), a

residual response was apparent for all other agonists, even at 30 μM AH-7614 (**Figure 5A,

5B and 5D**). Importantly, the agonist responses were similar in the presence of between 3

and 30μM AH-7614, indicating that the inhibitory properties of AH-7614 were saturable, a

hallmark of allosteric modulators.

In examining the allosterism between TUG-1506 and the various FFA4 agonists

(**Figure 5E-H**), much more striking 'probe dependence' was apparent. Most notably, while

TUG-1506 primarily shifted the potency of TUG-891 (**Figure 5E**) and Cpd A (**Figure 5H**)

with little effect on maximal response, for TUG-1197 and GSK137647A the opposite was

true, where TUG-1506 depressed the maximal response without effecting agonist potency.

This was reflected in the operational model curve fit parameters for these experiments, where

a large negative logα was observed for TUG-891 and Cpd A (**Table 1, Figure 5K**) but

instead a large negative logβ was observed for TUG-1197 and GSK137647A (**Table 1,

Figure 5L**). This is a clear indication of the 'probe dependence' in the NAM properties of

TUG-1506. Interestingly, this may be related to the chemical nature of the agonists, with

TUG-891 and Cpd A both being carboxylate-based FFA4 agonists, while TUG-1197 and

GSK137647A are each sulfonamide based-agonists. Importantly, once again, in all cases the

inhibitory effects of TUG-1506 were saturable, entirely consistent with defining this

compound as a NAM at FFA4. Similar experiments were also conducted with TUG-1387

against each FFA4 agonist, although in this case, given that TUG-1387 had already been

18

shown to lack activity at FFA4, only a single high concentration of TUG-1387 was used (**Figure 6A- 6D**). TUG-1387 did not produce a NAM effect on TUG-891, TUG-1197 or Cpd A, and only a very modest effect on GSK137647A. Overall, these results are consistent either with TUG-1387 having no affinity for FFA4, or it being a silent allosteric modulator, that even if it does bind the receptor, does not affect orthosteric agonist function.

To further assess the potential for using AH-7614 in combination with TUG-1387 to define biological functions of FFA4, we aimed to confirm the broader ability of AH-7614, but not TUG-1387, to block additional FFA4 signalling pathways. We explored both FFA4-mediated accumulation of inositol monophosphates (**Figure 7A**) and agonist-induced phosphorylation of Thr^{347} and Ser^{350} residues within the C-terminal of FFA4- detected with previously described phosopho-FFA4 antisera (Butcher et al., 2014, Prihandoko et al., 2016) (**Figure 7B**). In each case, the agonist response was functionally inhibited by AH-7614, but was not affected by TUG-1387. Importantly for studies on more physiologically relevant systems, we also found that TUG-1387 lacked activity at the related fatty acid receptor FFA1 in experiments measuring calcium release in 1321N1-hFFA1 cells (**not shown**).

Recently it has been suggested that FFA4 is able to act as a bi-potential regulator of osteogenic and adipogenic differentiation of bone marrow-derived mesenchymal stem cells (Gao et al., 2015). Furthermore, FFA4 has been proposed to be a key player in the activation of brown adipose tissue (Quesada-Lopez et al., 2016), further reinforcing a role for FFA4 in adipose tissue physiology. To assess further the role of FFA4 in adipogenic differentiation and to demonstrate the utility of the FFA4 NAM, AH-7164, when used in combination with TUG-1387 to interrogate FFA4 biology, we employed the murine mesenchymal stem cell line C3H10T1/2 (Tang et al., 2004, Lee et al., 2016, Hashimoto et al., 2016). Here,

differentiation towards an adipocyte phenotype was produced by maintaining the cells in the

presence of an induction mixture (IID) consisting of 100 nM insulin, 500 μM IBMX and 10

nM dexamethasone for 5 days. Oil Red O staining effectively visualized the development of

triglyceride deposits associated with adipogenesis (**Figure 8A**). In concert, exposure to the

IID-containing medium produced clear upregulation of mRNA for the adipogenic

development marker, peroxisome proliferator-activator receptor γ (PPARγ) (**Figure 8B**).

This effect was not produced simply by cell confluence on the tissue culture plate (**Figure**

8B). In parallel, levels of Runt-related transcription factor 2 (Runx2) mRNA, a key

transcription factor associated with osteoblast differentiation (Xu et al., 2015, Yuan et al.,

2016) was greatly reduced (**Figure 8C**). Over this time frame levels of mFFA4 mRNA were

also markedly increased by cell maintenance in the IID medium but, interestingly, an

equivalent upregulation of mFFA4 mRNA was also produced simply by maintaining

C3H10T1/2 cells at confluence for a 5 day period (**Figure 8D**). The ability of C3H10T1/2

cells to differentiate toward the adipocyte phenotype was clearly dependent upon signalling

from FFA4 because treatment of cells with AH-7614 along with the IID medium greatly

reduced visual observation of triglyceride stores as detected by Oil Red O staining and its

quantification (**Figures 9A, 9B**). Treatment with AH-7614 also limited IID-induced PPARγ

mRNA induction (**Figure 9C**), without affecting the associated down-regulation of Runx2

expression (**Figure 9D**). In contrast, treatment of C3H10T1/2 cells with TUG-1387 during

the induction period with the IID-containing medium had no significant effect on the

development of Oil Red O staining (**Figures 9A and 9B**). Taken together, these data indicate

that TUG-1387, although structurally related, lacks activity at both FFA4 and the related

FFA1, and therefore indicates this compound will be a useful chemical control to confirm

that biological effects produced by AH-7614 and related compounds such as TUG-1506 are in fact FFA4 mediated.

Discussion

The availability of pharmacological tool compounds is central to efforts to define functions of many cellular proteins, including GPCRs, and to assess such proteins as potential therapeutic targets. However, for poorly- and recently-characterized receptors such tool compounds are often lacking, described largely in the chemical/patent literature and, therefore, often of limited availability, and/or are poorly characterized in terms of selectivity and potential off-target effects. Within the group of GPCRs that respond to non-esterified, or 'free', fatty acids, to date the most broad-reaching pharmacopoeia targets the long-chain fatty acid receptor FFA1 (Milligan et al., 2017). This reflects that FFA1 has been targeted therapeutically and in human clinical trials to assess whether activation of this receptor might provide an approach to treat type II diabetes (Watterson et al., 2014, Li et al., 2016, Mohammad, 2016, Ghislain and Poitout , 2017). Although the second GPCR that is activated by longer-chain free fatty acids, FFA4, is also frequently suggested as a potential therapeutic target for diabetes and other metabolic disorders (Li et al., 2016, Moniri, 2016, Hansen and Ulven, 2017), this lags behind studies on FFA1 and no synthetic ligands with activity at this receptor have yet entered clinical studies (Milligan et al., 2017). As a result, even tool compounds useful to assess roles of this receptor are less widely available or understood (Hansen and Ulven, 2017). Moreover, as clear consensus is based on the therapeutic potential of agonism of FFA4 (Li et al., 2016, Moniri, 2016), little effort has been expended to identify, report or characterize antagonists of this receptor, despite antagonist tool compounds being a traditional touchstone for detailed pharmacological analysis.

The only inhibitor of FFA4 function reported to date is AH-7614 (Sparks et al., 2014). Whilst Sparks et al., (2014) suggested that this compound would likely act as a competitive antagonist of FFA4, the limited data presented in their report did not actually suggest such a mode of action, which appeared to be non-competitive, at least in assays measuring regulation of intracellular Ca^{2+} levels. The studies reported here expand on this, defining AH-7614 as a NAM of FFA4 function. This conclusion is supported by several hallmarks of allosterism (May et al., 2007), including that the ability of AH-7614 to inhibit FFA4 function is saturable, and that AH-7614 displays some level of 'probe dependence'. More striking, however, in this regard is the chemical derivative of AH-7614, TUG-1506, described for the first time in these studies ,which shows clear 'probe dependence' depending on the chemical nature of the FFA4 agonist it is modulating. Specifically, this compound is a NAM primarily of ligand affinity for the two carboxylate containing agonists tested, but primarily a NAM of efficacy for the non-carboxylate, sulphonamide-based agonists. Such changes in allosteric modulation are commonly observed with small chemical changes in allosteric ligands (Wood et al., 2011, Hudson et al. 2014) and, indeed, the observed pattern of inhibition by both AH-7614 and TUG-1506 can only be explained by an allosteric mode of action.

Whilst Sparks et al., (2014) assessed more broadly the selectivity of the FFA4 agonist, GSK137647A, described in their study, they did not extend this to AH-7614. Therefore, having identified in the current work TUG-1387 as a close structural analog of AH-7614 that lacks activity at FFA4, we used each of these ligands to define specific roles for FFA4. Indeed, the FFA4 NAM AH-7614 prevents differentiation of a mouse mesenchymal stem cell line toward an adipocyte phenotype, and does so in a manner that is consistent with blockade of endogenous signalling via FFA4.

22

MOL 107821

We describe AH-7614 and TUG-1506 as the first allosteric ligands for the FFA4 receptor. Despite only a minor structural difference between these two compounds substantial differences in the nature of their allosterism were observed, perhaps suggesting that further examination of the structure activity relationship within the AH-7614 chemical series may yield novel allosteric FFA4 ligands with diverse pharmacological properties. The fact that the observed NAM properties within the AH-7614 series were clearly 'probe dependent' indicates that care must be taken when designing future studies using these ligands. Overall, with appropriate caution, AH-7614, should prove to be a valuable tool in further unravelling functions of the FFA4 receptor.

Authorship Contributions

Participated in Research Design Watterson, Hudson, Ulven, Yarwood, Milligan

Conducted Experiments: Watterson, Alvarez-Curto, Raihan, Hudson, Dunlop

Contributed New Reagents, Hansen, Azevedo, Martin, Ulven

Performed Data Analysis, Watterson, Alvarez-Curto, Hudson

Wrote the manuscript, Milligan, Watterson, Alvarez-Curto, Hudson

23

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24

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26

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MOL 107821

Footnotes

29

MOL 107821

Figure Legends

Figure 1: Chemical structures of AH-7614, and range of FFA4 agonists

The chemical structures of the FFA4 antagonist AH-7614 and a range of FFA4 agonists

(TUG-891, Cpd A, GSK137647A and TUG-1197) are shown.

Figure 2: AH-7614 is a potent and selective inhibitor of both human and murine FFA4 function

Flp-In^{TM} T-REx^{TM} 293 cells induced to express hFFA4-eYFP were pre-treated with either

vehicle (1% (v/v) DMSO) or increasing concentrations of AH-7614 for 15 mins after which

time they were treated with either 50µM aLA (**A**) or 500 nM TUG-891 (**B**). Both aLA and

TUG-891-induced calcium release that was potently inhibited in the presence of AH-7614

(aLA, pIC_{50} 7.51 ± 0.08, n = 3, TUG-891, pIC_{50} 8.13 ± 0.08, n = 3). HEK293T cells

transiently expressing hFFA4-eYFP and β-arrestin-2-Rluc were pre-incubated with either

vehicle (1% (v/v) DMSO) or increasing concentrations of AH-7614. The cells were then

treated with either 50 µM aLA (**C**) or 500 nM TUG-891 (**D**) and β-arrestin-2 recruitment was

subsequently determined using a BRET-based assay. Both aLA and TUG-891 promoted β-

arrestin-2 recruitment that was potently inhibited (aLA, pIC_{50} = 7.66 ± 0.05, n = 5, TUG-891,

pIC_{50} = 7.55 ± 0.07, n = 5) in the presence of increasing concentrations of AH-7614. mFFA4-

dependent β-arrestin-2 recruitment in the presence of either 50 µM aLA) (**E**) or 500 nM

TUG-891 (**F**) was also potently inhibited (aLA, pIC_{50} = 8.05 ± 0.08 n = 3, TUG-891, pIC_{50}

= 7.93 ± 0.06, n = 3) by AH-7614. AH-7614 had no effect however on TUG-770 induced

calcium release in 1321N1 cells stably transfected with hFFA1 receptor, indicating that AH-

30

7614 is not an antagonist at this receptor. Data represent experiments performed in triplicate three times or more.

Figure 3 AH-7614 blocks agonist-induced receptor internalization of the human FFA4 receptor

Flp-In^{TM} T-REx^{TM} 293 cells induced to express hFFA4-mVenus were pre-treated with 10μM AH-7614 for 15 min after which time cells were further treated with either vehicle (0.1% (v/v) DMSO) or TUG-891 (3 μM) for 30 min (**A**) and subsequently fixed. AH-7614 prevented TUG-891 induced receptor internalization (pIC$_{50}$ 7.70 ± 0.10). Cells were pre-treated with varying concentrations of AH-7614 and exposed to increasing concentrations of TUG-891 (**B**) for 30 min after which they were fixed. Cells were imaged using a Cellomics ArrayScan II and the extent of internalization of the receptor construct quantified.

Figure 4 TUG-1387 and TUG-1506 are chemical derivatives of AH-7614 with varying activity at FFA4

Chemical structures of AH-7614, TUG-1387 and TUG-1506 are shown in **A**. HEK293T cells transiently co-expressing hFFA4-eYFP and β-arrestin-2-Rluc were pre-incubated with vehicle (0.1% DMSO) or increasing concentrations of either AH-7614, TUG-1387 or TUG-1506. β-arrestin-2 recruitment to the receptor was then determined in the presence 500 nM TUG-891 (**B**). All data represent experiments carried out in triplicate at least three times.

Figure 5 AH-7614 and TUG-1506 are probe-dependent negative allosteric modulators of FFA4

The nature of AH-7614 (**A-D**) and TUG-1506 (**E-H**) allosteric modulation of FFA4 function was probed by their ability to limit recruitment of β-arrestin-2 to hFFA4-eYFP induced by varying concentrations of four chemically distinct FFA4 agonists: TUG-891 (**A, E**), Merck compound A (Cpd A) (**B, F**), GSK137647A (**C, G**) and TUG-1197 (**D, H**) using HEK293T cells transiently co-expressing hFFA4-eYFP and β-arrestin-2-Rluc. Data are fitted to an operational model of allosterism (Hudson et al., 2014). Estimated parameters for log-α and log-β are shown for each of AH-7614 (**I-J**) and TUG-1506 (**K-L**) when assessed at each of the 4 agonists. Data shown are from representative experiments (N numbers for each agonist-NAM combination are reported in Table 1) fit to an operational model of allosterism (Hudson et al., 2014).

Figure 6 TUG-1387 does not antagonize the effects of distinct FFA4 agonist chemotypes

HEK293T cells transiently expressing mFFA4-eYFP and β-arrestin-2-Rluc constructs were pre-treated for 30 min with vehicle, 10μM AH-7614 or 10μM TUG-1387. They were then treated with TUG-891 (**A**), Cpd A (**B**), GSK137647A (**C**) or TUG-1197 (**D**) to assess receptor-mediated arrestin recruitment. AH-7614 was able to block β-arrestin-2 recruitment in all cases whereas TUG-1387 did not have any effect except to produce a small but significant (** $p<0.001$) reduction of the efficacy of GSK137647A (**C**). Data represent experiments performed in triplicate three/four times.

MOL 107821

Figure 7 AH-7614 but not TUG-1387 blocks agonist-induced elevation of intracellular inositol monophosphates and phosphorylation of FFA4

FFA4-mediated inositol monophosphate (IP1) production was measured in cells expressing hFFA4-mVenus (**A**). TUG-891 (closed circles) promoted accumulation of IP1. Pre-treatment with either 10µM AH-7614 (inverted triangles) or increasing concentrations of this molecule (open squares) blocked IP1 accumulation. By contrast pre-treatment with TUG-1387 was without effect (open diamonds). (**B**). mFFA4-eYFP cells were pre-treated with vehicle, or various concentrations of AH-7614 or TUG-1387 for 30 min followed by treatment with 10µM TUG-891 for 5 min. Cell lysates were prepared and resolved by SDS-PAGE and immunoblotted with a mFFA4 specific phospho-antiserum that recognises phosphorylation of residues Thr^{347} and Ser^{350} (Prihandoko et al., 2016). Quantification of a series of such immunoblots is shown in the right hand panel.

Figure 8 mRNA levels of FFA4 and PPARγ are upregulated in C3H10T1/2 cells

by differentiation towards the adipocyte phenotype C3H10T1/2 cells were differentiated toward an adipocyte phenotype by treatment with IID medium (**A**). Confirmation of effective differentiation after 5 days of treatment was obtained by staining for triglyceride droplets within the cells using Oil Red O (**A**). Q-RT-PCR was performed to assess changes in expression levels of PPARγ, (**B**), Runx2, (**C**) and mFFA4 (**D**) under conditions of subconfluence, confluence, 5 days post-confluence and 5 days post-confluence in the

33

presence of IID. Data are presented as the group mean ± SEM of 3 independent experiments.

(* different at p< 0.05, ** p<0.01 and *** p < 0.001). Scale bar in A = 400μm.

Figure 9 Adipocyte differentiation of C3H10T1/2 cells requires FFA4 signalling

C3H10T1/2 cells were differentiated (IID) in the presence of either vehicle (0.1% DMSO);

10μM AH-7614 or 10 μM TUG-1387 compared to a non-differentiated control and then

stained with Oil Red O (**A**). Oil Red O staining was subsequently dissolved in isopropanol

and then quantified by measuring absorbance at 405 nm (**B)** (n = 10, data presented as mean

± SEM). Quantitative PCR analysis of PPARγ (**C**, n = 5) and Runx2 (**D**, n = 4) levels was

then performed on differentiated (IID) and non-differentiated (vehicle) C3H10T1/2 cells in

the presence or absence of 10μM AH-7614. Data are presented as mean ± SE (* different at

p< 0.05, ** p<0.01 and *** p < 0.001). Scale bar in A = 400μm.

MOL 107821

Table 1: Operational Model Curve-fit Parameters for AH-7614 and TUG-1506

AH-7614

Agonist	$\log\alpha$	$\log\beta$	pK_A	pK_B	N
TUG-891	-0.80 ± 0.11	-0.77 ± 0.09	6.77 ± 0.14	7.98 ± 0.24	4
TUG-1197	-0.52 ± 0.09	-1.08 ± 0.10	6.44 ± 0.05	7.82 ± 0.26	5
GSK137647A	-0.58 ± 0.09	-1.54 ± 0.14	6.32 ± 0.04	8.14 ± 0.21	3
Cpd A	-0.80 ± 0.14	-0.66 ± 0.15	6.31 ± 0.22	8.17 ± 0.26	3

TUG-1506

Agonist	$\log\alpha$	$\log\beta$	pK_A	pK_B	N
TUG-891	-1.68 ± 0.17	-0.18 ± 0.10	6.79 ± 0.11	7.46 ± 0.06	4
TUG-1197	-0.48 ± 0.12	-1.34 ± 0.13	6.48 ± 0.08	6.90 ± 0.04	3
GSK137647A	-0.44 ± 0.07	-1.24 ± 0.11	6.44 ± 0.03	7.04 ± 0.10	3
Cpd A	-2.15 ± 0.36	0.06 ± 0.11	6.46 ± 0.18	7.28 ± 0.02	3

Figure 1

AH-7614

TUG-891

TUG-1197

Cpd A

GSK137647A

Figure 2

37

Figure 3

A

B

Figure 4

A

AH-7614   TUG-1506   TUG-1387

B

Figure 5

Figure 6

A

B

C

D

Figure 7

Figure 8

A

No treatment   IID

B

*

*

*

PPARγ expression
(fold change)

5.0

4.0

3.0

2.0

1.0

0.0

subconfluent   confluent   5 days confluent   5 days confluent + IID

C

***

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*

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*

Runx2 expression
(fold change)

1.5

1.0

0.5

0.0

subconfluent   confluent   5 days confluent   5 days confluent + IID

D

**

**

*

*

mFFA4 expression
(fold change)

15.0

10.0

5.0

0.0

subconfluent   confluent   5 days confluent   5 days confluent + IID

Figure 9

A

B

C

D