Andrew J. Brown, Christina Tsoulou, Emma Ward, Elaine Gower, Nisha Bhudia, Forhad Chowdhury, Tony W. Dean, Nicolas Faucher, Akanksha Gangar, Simon J. Dowell. Pharmacological properties of acid *N*-thiazolylamide FFA2 agonists. Pharmacology, Research and Perspectives.

Supplementary Table 1

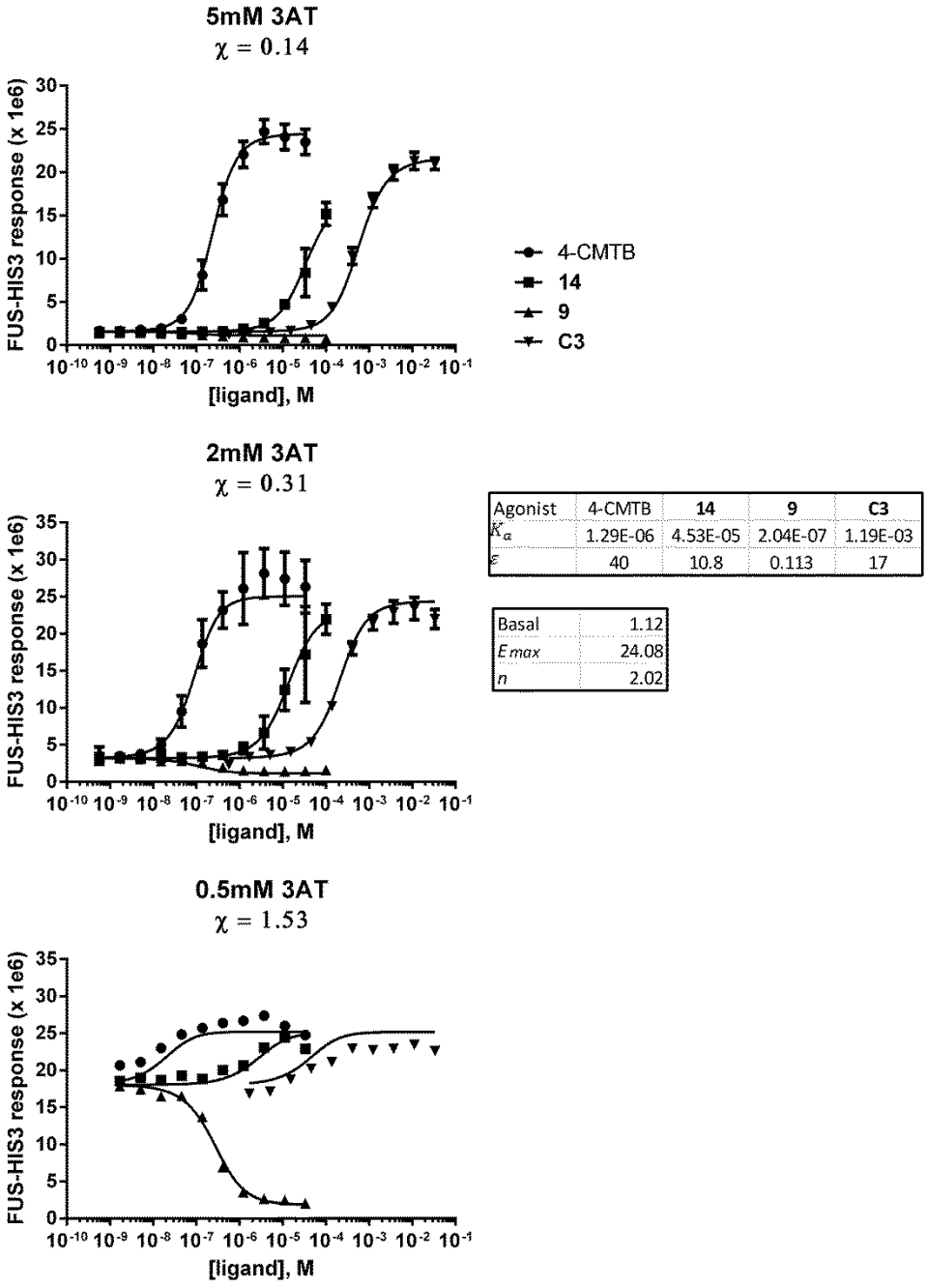
N-CBT (N-(4-Chlorobenzoyl)-L-tryptophan) is an antagonist selective for human FFA2 with no activity at rat FFA2.

Receptor	**Expression host (G_α-subunit)**	**Test Mode** (blockade of EC_{80} C3)	**N-CBT pIC50**	**Estimated pK_B**
hFFA2	Yeast (Gpa1/$G_{\alpha i1}$)	Reporter gene antagonist	5.8 ± 0.08 (n=8)	6.3±0.16 (n=8)
rFFA2	Yeast (Gpa1/$G_{\alpha i3}$)	Reporter gene antagonist	<4.6 (n=6)	
hFFA2	HEK293T ($G\alpha_{o1}$)	[^{35}S]GTPγS-binding antagonist	5.8 ± 0.24 (n=12)	6.3±0.27 (n=12)
hFFA3	HEK293T ($G\alpha_{o1}$)	[^{35}S]GTPγS-binding antagonist	<4.5 (n=10)	
hFFA3	U2OS ($G\alpha_{qi5}$)	Ca^{2+} mobilisation, antagonist (FLIPR)	<4.6 (n=6)	

N-CBT (N-(4-Chlorobenzoyl)-L-tryptophan; CAS Number: 56116-62-2) was identified by screening a large diverse collection of drug-like molecules (high-throughput screening) for compounds able to block the agonist effect of **C3** on yeast expressing hFFA2. N-CBT is chemically similar to another reported hFFA2 antagonist, CATPB (Hudson et al., 2012). N-CBT antagonises hFFA2 (Fig.4) but no antagonism of rFFA2 was detected up to the top concentration tested of 25 μM (pIC_{50}<4.6). Specificity for human over rodent FFA2 orthologues may be a feature across this chemical class since CATPB has been shown not to bind mouse FFA2 (Hudson et al., 2012). N-CBT antagonised hFFA2 in an assay of [^{35}S]GTPγS incorporation using HEK293T cell membranes containing hFFA2 and $G\alpha_o$ and using **C3** as agonist (Brown et al., 2003), but failed to antagonise hFFA3 in an equivalent assay. pIC50 values for inhibition of **C3** (EC_{80} concentration) were converted to estimated pK_B values by the method of Cheng-Prusoff. Lack of activity at hFFA3 was further confirmed in a FLIPR assay of calcium mobilisation. N-CBT also inhibited **C3**-evoked intracellular Ca^{2+} mobilisation in primary human PBMCs (see Fig.5). No evidence of partial agonism at either FFA2 or FFA3 was detected in any assay format (data not shown). N-CBT has previously been described as a CCK antagonist in patent literature (Bill, 1990).

Supplementary Figure 1

Operational modelling of yeast hFFA2 agonist and inverse agonist concentration-response data.



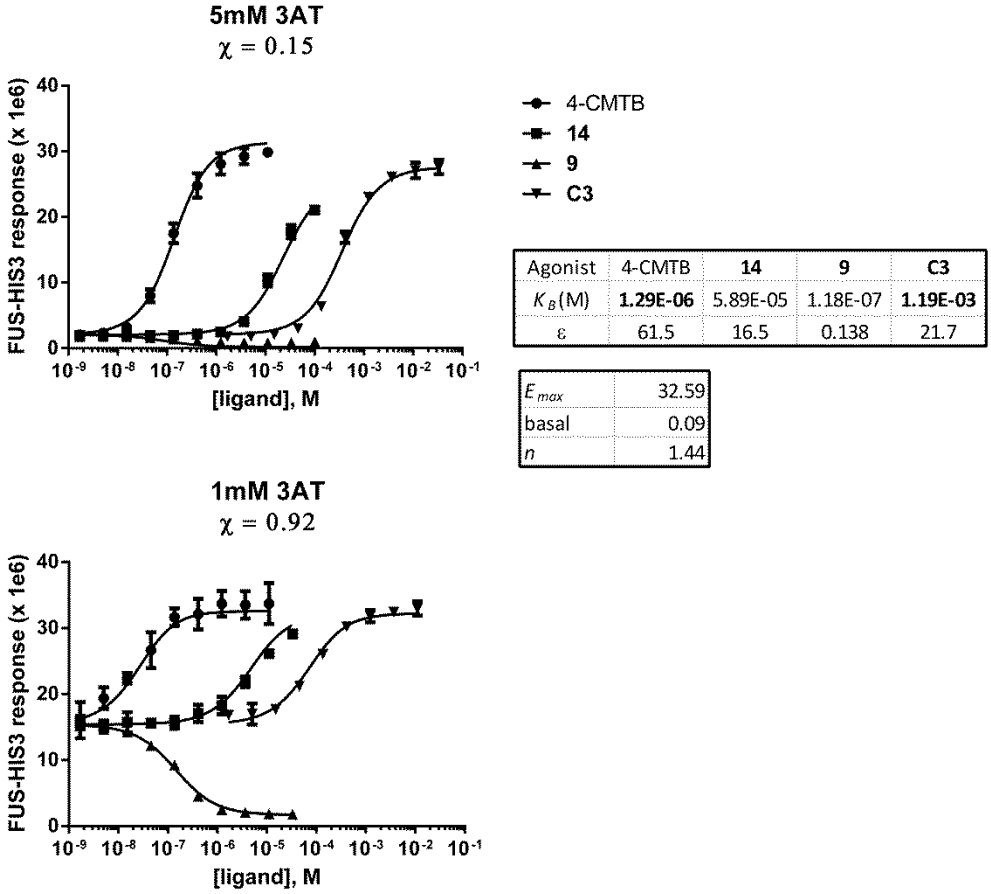
Agonist	4-CMTB	**14**	**9**	**C3**
K_a	1.29E-06	4.53E-05	2.04E-07	1.19E-03
ε	40	10.8	0.113	17

Basal	1.12
E max	24.08
n	2.02

Data were generated using yeast expressing hFFA2 with three agonists (4-CMTB, **C3** and **14**) and one inverse agonist (**9**) under conditions of low, intermediate and high receptor constitutive activity (5 mM, 2mM and 0.5 mM 3AT, respectively). The operational model of Slack and Hall (2012) was applied simultaneously to all data and resulting curve-fits and estimates K_a and ε, for each ligand, χ for each 3AT concentration and *n*, E_{max} and basal for the yeast hFFA2 assay, are shown. Error bars have been omitted from bottom panel for clarity (0.5 mM 3AT).

Supplementary Figure 2

Operational modelling of yeast hFFA2 agonist and inverse agonist concentration-response (data from Fig. 2E-G).



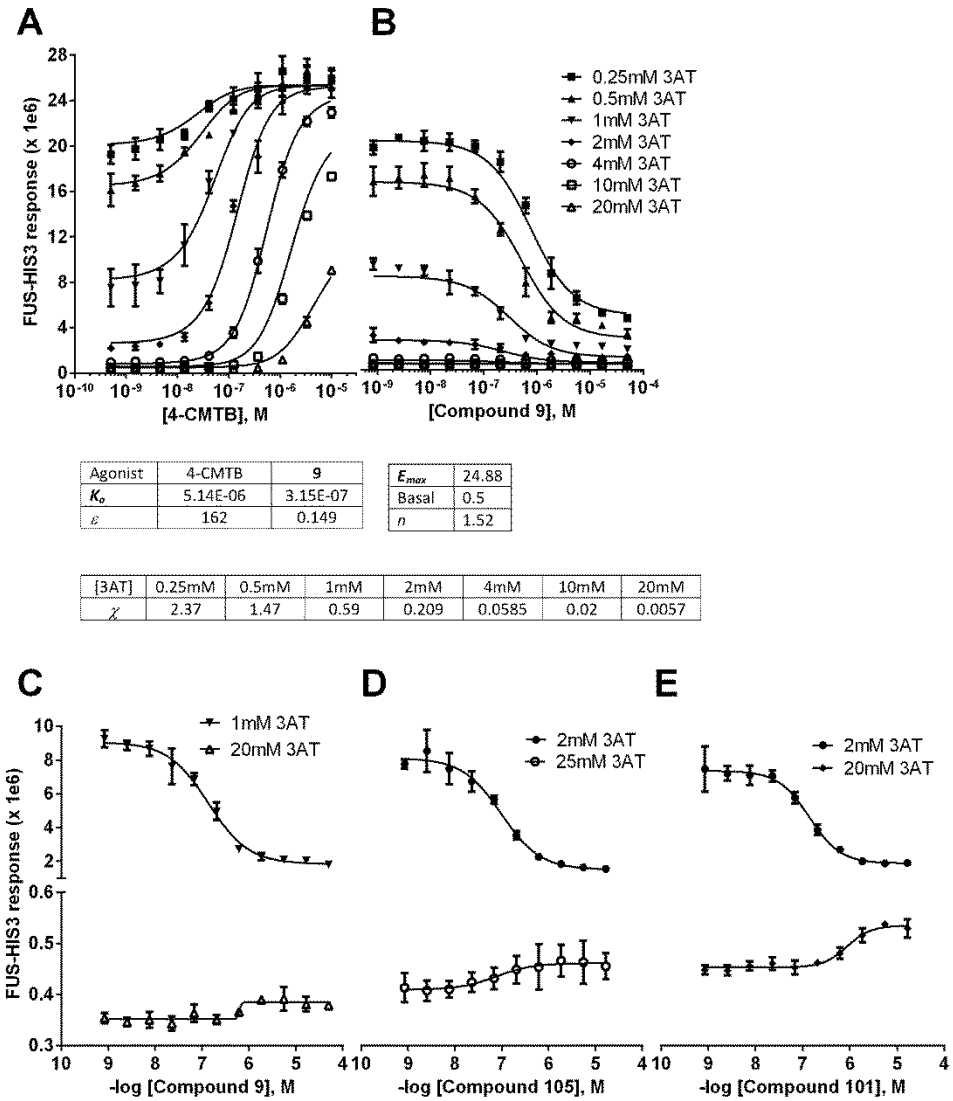
Agonist	4-CMTB	**14**	**9**	**C3**
K_B(M)	**1.29E-06**	5.89E-05	1.18E-07	**1.19E-03**
ε	61.5	16.5	0.138	21.7

E_{max}	32.59
basal	0.09
n	1.44

Data were generated and analysed as described in Supplementary Figure 1 except that K_a values for 4-CMTB and C3 were constrained to the values shown in bold in the inset table. Without this constraint divergent values for ε and K_a were obtained, suggesting that only two datasets may be insufficient to fully define variables in the model by this approach.

Supplementary Figure 3

Protean agonist properties of acid N-thiazolylamide FFA2 ligands.



Agonist	4-CMTB	9
K_a	5.14E-06	3.15E-07
ε	162	0.149

E_{max}	24.88
Basal	0.5
n	1.52

[3AT]	0.25mM	0.5mM	1mM	2mM	4mM	10mM	20mM
χ	2.37	1.47	0.59	0.209	0.0585	0.02	0.0057

3-Aminotriazole (3AT), which is an inhibitor of the product of the *FUS1-HIS3* gene-reporter, decreases apparent FFA2 constitutive activity in yeast. Hence, pEC_{50} of the agonist 4-CMTB decreases (A), and pIC_{50} of the inverse agonist compound **9** increases (B), at increasing 3AT concentration. Values in the tables and fitted curves were derived by simultaneous operational modelling of datasets (A) and (B) using the Slack/Hall equation, allowing all parameters to vary. At sufficiently high 3AT concentrations, compounds **9**, **105** and **101** (C to E) behave as protean FFA2 agonists, exhibiting weak positive efficacy (partial agonism; $P<0.05$; one-way ANOVA) in the yeast assay (<3% of the effect of 4-CMTB under the same conditions).