An Examination of the Mechanism of Action of Fenfluramine in Dravet Syndrome: A Look Beyond Serotonin

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INTRODUCTION

- Racemic fenfluramine (FFA) has provided unique, unexpected seizure control with a prolonged duration of effect in children and young adults with Dravet syndrome (DS),[1] which is unusual in epilepsy
- FFA is widely known to produce serotonergic effects via release of serotonin (5-HT) by reversal of 5-HT transporter flux[2] and direct activation of 5-HT receptors.[3,4] However, it is highly unlikely that these effects are solely responsible for the unique clinical profile:
  - Limited data in the preclinical and clinical literature[5-11] suggest 5-HT agonism might provide some benefit against seizures
  - Conversely, antidepressants have also been reported to lower seizure threshold[12] in some instances
  - The incidence of depression and anxiety is reported as high as 24% in people with epilepsy[13] and treatment with selective serotonin reuptake inhibitors (SSRIs) for depression in epilepsy is common, suggesting that a clear anticonvulsant effect would have been reported in the literature; this is not the case
  - After the first published case series using FFA,[1] pediatric epileptologists have prescribed SSRIs for DS, but without benefit [internal communication]
- Recently, Martin and colleagues[14] found that FFA alters activity at the sigma receptor at concentrations that are physiologically relevant. Based on their experiments:
  - FFA did not show classic agonist or antagonist activity
  - FFA and its enantiomers potentiated the sigma 1 agonist (+)-SKF-10,047, suggesting that FFA acts as a positive allosteric modulator
  - Racemic norfenfluramine and its enantiomers appeared to behave as inverse agonists
- Sourbron et al,[5] using a zebrafish model of DS, determined that FFA acts as an agonist at 5-HT1D, 5-HT2A, and 5-HT2C receptors and as an antagonist at the sigma 1 receptor (Sig-1R), to reduce seizure activity
- Given the above observations, we have conducted additional studies to examine and better understand the effect of FFA on Sig-1Rs
- Lorcaserin also has 5-HT2C receptor agonist activity,[15] although unlike FFA, it has shown only limited and short-term effectiveness in five patients with DS.[16] Therefore, we also examined the effect of lorcaserin on the Sig-1R

METHODS

- Receptor binding
  - Binding of FFA, norfenfluramine, and their enantiomers was evaluated using radioligand binding assays with recombinant human receptors or with rodent, bovine, or rabbit receptors when human receptors were not available (Sekisui XenoTech,LLC, Kansas City, KS)
    - Data were expressed as Ki values in molar concentration
- The Binding immunoglobulin Protein (BiP) assay was used to measure the activity of FFA and lorcaserin at Sig-1Rs
  - In the dormant state, the Sig-1R forms a complex with BiP
    - Sig-1R agonists cause a dissociation of the complex so that the receptor becomes active
    - Sig-1R antagonists inhibit the action of agonists by preventing the chaperone activity and mediation of proper folding of membrane proteins of sigma 1 agonists[17]
    - Sig-1R allosteric modulators alter the response to an agonist or antagonist
- Experimental methods: cell culture and ELISA assay
  - CHO cells were grown in 24-well plates and treated with compounds in culture medium at 37°C for 30 min at 1 μM and 10 μM final concentrations
  - Reaction was stopped by medium removal and adding 3 mL PBS at 37°C
  - CHO cells were harvested and suspended in 50 mM Hepes (pH 7.4) followed by cross-linking with 50 μg/ml of dithiobis succinimidyl propionate
  - Reaction was stopped by adding Tris-HCl (pH 8.8, final 50 mM)
  - Fifteen min after incubation on ice, cells were lysed with RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.3% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail]
  - After centrifugation at 12,000 g, 1 min, the supernatant was incubated overnight at 4°C with Sig-1R antibody
  - The cell lysate was incubated with Sepharose protein-A
  - After successive centrifugations, supernatants were analyzed by ELISA assay
- Experimental compounds: fenfluramine and lorcaserin
- Reference compounds: PRE084 (sigma 1 agonist) and NE 100 (sigma 1 antagonist)

RESULTS

Receptor Binding

Table 1. Enantiomers of FFA and Norfenfluramine: Ki Values (mol/L) Demonstrating >30% Binding Based on Percent Inhibition

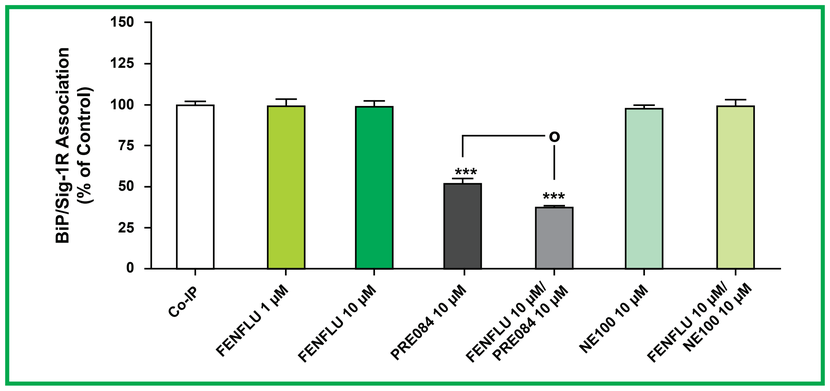
Receptor	(+) FFA	(-) FFA	(+) Norfen	(-) Norfen	Positive Control
β Adrenergic (non-selective)	1.61×10^{-5}	1.36×10^{-5}	9.76×10^{-6}	8.48×10^{-6}	3.53×10^{-9} (propranolol)
β2 Adrenergic	8.84×10^{-6}	1.40×10^{-5}	8.60×10^{-6}	5.56×10^{-6}	3.97×10^{-10} (propranolol)
M_1 Muscarinic	8.30×10^{-6}	1.15×10^{-5}	3.27×10^{-6}	4.00×10^{-6}	7.71×10^{-10} (atropine)
Sigma (non-selective)	1.63×10^{-7}	3.51×10^{-7}	1.80×10^{-6}	2.30×10^{-6}	1.84×10^{-9} (haloperidol)
Sigma 1	1.09×10^{-7}	5.02×10^{-7}	2.61×10^{-6}	4.60×10^{-6}	1.20×10^{-8} (pentazocine)
Sigma 2	4.31×10^{-7}	8.00×10^{-7}	2.98×10^{-6}	3.21×10^{-6}	5.18×10^{-9} (haloperidol)

- 47 receptor subtypes associated with epilepsy were identified and examined for fenfluramine binding[14]
- Receptors that showed binding >30% as measured by percent inhibition are shown in **Table 1** (enantiomers of FFA and norfenfluramine)
- Binding and activity at the Sig-1R receptor, but not adrenergic or muscarinic receptors, occurred at physiologically relevant concentrations

Agonist and Antagonist Assays

Fenfluramine

Figure 1. Effect of FFA on BiP – Sig-1R after 30 min of incubation in culture medium



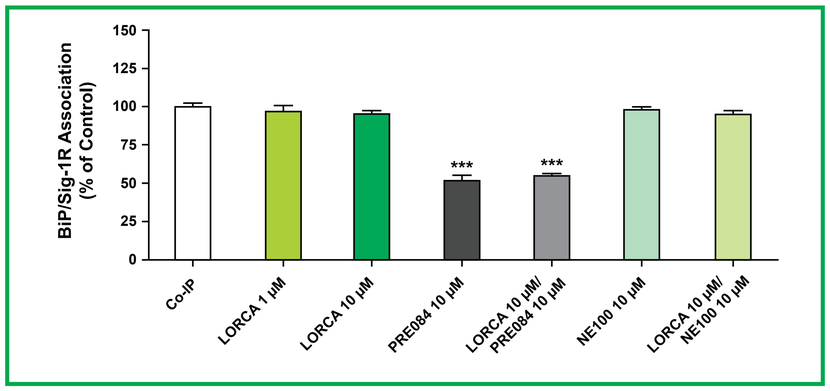
Abbreviations: Co-IP, co-immunoprecipitation; doses are expressed in micromolar; n=6 per condition. ***p<0.0001 vs Co-IP-treated group, °p<0.05 vs fenfluramine 10 μM group, by Dunnett's test.

Statistical analysis: $F_{(6;41)} = 67.62$, $p<0.0001$

- FFA alone had no effect on the BiP – Sig-1R dissociation and therefore did not act as an agonist or an antagonist
- FFA potentiated the effect of the agonist PRE084 and therefore acted as a positive allosteric modulator at a concentration of 10 μM
- However, FFA had no effect on the antagonist NE 100 and therefore did not act as a negative allosteric modulator

Lorcaserin

Figure 2. Effect of lorcaserin on BiP – Sig-1R after 30 min of incubation in culture medium



Abbreviations: Co-IP, co-immunoprecipitation; doses are expressed in micromolar; n=6 per condition. ***p<0.0001 vs Co-IP-treated group, by Dunnett's test.

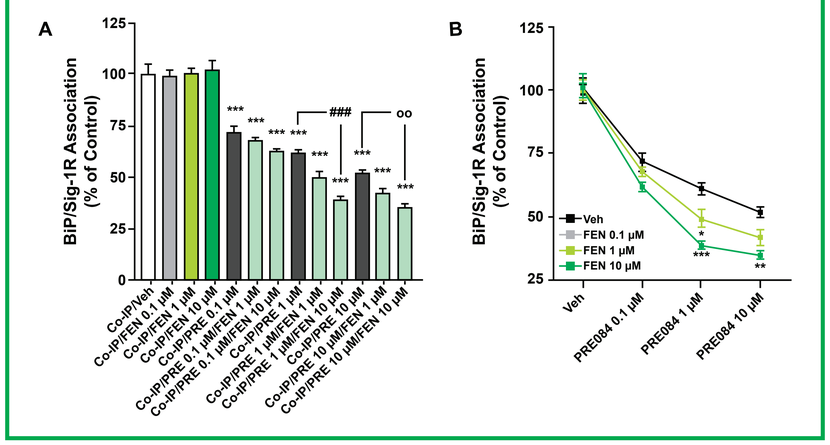
Statistical analysis: $F_{(6;41)} = 58.45$, $p<0.0001$

- Lorcaserin had no effect on the BiP – Sig-1R dissociation and therefore did not act as an agonist or an antagonist
- Since it did not alter the response of the agonist PRE084 or the antagonist NE 100, lorcaserin did not act as an allosteric modulator

Allosteric Modulation

Fenfluramine as a positive allosteric modulator

Figure 3A and 3B. Dose-effect study to characterize the positive allosteric modulatory effect of fenfluramine on PRE084-induced BiP – Sig-1R dissociation after 30 min of incubation in culture medium



Abbreviations: Co-IP, co-immunoprecipitation; doses are expressed in micromolar; n=6 per condition.

Figure 3A: one-way ANOVA and Dunnett's test. ***p<0.0001 vs Co-IP/vehicle group, ###p<0.0001 vs Co-IP/PRE 1, °°p<0.01 vs Co-IP/PRE 10.

Figure 3B: two-way ANOVA and Bonferroni test. *p<0.05, **p<0.01, ***p<0.0001 vs PRE084/veh groups (black curve).

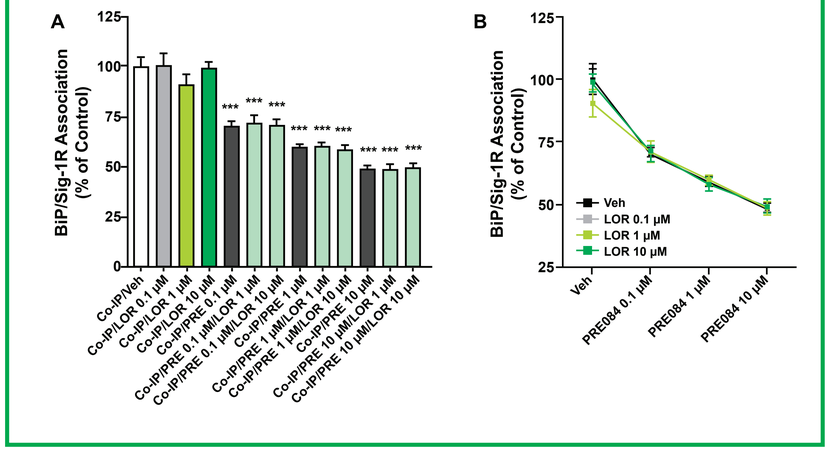
Statistical analysis: Figure 3A: $F_{(10;65)} = 67.58$, $p<0.0001$

Figure 3B: $F_{(2;60),fenfluramine} = 14.47$, $p<0.0001$

- As shown in **Figure 3A**, FFA has no effect by itself. However, FFA significantly increased the effect of PRE084, with dose-response relationships exhibited for both FFA and PRE084
- This is shown more clearly in **Figure 3B**. By representing the data as an activity curve, FFA can be seen to produce a dose-dependent increase in the activity of PRE084 on the BiP – Sig-1R association

Lorcaserin

Figure 4A and 4B. Effect of lorcaserin on PRE084 – induced BiP – Sig-1R dissociation after 30 min of incubation in culture medium



Abbreviations: Co-IP, co-immunoprecipitation; doses are expressed in micromolar; n=6 per condition.

Figure 4A: one-way ANOVA and Dunnett's test. ***p<0.0001 vs Co-IP/vehicle group.

Figure 4B: two-way ANOVA.

Statistical analysis: Figure 4A: $F_{(10;65)} = 29.59$, $p<0.0001$

Figure 4B: $F_{(2;60),lorcaserin} = 0.36$, $p>0.05$

- As shown in **Figure 4A**, lorcaserin has no effect by itself or when given in combination with PRE084
- This is shown more clearly in **Figure 4B**. By representing the data as an activity curve, it is clearly seen that lorcaserin has no activity on PRE084 in the BiP – Sig-1R association

SUMMARY OF RESULTS

- Fenfluramine binds to sigma receptors
- Fenfluramine, but not lorcaserin, acts as a positive allosteric modulator of the Sig
- Lorcaserin did not show activity on the Sig-1R

CONCLUSION

- FFA is widely known as a "serotonergic" drug, yet the unexpected meaningful and prolonged reduction in seizure frequency observed after its use in DS patients distinguishes it from other serotonergic drugs such as SSRIs and lorcaserin
- Recently Martin et al[14] evaluated 47 receptors associated with epilepsy as possible sites for fenfluramine activity that could explain its unique effectiveness in DS and possibly other pediatric epilepsies. In this investigation, FFA demonstrated binding to Sig-1Rs
- Sig-1R effects may play a role in the anticonvulsant activity of FFA
  - Activation of the Sig-1R attenuates seizures[18-20]
  - The Sig-1R dimerizes with other receptors, and it modulates serotonergic neurotransmission[21]
- FFA acts as a positive allosteric modulator of the Sig-1R
- Lorcaserin did not show activity on the Sig-1R — neither agonism, antagonism, nor allosteric modulation

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