Rescue of *Fmr1*[KO] phenotypes with mGluR$_5$ inhibitors: MRZ-8456 *versus* AFQ-056

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A R T I C L E   I N F O

A B S T R A C T

Metabotropic glutamate receptor 5 (mGluR$_5$) is a drug target for central nervous system disorders such as fragile X syndrome that involve excessive glutamate-induced excitation. We tested the efficacy of a novel negative allosteric modulator of mGluR$_5$ developed by Merz Pharmaceuticals, MRZ-8456, in comparison to MPEP and AFQ-056 (Novartis, a.k.a. mavoglurant) in both *in vivo* and *in vitro* assays in a mouse model of fragile X syndrome, *Fmr1*[KO] mice. The *in vivo* assays included susceptibility to audiogenic-induced seizures and pharmacokinetic measurements of drug availability. The *in vitro* assays included dose response assessments of biomarker expression and dendritic spine length and density in cultured primary neurons. Both MRZ-8456 and AFQ-056 attenuated wild running and audiogenic-induced seizures in *Fmr1*[KO] mice with similar pharmacokinetic profiles. Both drugs significantly reduced dendritic expression of amyloid-beta protein precursor (APP) and rescued the ratio of mature to immature dendritic spines. These findings demonstrate that MRZ-8456, a drug being developed for the treatment of motor complications of L-DOPA in Parkinson's disease and which completed a phase I clinical trial, is effective in attenuating both well-established (seizures and dendritic spine maturity) and exploratory biomarker (APP expression) phenotypes in a mouse model of fragile X syndrome.

1. Introduction

Fragile X syndrome (FXS)[1], the most common form of inherited intellectual disability, is characterized by moderate to severe cognitive impairment, sensory integration problems, autistic behaviors, hyperactivity, attention deficit, anxiety and seizures (Hagerman and Hagerman, 2002). At the neuroanatomical level, FXS is distinguished by an overabundance of long, thin, tortuous dendritic spines with prominent heads and irregular dilations resembling the spines observed during normal, early neocortical development (Rudelli et al., 1985; Wisniewski et al., 1991). This FXS pathology suggests a breakdown in normal dendritic spine maturation or pruning and is also observed in *Fmr1*[KO] mice (Comery et al., 1997). In the vast majority of cases, FXS is caused by a trinucleotide repeat expansion (CGG) in the promoter region of the fragile X mental retardation (*FMR1*) gene (Fu et al., 1991). *FMR1* mRNA codes for the fragile X mental retardation protein (FMRP), which is a messenger RNA (mRNA) binding protein that represses the translation of a subset of dendritic mRNAs whose products affect synaptic plasticity and function (Darnell et al., 2001; Laggerbauer et al., 2001; Li et al., 2001). Its absence in FXS leads to excessive synaptic protein synthesis of numerous dendritic mRNAs, which likely contributes to disease phenotypes.

Table 1
Summary of mGluR$_5$ inhibitor results in *Fmr1^{KO}* preclinical studies. A review of the literature regarding findings related to the testing of MPEP, CTEP, fenobam and AFQ-056 in *Fmr1^{KO}* mice is provided with corresponding citations.

mGluR$_5$ NAM	Effect in *Fmr1^{KO}*	Citation
MPEP	Rescued AGS and open field deficits.	(Yan et al., 2005)
	Rescued axonal branching defect.	(Tucker et al., 2006)
	Did not alter reduced potentiation in the neocortex.	(Wilson and Cox, 2007)
	Rescued aberrant internalization of GluR1.	(Nakamoto et al., 2007)
	Rescued PPI startle response.	(de Vrij et al., 2008)
	Increased inhibitory serine-phosphorylation of brain GSK3.	(Yuskaitis et al., 2010)
	Rescued mEPSC frequency but not amplitude in the amygdala. Did not rescue LTP or surface GluR1 in the amygdala.	(Suvrathan et al., 2010)
	Rescued spontaneous EPSC amplitude and charge at 2 weeks of age.	(Meredith et al., 2011)
	Rescued dendritic spine phenotypes.	(de Vrij et al., 2008; Su et al., 2011)
	Reduced surface K4.2 levels.	(Gross et al., 2011)
	Decreased marble burying, had no effect on activity or PPI, improved motor learning, and decreased AGS.	(Thomas et al., 2012)
	Potentiated brain stimulation reward (BSR).	(Fish et al., 2013)
	Rescued maze learning.	(Gandhi et al., 2014)
	Rescued clustering and morphological defects in mouse neurospheres.	(Achuta et al., 2017)
CTEP	Rescued protein synthesis, LTP, learning and memory, hypersensitivity to sensory stimuli, elevated locomotor activity, AGS, dendritic spine phenotypes, intracellular signaling, and local alterations of brain activity.	(Michalon et al., 2012; Michalon et al., 2014)
Fenobam	Rescued AGS.	(Westmark et al., 2011)
	Rescued dendritic spine phenotypes.	(de Vrij et al., 2008)
	Rescued associative motor learning and avoidance behavior.	(Vinueza Veloz et al., 2012)
	Rescued some synaptic protein distribution.	(Wang et al., 2014)
AFQ-056	Rescued dendritic spine phenotypes.	(Levenga et al., 2011; Pop et al., 2014)
	Restored social behavior.	(Gantois et al., 2013; de Esch et al., 2015)
	Rescued PPI.	(Levenga et al., 2011)

In 2004, Bear and colleagues proposed the metabotropic glutamate receptor (mGluR) theory of FXS in which they hypothesized that overactive signaling through group 1 mGluRs contributed to many of the symptoms of FXS (Bear et al., 2004). Group 1 mGluRs (mGluR$_1$ and mGluR$_5$) are glutamate-activated, G-protein-coupled receptors that are widely expressed in the central nervous system (CNS) and are attractive therapeutic targets in numerous neurological disorders (Gravius et al., 2010). Signaling *via* these receptors causes pulsatile translation of postsynaptic mRNAs by temporarily blocking FMRP (Todd et al., 2003). Over the past decade, substantial evidence has accumulated to support the mGluR theory of FXS. First, pharmacological treatment with mGluR$_5$ antagonists rescues FXS phenotypes in mouse (*Mus musculus*), fly (*Drosophila melanogaster*) and zebrafish (*Danio rerio*) disease models (Achuta et al., 2017; de Vrij et al., 2008; Fish et al., 2013; Gandhi et al., 2014; Gantois et al., 2013; de Esch et al., 2015; Gross et al., 2011; McBride et al., 2005; Meredith et al., 2011; Michalon et al., 2012; Michalon et al., 2014; Pop et al., 2014; Su et al., 2011; Suvrathan et al., 2010; Thomas et al., 2012; Tucker et al., 2006; Vinueza Veloz et al., 2012; Wang et al., 2014; Westmark et al., 2011; Yan et al., 2005; Yuskaitis et al., 2010). Second, mutant *Fmr1^{KO}* mice that express 50% fewer mGluR$_5$ receptors exhibit rescue of phenotypic and behavioral abnormalities associated with FXS (Dolen et al., 2007). And third, initial FXS clinical trials with mGluR$_5$ inhibitors showed promise in improving behavioral phenotypes (Berry-Kravis et al., 2009; Jacquemont et al., 2011), although recent clinical trials have failed in meeting significant improvement in abnormal behaviors compared to placebo (Bailey Jr et al., 2016; Berry-Kravis et al., 2016; Berry-Kravis et al., 2017; Youssef et al., 2018). There is much work remaining in selecting and validating the appropriate outcome measures for FXS clinical trials and in testing combination therapies (Berry-Kravis et al., 2013; Davenport et al., 2016).

We identified amyloid-beta protein precursor mRNA (*App*) as a synaptic target that is translationally regulated through mGluR$_5$- and FMRP-dependent signaling (Westmark and Malter, 2007). *App* mRNA codes for amyloid-beta protein precursor (APP), which is cleaved by β- and γ-secretase to produce amyloid-beta (Aβ), the most prevalent protein found in the senile plaques in Alzheimer's disease. FMRP binds to a guanine (G)-rich region in the coding region of *App* mRNA.

Activation of group 1 mGluR signaling with (*S*)-3,5-dihdroxyphenylglycine (DHPG) leads to the release of the translational repressor FMRP from *App* mRNA accompanied by increased APP synthesis, which can be blocked by the mGluR$_5$ antagonist 2-methyl-6-(phenylethynyl)pyridine (MPEP) (Westmark and Malter, 2007). Altered levels of APP and Aβ are observed in brain tissue from mice and humans with FXS (Westmark and Malter, 2007; Westmark et al., 2011). APP and Aβ play important roles in synapse formation and apoptosis during development and their dysregulation likely contributes to the seizure, behavioral, electrophysiology and dendritic spine phenotypes characteristic of FXS. Consistent with this hypothesis, audiogenic-induced seizures (AGS), anxiety, mGluR-mediated long-term depression (LTD), neocortical UP states, duration of hippocampal ictal discharges, and the ratio of mature to immature dendritic spines are partially or completely reverted to normal in *Fmr1^{KO}* mice after removal of one *App* allele, ie. normalization of APP levels (Westmark et al., 2011; Westmark et al., 2016).

Treating FXS with mGluR$_5$ antagonists is an attractive therapeutic strategy because it targets the underlying molecular defect by downregulating excessive protein synthesis (Hagerman et al., 2014). Preclinical validation of novel mGluR$_5$ inhibitors is required to move the most effective compounds into the clinic. These compounds typically undergo preclinical testing in *Fmr1^{KO}* mice, which are currently the best validated FXS model system. Preclinical studies with MPEP, CTEP [2-chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine], fenobam [1-(3-chlorophenyl)-3-(3-methyl-5-oxo-4H-imidazol-2-yl)urea] and AFQ-056 [methyl (3a*R*,4*S*,7a*R*)-4-hydroxy-4-[(3-methylphenyl)ethynyl]octahydro-1*H*-indole-1-carboxylate] show rescue of AGS, hyperactivity, inhibition of the startle response, social behavior, learning and memory, excessive protein synthesis and/or elongated dendritic spines in *Fmr1^{KO}* (Table 1). This study compared the pharmacokinetics and efficacy of a novel mGluR$_5$ negative allosteric modulator (NAM) developed by Merz Pharmaceuticals, MRZ-8456 [6-bromo-pyrazolo[l,5-a]pyrimidin-2-yl)-(l(*R*)-methyl-3,4-dihydro-lH-isoquinolin-2-yl)-methanone] (Danysz et al., 2007) with MPEP and AFQ-056 in *Fmr1^{KO}* mice with the goal of verifying whether FXS may be a further indication for this agent. It is of particular interest to study MRZ-8456 because this novel mGluR$_5$ NAM

exhibited extended pharmacokinetics with a flat curve over many hours and superior antidyskinetic action in rats compared to previously studied mGluR$_5$ antagonists (Dekundy et al., manuscript in preparation). The superior activity principally results from its chemical structure. MRZ-8456 does not have a triple bond and thus has no issues with reactivity or the generation of protein or glutathione adducts. The affinity of MRZ-8456 to its target receptor is comparable with MPEP and solubility problems are less pronounced than many other mGluR$_5$ NAM. Thus, novel insights about the pathophysiology and management of FXS may be gained by assessment of MRZ-8456.

2. Materials and methods

2.1. Test compounds

Merz Pharmaceuticals identified novel pyrazolopyrimidines as potent and selective NAMs of mGluR$_5$ through rational drug design methods. The compounds were synthesized as described in patent #EP2295439A1 (Danysz et al., 2007) and evaluated for pharmacokinetic and pharmacodynamic properties after oral administration. Based on these data, an initial hit compound, MRZ-8456 with a potency of IC$_{50}$ = 13 nM in a functional assay and Ki of 27 nM in a binding assay, was selected for efficacy testing in comparison to the lead Novartis mGluR$_5$ NAM AFQ-056 and the research grade mGluR$_5$ inhibitor MPEP (chemical characteristics, Table 2).

2.2. Drug preparation

Test compounds were provided by Merz Pharmaceuticals: MRZ-8456 (batch #MRZ-0008456-51), AFQ-056 (batch #MRZ-0014901-02), and MPEP (batch #MRZ-10). For the *in vivo* work, the test compounds were prepared as a fine suspension in 1% hydroxypropyl methylcellulose (HPMC)/1% Tween-80 using a IKA-Ultra Turrax mill. For the *in vitro* work, the test compounds were dissolved in a small volume of dimethyl sulfoxide (DMSO) and then diluted in Hank's buffered salt solution (HBSS) prior to treating the neuronal cells. The final concentration of DMSO in the cell media was 0.025% and there was no evidence of the drugs precipitating out of solution upon dilution of the DMSO stocks with HBSS. Persons conducting the experiments were blinded to the identity of the compounds until after data acquisition and analysis.

2.3. Toxicology testing

Irwin-like toxicity screening was conducted in wild type male mice (*n* = 5; 20-25 g). MRZ-8456 was prepared in 1% HPMC and orally dosed at 10 mL/kg at doses of 36 mg (1.44–1.8 mg/kg), 108 mg (4.32–5.4 mg/kg) and 324 mg (13.0–16.2 mg/kg). Testing occurred 180 min post-drug administration and mice were screened for mortality, ataxia, tremor, tonic seizures, clonic seizures, ptosis,

piloerection, stereotypy, straub tail, loss of activity, excitation, loss of exploration, loss of pinnar reflexes, loss of righting reflex, mydriasis, catalepsy, loss of grasping reflex, rotarod, tonic-MES, clonci-MES, death after MES, mortality after 24 h, and analgesia. There were minimal adverse effects at all doses tested and therapeutic-like actions in terms of analgesia (**Supplementary Table 1**).
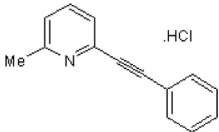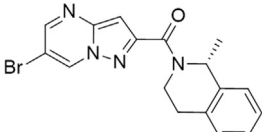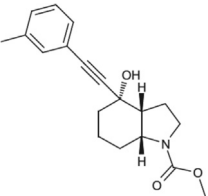
2.4. Animal husbandry

The *Fmr1^{KO}* mice were originally developed by the Dutch-Belgian FXS Consortium and backcrossed > 11 times to FVB mice (Dutch-Belgian Fragile X Consortium, 1994). They were backcrossed into the C57BL/6 background by Dr. Bill Greenough's laboratory (University of Illinois at Urbana-Champaign) and distributed to other laboratories. We have maintained the *Fmr1^{KO}* mice in the C57BL/6 background at the University of Wisconsin-Madison for over 10 years with occasional backcrossing with C57BL/6 J mice from Jackson Laboratories to avoid genetic drift. Mice were housed in static microisolator cage on a 6 a.m.–6 p.m. light cycle with *ad libitum* access to food (Purina 5015 mouse diet) and water. The cages contained seeds and a nestlet as the only sources of environmental enrichment. All animal husbandry and euthanasia procedures were performed in accordance with NIH and an approved University of Wisconsin-Madison animal care protocol administered through the Research Animal Resources Center with oversight from the Institutional Animal Care and Use Committee (IACUC). *Fmr1* genotypes were determined by PCR analysis of DNA extracted from tail biopsies. For the *in vitro* experiments, primary neurons were prepared from embryos harvested from pregnant *Fmr1^{KO}* female mice (age 3 months). For the *in vivo* studies, male and female mice were tested for AGS at age postnatal day 21 (P21) (weight range: 5–12.25 g).

2.5. Audiogenic seizures

Litters of mice were allocated to treatment groups [2 litters of mice were tested for the 1 mg/kg AFQ-056 cohort; all other treatment cohorts contained a minimum of 3 litters]. Individual mice were not randomized to drug treatments. *Fmr1^{KO}* in the C57BL/6 background have peak sensitivity to AGS at P21 (Yan et al., 2004). Thus, mice were treated with vehicle or the indicated dose of drug by intraperitoneal (I.P.) injection at P21 and 30 min later transferred to a Plexiglas box (13"L X 8"W X 7"H) and exposed to a high-pitched siren (118 dB) from a personal body alarm (LOUD KEY™). The number of mice exhibiting wild running (WR), tonic seizures (AGS) and death were scored. The treatment groups included: (1) 1% HPMC/1% Tween-80 vehicle; (2) 10 and 30 mg/kg MPEP; (3) 1, 3 and 10 mg/kg MRZ-8456; and (4) 1, 3 and 10 mg/kg AFQ-056. A dosing volume of 20 mL/kg was used, and dosing levels took into account the established concentration of MPEP known to reduce AGS. Mice weighed in the range 5.00–12.25 g. Treatment groups were compared by the Barnard exact test. After AGS testing, mice were anesthetized with isoflurane and the blood removed from the

Table 2
Compound characteristics. The molecular weight, Ki, IC$_{50}$ and structures are provided for MPEP, MRZ-8456 and AFQ-056.

Compound	MPEP	MRZ-8456	AFQ-056
Mol Wt (g/mol)	229.71	371.24 g/mol	313.39
Ki (binding displacement assay)	–	27 nM	47 nM
IC$_{50}$ (PI hydrolysis assay)	–	13 nM	30 nM
Chemical structure			

abdominal aortic artery with a 23 g needle and mixed with 20 μL of 10 mg/mL sodium heparin to prevent coagulation. The mice were then decapitated and the brains dissected, cut in half, and frozen in dry ice. After blood samples were collected, tubes were spun at 5000 rpm for 10 min. The upper plasma layer was removed and frozen on dry ice. Plasma samples were used for pharmacokinetic analyses of compound levels.

2.6. Preparation and treatment of primary cultured neurons

Pregnant females (embryonic day 18) were anesthetized with iso-flurane prior to decapitation and transfer of the uterine sac to ice-cold HBSS. Cortices were removed, washed with ice-cold HBSS, lysed with 0.5 mg/mL trypsin for 25 min at 37 °C, washed with HBSS, suspended in NeuroBasal medium (supplemented with 2% B27 supplement, penicillin/streptomycin, 0.5 mM glutamine), triturated $70\times$ with a 10 mL pipet and passed through a 70 μm cell strainer. Cells were counted by trypan blue dye exclusion and plated at 1.3×10^5 cells/mL on poly(D)-lysine coated glass coverslips in 12-well tissue culture dishes and cultured for 15 days at 37 °C/5% CO_2. Cells were treated with the indicated doses of mGluR$_5$ inhibitor in NeuralBasal culture media containing B27 supplement for the indicated times. The treatment groups for the *in vitro* studies are listed in Supplementary Tables 2 and 3. Dosing levels of MRZ-8456 and AFQ-056 were based on existing relevant data attained by Merz Pharmaceuticals and took into account the established concentration of MPEP known to reduce dendritic APP levels and dendritic spine length. The cells were dosed *in vitro* at a constant dose volume of 1 mL dosing solution per well.

2.7. APP staining, confocal microscopy and image analysis

To assess dendritic APP levels, treated neuronal cells were fixed and stained with anti-APP antibody. For fixation, treated cells were washed with Dulbecco's phosphate buffered saline (DPBS), fixed in 4% paraformaldehyde for 10 min at room temp and permeabilized with methanol (-20 °C) for 15 min. Fixed, permeabilized cells were stained with anti-22C11 antibody targeted against the amino-terminus of APP (Chemicon #mAB348, Temecula, CA) (1:2000, overnight) and visualized with goat anti-mouse rhodamine-conjugated secondary antibody (Invitrogen, Carlsbad, CA) (1:500 for 20 min in the dark). Washes and antibody dilutions were in DPBS containing 2% fetal bovine serum (FBS). Coverslips were fixed to slides with 12 μL ProLong Gold Antifade (Invitrogen, Carlsbad, CA) and dried overnight. Images were acquired with a Nikon C1 laser scanning confocal microscope (Nikon Eclipse E600 upright microscope) using the 543 Diode (1mw Mellet Griot) laser, the Nikon Plan Apo $60\times/1.40$ oil objective with Zeiss Immersol™ 518F oil at ambient temperature, and Nikon EZ-C1, v3.91 software (Nikon Corp, Tokyo, Japan). APP levels in the puncta of 4–7 dendrites per sample were quantitated with IMAGE J software using the Analyze Particles function (Rasband, W.S., Image J, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2006). Treatment groups were compared by ANOVA and post-hoc Student *t*-tests using Prism 5.0d and Excel software, respectively.

2.8. Assessment of dendritic spine length and density

To assess dendritic spine phenotypes, treated neuronal cells were fixed with 4% paraformaldehyde and stained with DiI dye (Gibco Life Technologies, catalog #D282). DiI is a lipophilic, orange-red fluorescent, membrane stain that diffuses laterally to stain the entire cell. For the staining, the wells were aspirated and sprinkled with DiI crystals and a small amount of DBPS was added to the edge of the wells to prevent dehydration of the cells. Cells were stained for 10 min, copiously washed with DPBS to remove all crystals and fixed to slides with ProLong Gold Antifade (Life Technologies Corporation, Carlsbad, CA, USA). Slides were allowed to set for at least 3 days to allow complete

migration of the DiI into dendritic spines. Dendritic spines were imaged on a Zeiss Axioplan 2 Imaging Photomicroscope equipped with a MBF Biosciences automated XYZ stage and MicroFire A/R camera. Images were taken using the $100\times$ objective (Zeiss FLUAR $100\times/1.30$ oil) and Zeiss Immersol™ 518F oil at ambient temperature. Spine length was quantitated with StereoInvestigator v9 software. Contours were drawn around the protrusions and the feret max (length) and feret min (widest width) of the contours were calculated. A minimum of 2 coverslips were analyzed per neuronal cell prep and images of neurons were taken from multiple areas of those coverslips. Spines (333–592) were quantitated per condition. The feret width was divided by feret max and protrusions having a ratio < 0.5 were classified as filopodia and those with a ratio greater than or equal to 0.5 were classified as spines. Treatment groups were compared by ANOVA and post-hoc Student *t*-tests using Prism 5.0d and Excel software, respectively.

3. Results

NAMs of mGluR$_5$ are under intense investigation for the treatment of FXS. Herein, we compared the efficacy of Merz' mGluR$_5$ inhibitor MRZ-8456 with the research grade mGluR$_5$ inhibitor MPEP and with Novartis' lead mGluR$_5$ NAM, AFQ-056 (Gomez-Mancilla et al., 2014), side-by-side in both *in vivo* and *in vitro* assays in *Fmr1^{KO}* mice and cells, respectively. *In vivo* testing included AGS susceptibility and quantification of drug levels in blood plasma. The *Fmr1^{KO}* mice are highly sensitive to AGS, which is currently the gold standard phenotype for drug efficacy testing in this model. The mice were treated intraperitoneally with MRZ-8456 and AFQ-056 (1, 3 or 10 mg/kg) or MPEP (10 and 30 mg/kg) and monitored for adverse reactions. The mice took a few minutes to recover after the injections and then exhibited normal home cage activity. Recovery time was comparable regardless of the compound and dose. There was no evidence for any adverse effects (motor coordination, behavior, *etc.*) following the first few minutes. The mice were tested for AGS susceptibility 30 min post-injection. Both MRZ-8456 and AFQ-056 attenuated WR and AGS at doses of 3 and 10 mg/kg in *Fmr1^{KO}* mice, but neither was effective at 1 mg/kg (Fig. 1). Thus, MRZ-8456 and AFQ-056 were both effective in attenuating seizures in *Fmr1^{KO}* mice. The lowest dose of MPEP tested was 10 mg/kg, which also significantly reduced both WR and AGS. There were no statistically significant differences in mortality rates between treatment groups due to the low incidence of audiogenic-induced deaths. Plasma samples were collected directly following the AGS testing for measurement of drug levels (Fig. 2, Table 3). There appeared to be a dose-dependent increase in plasma drug levels in both WT and *Fmr1^{KO}* mice for all three test drugs as well as elevated drug plasma levels in WT compared to *Fmr1^{KO}* mice at the higher doses; however, there was large variability between animals within groups.

The *in vitro* assays included dose response assessments of MRZ-8456 and AFQ-056 efficacy in reducing dendritic APP expression and dendritic spine length and density in *Fmr1^{KO}* primary neurons. There were trends for reduced APP expression at all concentrations of MRZ-8456 and AFQ-056 tested (0.0625–2.5 μM) with both drugs reaching a maximal reduction of 50% within the 60 min treatment (Fig. 3). MRZ-8456 significantly reduced dendritic expression of APP at concentrations of 0.25, 0.5 and 1.0 μM, and AFQ-056 significantly reduced APP expression at 0.0625, 0.25, 0.5, 1.0 and 2.5 μM (ANOVA, $P = 0.0007$, F = 2.75, $R^2 = 0.002$). Thus, the lowest effective dose of MRZ-8456 was 0.25 μM and that of AFQ-056 was perhaps 0.0625 μM although the aberrant result at 0.125 μM AFQ-056 occludes a definitive conclusion. The 0.125 and 2.5 μM doses of MRZ-8456 approached statistical significance in reducing APP expression ($P \leq 0.08$). MPEP was not effective in this assay, which is contrary to our previous results that demonstrated a 40% decrease in dendritic APP levels with MPEP (2–10 μM) treatment (unpublished data). The lack of effect with MPEP could be due to the difference in solvents used to dissolve the drug.
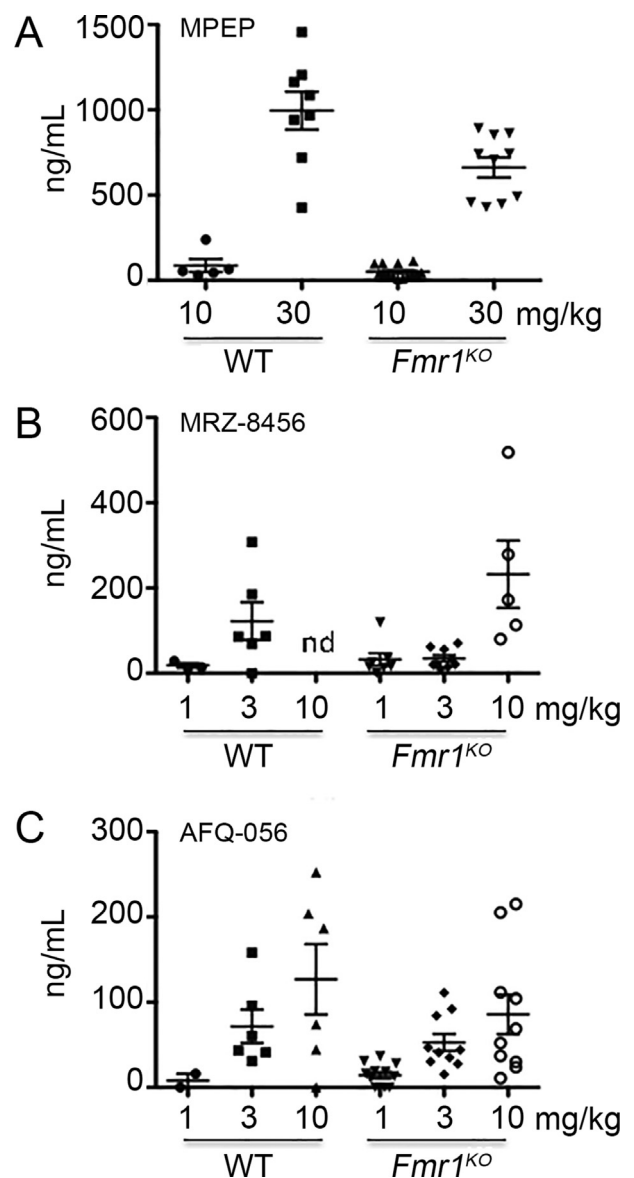
Both MRZ-8456 and AFQ-056 significantly decreased dendritic
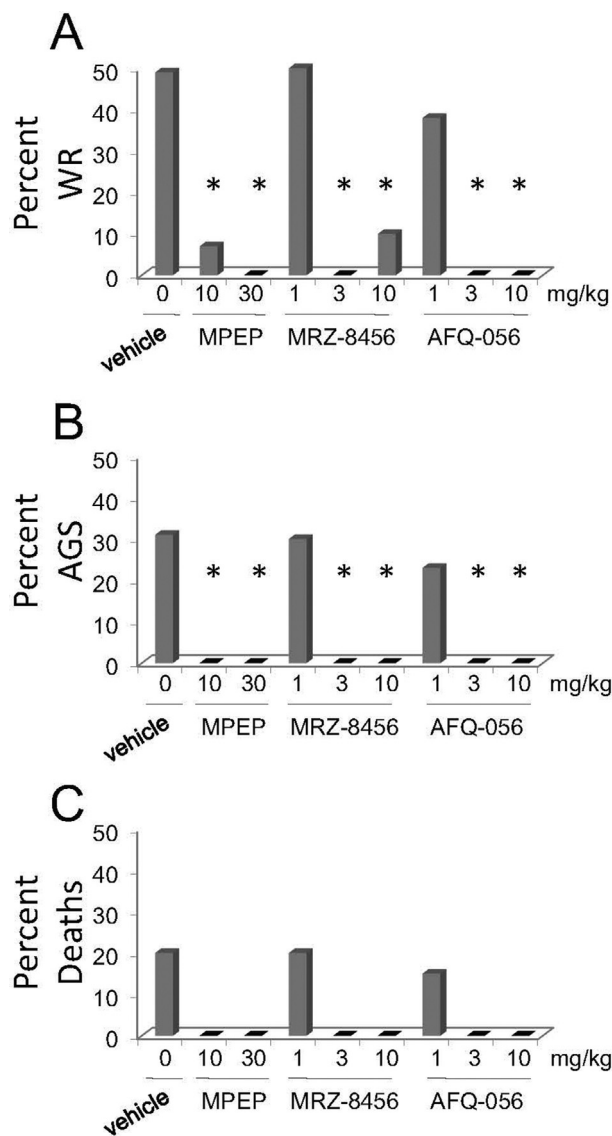
Fig. 1. mGluR$_5$ NAMs attenuate AGS in *Fmr1^{KO}* mice. WR, AGS and death rates were assessed in *Fmr1^{KO}* mice (age P21) treated with vehicle ($n = 35$; 9.44 g $\pm$ 1.26 g), 10 mg/kg MPEP ($n = 14$; 8.44 g $\pm$ 0.56 g), 30 mg/kg MPEP ($n = 10$; 9.17 g $\pm$ 1.45 g), 1 mg/kg MRZ-8456 ($n = 10$; 9.25 g $\pm$ 1.48 g), 3 mg/kg MRZ-8456 ($n = 10$; 9.22 g $\pm$ 1.09 g), 10 mg/kg MRZ-8456 ($n = 10$; 8.21 g $\pm$ 0.84 g), 1 mg/kg AFQ-056 ($n = 13$; 7.84 g $\pm$ 1.39 g), 3 mg/kg AFQ-056 ($n = 10$; 9.85 g $\pm$ 1.70 g), and 10 mg/kg AFQ-056 ($n = 10$; 9.81 g $\pm$ 0.90 g). Asterisks denote statistically significant differences in seizure phenotypes from placebo-treated mice by Barnard's exact test ($P < 0.05$).

Fig. 2. Plasma concentrations of (A) MPEP (10 and 30 mg/kg), (B) MRZ-8456 (1, 3 and 10 mg/kg), and (C) AFQ-056 (1, 3 and 10 mg/kg) in WT and *Fmr1^{KO}* mice as measured 30 min after i.p. administration. Graphical symbols represent values for individual animals. ND = not determined.

spine length by 2-fold with both 15 and 75 min treatments with 0.25 μM drug (ANOVA, $P < 0.0001$, F = 40, R^2 = 0.07) (Fig. 4). The AFQ-056 also reduced dendritic spine length by ~2-fold with the 5 min treatment, whereas there was a significant but smaller effect with the MRZ-8456. The 0.25 μM dose was chosen as the lowest common effective dose so that the two drugs could be compared over time. The control *Fmr1^{KO}* cells exhibited an average spine length of 2.24 μm as expected. Based on previous studies, *Fmr1^{KO}* neurons have longer spines than WT neurons, which have an average spine length of 1 μm, and spine length in *Fmr1^{KO}* neurons is rescued to the WT phenotype with 2.5 μM MPEP (Westmark et al., 2011). Both drugs reduced the percentage of immature spines (filopodia) by 1.8–2.4-fold (Chi square analysis, $P = 0$). Spine density was highly variable with both placebo and MPEP treatment.

4. Discussion

For the past decade, mGluR$_5$ has been the major target for drug discovery in FXS. These glutamate receptors are generally postsynaptic in location and consist of a heptahelical domain in the membrane region, a large extracellular amino terminal domain where the glutamate binding site is found, and an intracellular carboxy terminal domain. The amino terminal domain has a bilobate Venus Flytrap domain where glutamate binds and the closed conformation of this domain is required for mGluR$_5$ activation. Competitive antagonists of mGluR$_5$ prevent complete closing of the bilobular Venus Flytrap domain whereas allosteric modulators are non-competitive ligands that bind to the transmembrane heptihelical domain. Thus, NAMs inhibit receptor activation without affecting agonist binding. MPEP, fenobam, AFQ-056 and MRZ-8456 are all selective and systemically active NAMs of mGluR$_5$ (Levenga et al., 2011; Pagano et al., 2000; Porter et al., 2005).

In this study, we compared the efficacy of Merz' novel mGluR$_5$ NAM, MRZ-8456, with MPEP and AFQ-056 in the *Fmr1^{KO}* mouse model.

Table 3

Pharmacokinetics of mGluR$_5$ NAMs in WT and *Fmr1^{KO}* mice. The mean and median plasma concentrations of MPEP, MRZ-8456 and AFQ-056 in WT and *Fmr1^{KO}* mice are provided.

Drug[a]	Dose	WT mice		*Fmr1^{KO}* mice	
		Mean plasma concentration (ng/mL) ± SEM	Median plasma concentration (ng/mL) (range)	Mean plasma concentration (ng/mL) ± SEM	Median plasma concentration (ng/mL) (range)
MPEP	10 mg/kg	86.4 ± 38.7	54.7 (28.6–239.1)	50.0 ± 9.7	38.2 (8.6–112.6)
	30 mg/kg	996.0 ± 111.4	1027.8 (426.5–1455.8)	662.5 ± 59.1	724.1 (429.6–891.8)
MRZ-8456	1 mg/kg	18.5 ± 5.1	14.2 (12.6–28.7)	32.2 ± 15.1	16.8 (0–119.1)
	3 mg/kg	122.3 ± 44.2	86.1 (0–307.5)	34.5 ± 7.5	21.6 (10.8–70.7)
	10 mg/kg	ND	ND	232.4 ± 79.0	172.1 (80.1–518.1)
AFQ-056	1 mg/kg	8.0 ± 8.0	8.0 (0–16)	14.4 ± 3.7	14.6 (0–36.5)
	3 mg/kg	71.7 ± 19.6	52.0 (30.8–158)	52.8 ± 10.0	42.6 (15.4–111.1)
	10 mg/kg	126.9 ± 41.2	130.4 (0–252.4)	85.9 ± 23.2	60.5 (10.8–215.1)

[a] To obtain the concentrations in nM, the values in ng/mL should be multiplied by a factor of ~4.4 for MPEP, ~2.7 for MRZ-8456, and ~3.2 for AFQ-056.
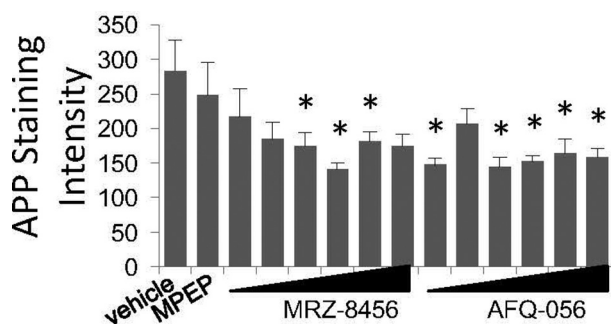

Fig. 3. mGluR$_5$ NAMs reduce neuronal APP expression in *Fmr1^{KO}* mice. Primary cultured *Fmr1^{KO}* neurons were treated with MPEP (2.5 μM) *versus* MRZ-8456 and AFQ-056 over concentration ranges of 0.0625–2.5 μM. Average APP staining intensities of 4–7 dendrites per cell for 6 cells (3 cells per slide, 2 slides per treatment) were plotted against drug treatment. Statistical significance was determined by ANOVA ($P = 0.0007$, F = 2.75, R^2 = 0.002) and post-hoc *t*-test analysis (vehicle *versus*: 2.5 μM MPEP, $P = 0.59$; 0.0625 μM MRZ-8456, $P = 0.27$; 0.125 μM MRZ-8456, $P = 0.056$; 0.25 μM MRZ-8456, $P = 0.014$; 0.5 μM MRZ-8456, $P = 0.00074$; 1.0 μM MRZ-8456, $P = 0.022$; 2.5 μM MRZ-8456, $P = 0.082$; 0.0625 μM AFQ-056, $P = 0.0022$; 0.125 μM AFQ-056, $P = 0.11$; 0.25 μM AFQ-056, $P = 0.019$; 0.5 μM AFQ-056, $P = 0.00032$; 1.0 μM AFQ-056, $P = 0.032$; 2.5 μM AFQ-056, $P = 0.0010$).

These mice are the most widely used animal model for FXS and exhibit many of the physical and behavioral characteristics of humans with the disorder including lower seizure threshold and abnormal dendritic spine morphology. The mouse model has good face validity in terms of FXS phenotypes, but poor predictive validity in translating promising preclinical pharmaceutical drugs to the clinic. MPEP is a research grade drug that reduces AGS, anxiety and dendritic spine protrusion phenotypes in *Fmr1^{KO}* mice (de Vrij et al., 2008; Yan et al., 2005) as well as repetitive self-grooming behavior in the BTBR mouse model of autism (Silverman et al., 2010). AFQ-056 is Novartis' lead mGluR$_5$ NAM (Gomez-Mancilla et al., 2014). MRZ-8456 is under development by Merz for the treatment of motor complications of L-DOPA in Parkinson's disease. MRZ-8456 interacts with human mGluR$_5$ at the same binding site as MPEP with a Ki of 27 nM and inhibits glutamate-stimulated phosphatidylinositol (PI) hydrolysis with an IC$_{50}$ of 13 nM (Merz, unpublished data). In comparison, AFQ-056 has a Ki of 47 nM for human mGluR$_5$ and an IC$_{50}$ of 30 nM in the PI turnover assay (Vranesic et al., 2014). Pharmacokinetic experiments in mice and rats show that MRZ-8456 has a half-life of c.a. 2 h in blood (i.v. administration) compared to AFQ-056 (0.2 h i.v.) with no detectable drug level 24 h after oral administration of 75 mg/kg (Levenga et al., 2011; Merz, unpublished data). Based on these data, the chosen concentrations of mGluR$_5$ antagonists MRZ-8456, AFQ-056 and MPEP were investigated in WT and *Fmr1^{KO}* mice. Plasma samples were collected ~30 min after substance administration, directly following behavioral testing (AGS). The study

confirmed the dose-dependent exposure of *Fmr1^{KO}* and WT C57/BL6 mice to MRZ-8456, AFQ-056 and MPEP. MRZ-8456 reached concentrations which were generally ~3 fold lower (ranging from ~50 to ~500 ng/mL, median ~200 ng/mL) than the ones observed with effective doses in rat models of neurological disorders (*e.g.*, L-DOPA-induced dyskinesia), typically reaching ~700 ng/mL (Merz, unpublished data). Nevertheless, the 2 highest doses of MRZ-8456 effectively suppressed AGS in *Fmr1^{KO}* mice. The apparent discrepancy may result from different pharmacokinetics of the substances in rats and mice. Moreover, it is conceivable that the pharmacodynamics of the drug may be different in various diseases and disease models. In particular, FXS patients and *Fmr1^{KO}* mice exhibit pathological alterations in mGluR$_5$ function and/or density (Dolen and Bear, 2008; Giuffrida et al., 2005; Jacquemont et al., 2011; Krueger and Bear, 2011). The pharmacokinetic data in Fig. 2 and Table 3 suggest that there may be higher mGluR$_5$ NAM levels in the plasma of WT mice compared to *Fmr1^{KO}*; however, due to high variability between animals, the trends were not statistically significant. With *in vivo* seizure testing, MRZ-8456 and AFQ-056 both attenuated wild running and AGS in *Fmr1^{KO}* mice.

Accumulating evidence suggests that dysregulated levels of APP and Aβ contribute to the impaired synaptic plasticity and seizure incidence observed in several neurological disorders including FXS (Westmark, 2013). We have demonstrated that mGluR$_5$ blockade inhibits the synthesis of APP (Westmark and Malter, 2007) and that several FXS phenotypes are rescued by genetic reduction of APP levels in mice (*Fmr1^{KO}/APP^{HET}*) (Westmark et al., 2011; Westmark et al., 2016). In addition, FXS subjects and *Fmr1^{KO}* mice exhibit altered levels of APP and metabolites (Westmark and Malter, 2007; Westmark et al., 2011; Ray et al., 2016; Westmark et al., 2016b), and APP levels can be modulated by acamprosate treatment in FXS patients (Erickson et al., 2014). APP functions in dendritic spine formation, neurite motility, synapse formation, synaptic transmission, and learning and memory (Hoe et al., 2012). Both *Fmr1^{KO}* mice and patients with FXS have long thin dendritic spines consistent with an immature spine phenotype that likely underlies defective synaptic plasticity. Published studies have shown rescue of immature spine phenotypes with fenobam, MPEP and AFQ056 (de Vrij et al., 2008; Levenga et al., 2011), and the ratio of immature to mature dendritic spines is rescued in *Fmr1^{KO}/APP^{HET}* mice (Westmark et al., 2011). We demonstrate that both MRZ-8456 and AFQ-056 significantly reduce dendritic APP expression as well as rescue dendritic spine length and the percentage of mature spines. Thus, APP is implicated in FXS pathogenesis and is a potential therapeutic target as well as biomarker for FXS.

MPEP was not effective in the current *in vitro* study, but was active in the *in vivo* AGS study. We expect that the different solvents used to dissolve/suspend the drugs between the *in vitro* and *in vivo* work affected the activity of the MPEP. The MRZ-8456 and AFQ-056 compounds are not aqueous soluble. For the *in vivo* work, these drugs as well as the MPEP were prepared as fine suspensions in 1% HPMC/1%
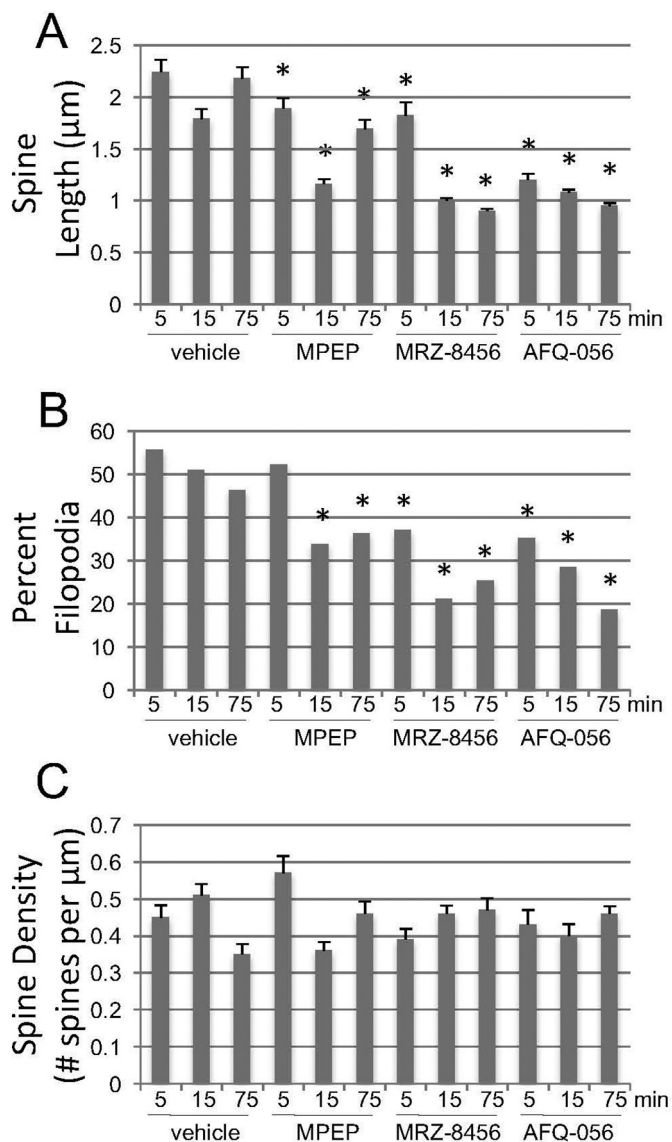
Fig. 4. mGluR$_5$ NAMs rescue dendritic spine phenotypes in *Fmr1*^{KO} mice. (A) The lengths of dendritic protrusions were quantitated with Stereo Investigator® software and plotted against compound treatment. Error bars represent SEM. Statistical significance was determined by ANOVA ($P < 0.0001$, F = 40, R2 = 0.07) and post-hoc *t*-tests (vehicle *versus* MPEP, MRZ-8456 and AFQ-056 at 5 min $P < 0.03$; vehicle *versus* MPEP, MRZ-8456 and AFQ-056 at 15 min, $P < 5E$-9; vehicle *versus* MPEP, MRZ-8456 and AFQ-056 at 75 min, $P < 0.001$). (B) The percentage of filopodia was plotted against compound treatment. Filopodia were defined as protrusions with a width-to-length ratio less than or equal to 0.5. Statistical significance was determined by Chi square analysis ($P = 0$ for all treatments marked with asterisks as compared to vehicle). (C) Spine density (# of spines per length of spine) was plotted as a function of compound treatment. Multiple areas of multiple cells were assessed for each treatment. Error bars represent SEM.

Tween-80 using a IKA-Ultra Turrax mill. For the *in vitro* studies, the detergent in the 1% HPMC/1% Tween-80 was expected to lyse the cells. Thus, the MRZ-8456, AFQ-056 and MPEP were dissolved in a small volume of DMSO and then diluted in HBSS prior to treating the neuronal cells. The final concentration of DMSO on the cells was 0.025% and there was no evidence of the drugs precipitating out of solution upon dilution of the DMSO stocks with HBSS. In previous studies treating primary cultured neurons, we have dissolved and diluted the MPEP in HBSS, but in this case, we prepared all of the drugs in DMSO, which was required to dissolve the MRZ-8456 and AFQ-056. Thus, we

speculate that the DMSO affected the activity of the MPEP and precluded comparison of the efficacy of the test drugs with MPEP in the *in vitro* studies. In the *in vivo* studies, the lowest dose of MPEP tested in the AGS protocol was 10 mg/kg, which was active. In the literature, 30 mg/kg MPEP is routinely used to inhibit AGS; thus, our data suggest that a dose response curve with lower concentrations of MPEP is required to determine if the new compounds are more effective than MPEP.

FXS clinical trials have been completed with fenobam, STX107, R04917523 (a.k.a. basimglurant) and AFQ-056 (Hagerman et al., 2014). An open-label pilot trial of fenobam in 12 patients with FXS showed improvement in prepulse inhibition (PPI) (Berry-Kravis et al., 2009). A phase I trial of STX107 in FXS passed safety testing, but a phase 2 trial to assess tolerability and pharmacokinetic outcomes was suspended. R04917523 showed a favorable safety profile in an initial phase 2 trial of 40 adults with FXS, but a 12-week, double-blind, parallel-group study of 183 adults and adolescents with FXS testing behavioral symptoms using the Anxiety Depression and Mood Scale showed did not demonstrate improvement over placebo (Youssef et al., 2018). A randomized, double-blind, two-treatment, two-period, crossover clinical trial of 30 male FXS patients ages 18–35 years indicated that AFQ-056 was associated with improvement in Aberrant Behavior Checklist-Community Edition (ABC-C) scores in an exploratory analysis of the subset of FXS patients with full methylation of the *FMR1* promoter (Jacquemont et al., 2011); however Novartis will no longer continue long-term extension studies of AFQ-056 in FXS because phase IIb/III studies did not meet the primary endpoint of significant improvement in abnormal behaviors compared to placebo (Scharf et al., 2015; Bailey Jr et al., 2016; Berry-Kravis et al., 2016).

Merz Pharmaceuticals completed phase I clinical trial testing of MRZ-8456 as part of profiling of this compound for the treatment of dyskinesia in Parkinson's disease patients. Dyskinesia is the uncontrolled, over-reactive movements that occur in patients with Parkinson's disease after years of treatment with levodopa. The coadministration of mGluR$_5$ NAMs and L-DOPA is a potential therapeutic strategy for reducing L-DOPA-induced dyskinesias (LIDs). The hypothesis is that mGluR$_5$ NAMs can increase the L-DOPA therapeutic window thus alleviating dyskinesia and allowing decreased dosing frequency of L-DOPA (Petrov et al., 2014). MRZ-8456 was well tolerated and exhibited a good pharmacokinetic profile (Dekundy et al., manuscript in preparation). Novartis discontinued clinical trials of AFQ-056 for the treatment of LID due to lack of efficacy in trials NCT01385592 and NCT01491529.

It is important to study multiple mGluR$_5$ inhibitors, which differ in their binding sites and efficacy, in both FXS and dyskinesia. The original mGluR$_5$ antagonist MPEP exhibits significant off-target effects precluding its use in humans. It is both a NAM of glutamate at mGluR$_5$ as well as a positive allosteric modulator (PAM) of L-AP4 at mGluR$_4$ (Mathiesen et al., 2003). AFQ-056 is the frontrunner among mGluR$_5$ NAMs currently being developed for FXS. AFQ-056 is a chemical derivative of MPEP that differs by the addition of a carbamate group, acetylene groups, and aromatic substitutions. It binds deeper with the 7-transmembrane bundle increasing selectivity within the common binding pocket and has a narrower range of side effects (Gregory and Conn, 2015). Thus, AFQ-056 is a vast improvement over MPEP; however, AFQ-056 may have limited tolerability. In the phase IIb NCT00986414 clinical trial in patients with PD and moderate-to-severe LID, there were serious adverse effects including the death of one patient, an event suspected to be treatment-related. The patient had been randomized to the 100 mg daily AFQ-056 cohort and he died suddenly on day 19 after having received AFQ-055 50 mg daily on day 1 and AFQ-056 100 mg daily since day 15. He reported side effects of visual hallucinations and insomnia (Stocchi et al., 2013). MRZ-8676 (6,6-dimethyl-2-phenylethynyl-7,8-dihydro-6H-quinolin-5-one) exhibits potent antidyskinetic effects in the rat model of LID whereas AFQ-056 only had a modest effect (Dekundy et al., 2011, Sagarduy et al., 2010). MRZ-8456 differs structurally from AFQ-056 and MRZ-8676 in that it

does not contain a triple bond and thus does not have reactivity issues. In addition, MRZ-8456 binds to mGluR$_5$ with comparable affinity as MPEP but has less pronounced solubility problems.

Considering the high costs of drug development, it is highly advantageous to both pharmaceutical companies and families with rare disorders if effective test compounds can be purposed for multiple disorders. Herein, we provide preclinical validation data comparing the efficacy of MRZ-8456 with AFQ-056 in *Fmr1^KO* mice. These data contribute to a rapidly growing body of preclinical data supporting the use of mGluR$_5$ NAMs in the treatment of FXS. In addition to the phenotypes rescued in the *Fmr1^KO* mice, mGluR$_5$ NAMs reduce repetitive behaviors and rescue social deficits in mouse models of autism (Silverman et al., 2010; Silverman et al., 2012) as well as rescue memory deficits and decrease Aβ oligomer concentrations and plaque formation in Alzheimer's disease mice (Hamilton et al., 2014; Hamilton et al., 2016). Thus, the development and validation of novel mGluR$_5$ NAMs may benefit multiple CNS disorders. It will be of interest to study behavioral alterations in future studies, particularly considering that Novartis stopped its clinical trial of AFQ-056 in FXS due to the lack of sufficient effects on abnormal behavior. Of note, AGS testing occurs in juvenile mice, and the younger age may correlate better with drug efficacy than behavioral studies in adult animals.

In conclusion, NAMs of mGluR$_5$ are under investigation for the treatment of a wide range of CNS disorders including anxiety, pain, depression, post-traumatic stress disorder, schizophrenia, FXS, Alzheimer's disease and Parkinson's disease (Gravius et al., 2010; Westmark, 2014). Regarding FXS, many mGluR$_5$ inhibitors have shown promise in clinical trials. Herein, we compared the efficacy of a novel NAM of mGluR$_5$ developed by Merz Pharmaceuticals, MRZ-8456, with Novartis' AFQ-056, the leading mGluR$_5$ NAM tested in clinical trials. Both MRZ-8456 and AFQ-056 were effective in attenuating *in vitro* and *in vivo* FXS phenotypes in a mouse model of the disorder. Both drugs were effective at 3 and 10 mg/kg in the *in vivo* study. With the *in vitro* work, AFQ-056 was more effective in rescuing spine length with a shorter treatment period than MRZ-8456 and may have been more effective in reducing APP expression at a lower drug dose.

Declaration of interest

Andrzej Dekundy, Andreas Gravius and Wojciech Danysz are employees of Merz Pharmaceuticals Gmbh. The study was single blinded. Persons conducting the experiments were blinded to the identity of the compounds until after data acquisition and analysis.

Author contributions

CW, AD, AG, WD conceived and designed the experiments. CW, PW acquired data. CW, AD interpreted data. CW drafted the manuscript.

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Acknowledgments

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