



# The Use of Peptides in the Treatment of Fragile X Syndrome: Challenges and Opportunities

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Fragile X Syndrome (FXS) is the most frequent cause of inherited intellectual disabilities and autism spectrum disorders, characterized by cognitive deficits and autistic behaviors. The silencing of the *Fmr1* gene and consequent lack of FMRP protein, is the major contribution to FXS pathophysiology. FMRP is an RNA binding protein involved in the maturation and plasticity of synapses and its absence culminates in a range of morphological, synaptic and behavioral phenotypes. Currently, there are no approved medications for the treatment of FXS, with the approaches under study being fairly specific and unsatisfying in human trials. Here we propose peptides/peptidomimetics as candidates in the pharmacotherapy of FXS; in the last years this class of molecules has catalyzed the attention of pharmaceutical research, being highly selective and well-tolerated. Thanks to their ability to target protein-protein interactions (PPIs), they are already being tested for a wide range of diseases, including cancer, diabetes, inflammation, Alzheimer's disease, but this approach has never been applied to FXS. As FXS is at the forefront of efforts to develop new drugs and approaches, we discuss opportunities, challenges and potential issues of peptides/peptidomimetics in FXS drug design and development.

**Keywords:** Fragile X syndrome, targeted therapy, peptides, peptidomimetics, drug development

## INTRODUCTION

Fragile X syndrome (FXS) was first described in 1943 and it is now established as the most common cause of inheritable intellectual disabilities (ID) (1). The molecular cause of FXS is the extensive repeat expansion of a CGG triplet (200 repeats in the full mutation) in the 5' untranslated region (UTR) and consequential hypermethylation of the *Fmr1* gene, finally leading to transcriptional silencing of the fragile X mental retardation protein (FMRP) (2–4). A small proportion of individuals affected by FXS with different levels of severity show deletion or point mutation in the *Fmr1* gene, that in turn cause the complete loss of FMRP or the production of a functionally deficient protein (5–7).

This disease affects 1:4,000 males and 1:6,000–8,000 females (8, 9) showing symptoms from moderate to severe ID (10). The clinical picture of the syndrome is complex; FXS phenotype displays characteristics in common with autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD), with general anxiety, social avoidance and hyperactive behaviors (11–13). Seizures, recurrent otitis media, strabismus and obesity are also often occurring in patients affected by FXS. Besides, about 10% of males with FXS display a Prader-Willi like phenotype (14).

The physical features of FXS comprehend elongated face, broad forehead, high palate, prominent ears, hyperextensible finger joints, flat feet and macroorchidism (15). All these behavioral, phenotypical and clinical characteristics of FXS, are due to the lack of FMRP, a well-characterized RNA-binding protein, showing crucial functions mainly related to mRNAs metabolism (16). Its main role is represented by the translational repression of numerous key mRNAs in pre- and postsynaptic neurons (17, 18). The FMRP deficiency results in increased protein synthesis, causing the upregulation of several signaling effectors, such as excitatory metabotropic glutamate receptor (mGluR) (19, 20),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (21), extracellular signal-related kinase (ERK1/2) (22–24), matrix metalloproteinase 9 (MMP-9) (25–27), brain-derived neurotrophic factor (BDNF) (28, 29), and mammalian target of rapamycin (mTOR) (30, 31). Moreover, functional impairment in gamma-aminobutyric acid (GABA) receptor and in the endocannabinoid system have been also documented in FXS (32–34). In healthy conditions all these machineries orchestrate neurotransmission and local protein synthesis that impact synaptic plasticity, learning and memory. Hence, in FXS pathology, the lack of FMRP leads to increased protein synthesis with a direct effect on dendritic spine dysgenesis and cognitive disabilities (9, 17), causing the majority of the FXS symptoms. Evidences from *Fmr1* knockout (KO) mice and from human post-mortem brain biopsies showed increased amount and length of dendritic spines, with an immature profile (35–37).

FMRP inhibits translation initiation through its interactions with eIF4E (Eukaryotic translation Initiation Factor 4E) and CYFIP1 (Cytoplasmic FMRP Interacting Protein 1) (4, 7, 38–40). eIF4E is the cap-binding protein known to be activated by the interaction with the scaffold protein eIF4G (Eukaryotic translation Initiation Factor 4G) or inhibited by 4E-binding proteins (4E-BPs), these last being a well-characterized group of proteins that repress protein synthesis (41, 42). The 4E-BPs and eIF4G compete for the same binding site on the eIF4E surface; thus, 4E-BPs inhibit the eIF4E-eIF4G complex formation by sequestering the unbound eIF4E (43, 44). CYFIP1 belongs to the 4E-BPs family and in neurons, mainly at synapses, the FMRP-CYFIP1-eIF4E inhibitory complex regulates protein synthesis during synaptic activity, playing a pivotal role in the modulation of long-term synaptic plasticity at synapses (18, 38). Moreover, the CYFIP1 paralog CYFIP2 is itself able to interact with FMRP and with the FMRP-related proteins FXR1P/2P, which are cytoplasmatic proteins that share with FMRP the functional domains deputed to promote homo- and heteromerization (45, 46).

## CURRENT STRATEGIES IN FXS TREATMENT

Recently, strong effort was dedicated to develop specific FXS pharmacological treatment that can lead to a possible cure, or at least alleviate symptoms (47–51). The most promising or studied treatments for FXS are listed in **Table 1**. However, although several therapeutic approaches are being tested on different FXS

animal models (i.e., *Fmr1* KO mouse, rat and zebrafish; *dFmr1* null mutant fly) and patients over the years, an approved and successful curative therapy for FXS is missing to date, and the management of the clinical aspects of the syndrome continue to focus on symptomatic treatment of psychiatric and behavioral problems, rather than the molecular causes (49, 50, 52).

One of the first approaches that was suggested for FXS treatment was the *Fmr1* gene activity restoration through changes in the DNA methylation levels and epigenetic modifications (53, 54). Although different compounds were tested and successfully achieving *in vitro* reactivation of the *Fmr1* gene, such as 5-azadeoxycytidine (5-azadC) (53), this strategy has not been tested with *in vivo* studies due to safety problems related to low reactivity and high toxicity of these chromatin-modifying enzymes inhibitors (55). Similarly, another strategy involves the use of non-coding RNAs to affect DNA methylation state and histones modification (56). Based on the promising results obtained in cancer and other diseases (57), several miRNAs and lncRNAs were identified and tested in different FXS models (58–60), but their potential use in clinical therapy is still far away similarly to the modern application of gene therapy methods to restore the *Fmr1* gene (61). Indeed, independent groups demonstrated the possibility to use viral-vectors or CRISPR-technology, with encouraging results in preclinical FXS models (62–67); however, the clinical application in patients is being debated for several undisclosed questions, as safety and brain-targeted delivery. Regarding this approach it is also important to consider that the reactivation of the *Fmr1* mRNA with the full mutation could be toxic, as it was demonstrated by a correlation detected between the *Fmr1* mRNA levels in blood and more severe autism features (68).

However, since several compounds are used to treat behavioral and mental problems, such as stimulants or antipsychotics (69), the majority of pharmacological efforts are employed to compensate the absence of FMRP. Among targeted treatment for FXS, several focused on the neurotransmission imbalance associated with FXS. Particular attention has been dedicated in testing the group 1 metabotropic glutamate receptors 5 (mGluR5) antagonists, such as AFQ056/Mavoglutarant, Fenobam, MPEP, STX107, CTEP, RO4917523 (9, 10, 17, 19), and GABA receptors (GABA<sub>A</sub> and GABA<sub>B</sub>) agonists (70). In *Fmr1* KO mice these agents showed improvement of several FXS features, including better behavioral abilities, restoration of normal levels of dendritic spines and reduction in protein synthesis (9, 17, 69). Despite these positive results, the transition from animal to human model did not give the same encouraging outcomes, since most of clinical trials failed (71, 72). The high placebo response and the imprecise design and methodology of the trials were the major causes of failure.

Most of drugs tested in FXS pharmacotherapy are compounds already employed or approved for other disorders. Sertraline is a serotonin reuptake inhibitor approved for treating anxiety and mental disabilities in young children and tested in *Fmr1*-KO mouse model. Sertraline normalizes serotonin and dopamine levels, with a rescue on synapse and dendritic formation (73, 74). Even in FXS patients, Sertraline showed favorable results, as several studies demonstrated improvements

in language, anxiety and social conduct (75, 76). Cannabidiol (CBD), a synthetic molecule active on cannabinoid receptors, has been used for the treatment of neurological disorders, such as Huntington, Parkinson's and Alzheimer's diseases, but also epilepsy, schizophrenia, autoimmune diseases. All these pathologies have in common altered endocannabinoid signaling pathway, condition confirmed to be deregulated also in FXS animal model (77, 78). Clinical studies indicated good results (79–81), albeit with tolerated side effects. The following FDA-approved drugs have been tested in FXS preclinical and clinical studied: acamprosate (for maintenance of alcohol abstinence), lovastatin (for hypercholesterolemia), minocycline (for acne) and metformin (for non-insulin diabetes mellitus). In particular lovastatin targets the RAS-MAPK-ERK1/2 pathway (82, 83) while minocycline inhibits the MMP-9 activity (25). Both compounds showed promising results in preclinical testing using different model systems, with a reduction in protein synthesis and beneficial cognitive and behavioral aspects (25, 82, 84, 85). Nevertheless, these encouraging data were followed by moderate effects in trials on FXS patients, also expressing the need for a more in-depth investigation on the tolerability of these compounds (86–88). To date, the anti-diabetes drug metformin could be considered as one of the most promising treatments for FXS (89, 90). It has different mechanisms of action, depending on dosage and treatment time, including inhibition of mammalian/mechanistic target of rapamycin complex 1 (mTORC1) and mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) pathways, both hyperactivated due to the lack of FMRP in FXS humans and mice (91, 92). As a consequence, metformin also affects the proteins downstream to these cascades, reducing specifically the eIF4E phosphorylation and the translation of MMP-9 (93), which in the pathological condition is the cause for the degradation of proteins essential for synaptic maturation and activity (27). Preclinical studies were performed on *Fmr1*-KO flies and mice models of FXS, showing a rescue of dendritic spine morphology, long-term depression (LTD) of synapses, but also improvement in cognitive, intellectual and social deficits (90, 94). These findings paved the way for treatments in humans, where clinical trials starting in 2018 have been conducted with promising benefits both in terms of behavior and safety of treatment (93, 95–97). Currently 3 trials aimed to evaluate safety, tolerability, and efficacy of metformin in FXS patients are ongoing (ClinicalTrials.gov Identifier: NCT04141163, NCT03862950, NCT03479476). Another interesting therapeutic target in FXS is represented by phosphodiesterases (PDEs), a family of enzymes that regulate the cellular levels of cAMP and cGMP. Among PDEs, PDE1A, PDE2A, and PDE10A have been identified as mRNA targets of FMRP (98). Accordingly, decreased cAMP levels were observed in fly and mouse FXS models and a deregulation of cAMP and cGMP was also identified as a molecular hallmark in FXS patients (99, 100). Hence, several inhibitors have been tested, starting from *Drosophila* model of fragile X, passing through *Fmr1*-KO mice, finally to human trials. Interestingly, inhibiting PDE4 (101, 102), PDE2A (103, 104), PDE4D (105, 106), or synergistically PDE2 and PDE4 (107)

demonstrated beneficial effects in terms of rescue of social and behavioral impairments and in dendritic spines morphology in fly and mouse models. Cognitive enhancements were pointed out from FXS trials (105, 106), suggesting that PDEs are candidate targets to develop FXS therapeutic strategy.

## PEPTIDES/PEPTIDOMIMETICS: A FEASIBLE STRATEGY FOR FXS TREATMENT

All the strategies mentioned so far target different pathways, whose uncontrolled activity seems to be crucial in the pathology of FXS, but also in other neurological disorders and types of cancer (108, 109), leading to pleiotropic effects. Accordingly, the lack of specificity and selectivity, together with bioavailability and safety problems, could be the main drawbacks of these approaches.

In this scenario, a novel and feasible option in FXS pharmacotherapy could be the use of peptides or peptidomimetics.

Since the last 30 years, and especially in the past decade, severe pathologies are being treated with peptides (110) and this class of molecules have attracted the attention of either academia researchers or pharmaceutical industries. Indeed, the global Peptide Therapeutics market reached USD 25.35 billion in 2018 and is expected to achieve USD 50.60 billion by the year 2026. To date, 400–600 peptides are in the preclinical phase of development and more than 60 peptides are FDA-approved (111). The main fields in which therapeutic peptides are currently in development are oncology, metabolic diseases and inflammation (110). Peptides represent an attractive pharmaceutical source due to their excellent properties, namely high selectivity, safety and tolerability (112). However, this kind of approach has never been applied to FXS to restore the imbalance in protein synthesis. Very recently novel structural information opened new possibilities for developing inhibitors acting on the mRNAs translation initiation complex with high specificity and efficiency.

Among these compounds, the 4EGI-1 is one of the most promising inhibitors of the translation activation complex and it has been already tested in different cancer models (113–116). This molecule was also proved to reduce the eIF4E-eIF4G interaction in a FXS mouse model (117). However, the lack of drug-like characteristics, such as poor target specificity and selectivity, high toxicity, several off-targets, severe side effects, poor metabolic stability, poor membrane permeability and rapid proteolysis, make this molecule fairly unsuitable candidates for therapeutic applications.

Furthermore, the putative molecules effective in disrupting the FMRP-CYFIP1-eIF4E or eIF4E-eIF4G complexes formation, are required to target protein-protein interaction (PPI) interfaces, that are large, flat and hydrophobic binding surfaces considered as “undruggable” by small compounds (118–120). One solution could be represented by antibodies, more powerful in targeting PPI, but anyway scarcely able to cross the cell membrane to perform their specific function. In light of this, peptides are

**TABLE 1** | Treatments for Fragile X syndrome.

Approach	Name	Agent class	Mechanism of action	
<i>Fmr1</i> gene activity restoration	5-azadeoxycytidine (5-azadC)	Chromatin-modifying enzymes inhibitor	Affects DNA methylation levels and epigenetic modifications	
		Non-coding RNA (miRNAs and lncRNAs)	Affect DNA methylation state and histones modification	
		Viral-vectors or CRISPR-technology	Gene editing or gene replacement	
Targeted therapy	AFQ056/Mavoglutarant, Fenobam, MPEP, STX107, CTEP, RO4917523	Group 1 metabotropic glutamate receptors 5 (mGluR5) antagonists	Block mGluRI signaling	
		OV101/gaboxadol, Ganaxolone	GABAa and GABAb agonists	Modulate GABA receptors
		Sertraline	Serotonin reuptake inhibitor	Normalizes serotonin and dopamine levels, stimulation of BDNF
	Cannabidiol	Cannabinoid receptors inhibitor	Regulates endocannabinoid signaling pathway	
	Lovastatin	RAS signaling inhibitor	Regulates RAS-MAPK-ERK1/2 pathway	
	Minocycline	Semi-synthetic tetracycline derivative	Regulates MMP-9 activity	
	Metformin	Derivative of guanidine	Normalizes mTOR and MAPK/ERK pathways, phosphorylates eIF4E, and lowers expression of MMP9	
Symptomatic treatment	Bay 60–7550, BNP14770, Roflumilast	PDE4, PDE2A, PDE4D inhibitors	Normalize cAMP and cGMP signaling	
	Risperidone and aripiprazole	Antipsychotics	Impacts dopaminergic and serotonergic neurotransmission to treat irritability	

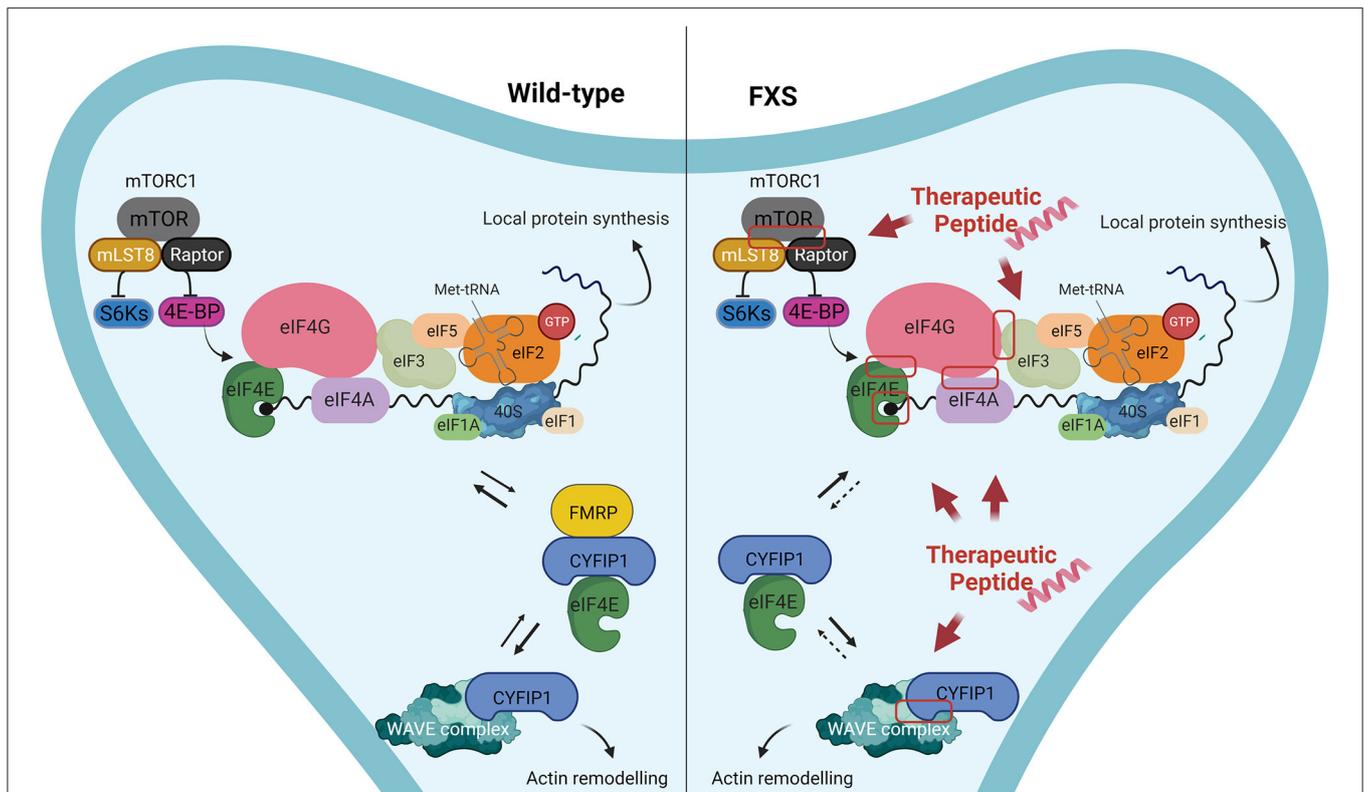
now considered as the most appropriate candidates to regulate disease-associated PPIs. However, peptides have intrinsic weak points, and they did not provide encouraging results *in vivo*, likely due to their physical, chemical and structural instability and low membrane permeability (112, 119, 121). To overcome these possible limitations several strategies have been developed, such as amino acids substitution with residues mimicry, termini protection or introduction of chemical modifications aimed at stabilizing their active conformation and increasing cellular permeability (119, 121). These advances in the peptides technology results in the development of an alternative class of compounds called peptidomimetics, that is recently emerging as a class of new potential therapeutic molecules able to target PPIs in the treatment of different pathologies (112). Peptidomimetics are organic molecules with physico-chemical features and structural characteristics comparable with classical oligopeptides, but guarantee enhanced protection against peptidases, improved systemic delivery and cellular uptake, high target specificity and poor immune response (122), and for these reasons their use is under investigation for the treatment of cancer, ischemia, Alzheimer's disease (123–127) and other neurodegenerative disorders (128–131).

On the contrary, the use of peptides/peptidomimetics has never been investigated in the FXS context, but it could represent a viable solution as it might result in a compensation of FMRP absence. Indeed, restoring the FMRP-CYFIP1 deficiency *via* a small chimeric peptide acting on the dysregulation of protein synthesis could be central for the new FXS pharmacological therapy development.

Although the 3D structure of FMRP-CYFIP1 or FMRP with other interacting proteins are still not available, there is a growing number of structures, from different organisms, of complexes

belonging to the translation initiation pathway, in particular eIF4G/eIF4E and eIF4E/4E-BPs, and of their regulatory proteins (43, 44, 132, 133). The plethora of structural information, together with the increasing power of computational facilities and refinement of binding prediction tools (110, 112, 119), open the possibility to design peptides/peptidomimetics able to target the translation complexes, with the aim to decrease protein synthesis of specific neuronal mRNAs and rescuing a healthy phenotype in individuals affected by FXS.

For instance, peptides could target and affect the assembly of the eukaryotic Initiation Factor 4F (eIF4F) complex (composed by eIF4E, the DEAD-box helicase eIF4A and the scaffold protein eIF4G) (41, 42), and the formation of 43S pre-initiation complex (43S PIC), composed by small ribosomal subunit 40S and the eukaryotic translation initiation factors (eIFs): eIF1, eIF1A, eIF3, eIF5 (41, 42) (**Figure 1**). In addition to those already mentioned, the PPIs that could be targeted and disrupted by peptides, could be for instance, eIF4E/cap, eIF4A/eIF4G or eIF4G/eIF3 dimers (**Figure 1**). Alternatively, peptides/peptidomimetics could be designed to target the eIF4E-upstream regulators, as the PI3K–mTOR pathway, likely inhibiting the interactions of mTORC1 with Raptor and other partners, affecting the activity of the downstream S6K or 4E-BP proteins (**Figure 1**) (134). Furthermore, not only protein synthesis but also actin dynamic imbalance concurs to the pathophysiology of FXS, leading to defects in dendritic spines morphology (135, 136). Although much information is still lacking, there are indications that Rac1–PAK pathway and FMRP are linked (137, 138). Active Rac1, the Rho-family of small GTPases, activates p21-activated kinases (PAKs) which in turn phosphorylates Cofilin, an actin-binding protein that regulates actin turnover. Rac1 also activates the Wave Regulatory Complex (WRC, composed by five proteins:



**FIGURE 1** | Proposed examples of protein-protein interactions that could be targeted by peptides/peptidomimetics in Fragile X Syndrome. Left panel: In wild-type neurons FMRP plays a key role in down-regulating the translation of FMRP targets, by forming a complex with CYFIP1 and the eukaryotic initiation factor 4E (eIF4E). Furthermore, CYFIP1 can bind and inhibit the WAVE regulatory complex, thereby regulating actin remodeling. Right panel: In FXS neurons the absence of FMRP leads to increased local protein synthesis in neurons, due to the lack of the formation CYFIP1-FMRP-eIF4E complex, that represses the translation initiation. Consequently, a bigger amount of eIF4E is bound to eIF4G, while CYFIP1 interacts mostly with WRC-Rac1-GTP, resulting in higher levels of protein synthesis and altered actin remodeling at dendritic spines. Examples of macromolecular complexes that could be disrupted by peptides/peptidomimetics with the aim to restore the FMRP activity are highlighted by a red box.

CYFIP1, NCKAP1, Abi2, HSPC300, and WAVE1) by directly binding CYFIP1 and leading to Arp2/3 complex-mediated actin polymerization (139–141). The release of the X-ray structure of WRC (140), together with data coming from computational analyses that highlighted structural-dynamical features of CYFIP1 (39, 40), may provide useful details to be exploited for the CYFIP1-based peptidomimetics design (Figure 1). To offer some realistic examples, using different regions of CYFIP1 as templates, CYFIP1-derived peptidomimetics could interfere with the eIF4F complex formation by sequestering eIF4E from the binding with eIF4G. Similarly, impeding the CYFIP1/Rac1 interaction or the CYFIP1/NCKAP1 dimer formation, could have a dual beneficial effect in concomitantly restoring normal levels of protein synthesis and actin dynamics, both processes being dysregulated in FXS. Aside from CYFIP1, other 4E-BPs structures in complex with eIF4E are available and represent an attractive template for peptides design. For example, 4E-BP1, 4E-BP2, and Angell1-based peptides were developed and tested in different cancer cell lines (142–144). Furthermore, Lama et al. developed a set of peptides with chemical modifications that increase the pharmacological properties and binding affinity to

eIF4E, providing a strong starting point for future oncological preclinical studies (145–147).

On the basis of the knowledge acquired in cancer research, we assume that the peptides/peptidomimetics approach could also be applied to other diseases, in particular in the FXS pharmacotherapeutic.

## DISCUSSION

In the last decade many efforts were employed in research and development of new pharmacological treatments of FXS and simultaneously great advances were made in developing peptides therapeutics against several diseases. With this perspective we speculate that these two roads might cross, starting a new era of the pharmacotherapeutic approach for patients with FXS. To date, chemical compounds that inhibit several pathways deregulated in FXS represent the most studied approaches for FXS management. However, the cause of FXS is a genetic defect (the *Fmr1* silencing) and to effectively correct the absence of FMRP protein is still a challenge. Moreover, although several

available treatments are effective in animal models, many clinical trials testified lack of success of these approaches. The emerging peptides technology, in combination with increasingly advanced computational approaches and number of proteins structures deposited in databases, provide an alternative and powerful method to develop a more specific and safe molecules targeting those protein complexes that could be considered as the major players in the FXS pathology. The main point of strength of the use of peptides include selectivity, tolerability, predictable metabolism, ability to target PPIs and lower synthesis complexity that in turn leads to lower production costs compared to others pharmaceutical molecules (119, 121). However, as mentioned above, peptides in general, and thus their possible application against FXS, have several weaknesses that is necessary to discuss. Poor *in vivo* stability, membrane impermeability, and toxicity are widely accounted as major drawbacks in peptides technology. Nevertheless, several of these aspects have been successfully sorted out over recent years through the new technologies available in peptides design field: new bioinformatics tools in combination with other approaches such as virtual screening, structure-based drug design, high throughput screening (HTS) and chemical strategies, provide a comprehensive pharmacological description of putative peptides, improving their chemical and physical features (119, 121, 148). Moreover, the treatment of neurodevelopmental disorders, as FXS, require the delivery of molecules to the brain, accounting for the crossing of the blood-brain barrier (BBB), that could limit the access of peptides to the central nervous system (CNS). In this respect, a strategy to overcome these issues could be the exploration of other drugs administration methods, e.g., the non-invasive intranasal delivery (129, 131). The direct access to CNS allows to overcome limitations linked to the degradation, bioavailability problems and also to possible systemic side effects

onset that occur if peptides are present in blood vessels after intravenous administration. Additionally, several strategies have been developed to specifically deliver peptides to target regions of the CNS, such as cyclodextrins, PEI or others (129), resulting in a lower dosage of peptides, also decreasing the toxicity issues which have been demonstrated toward eukaryotic cells (149, 150).

Furthermore, peptides therapy would not be a chronic intervention, but these molecules would be administered only in a limited period of time during the 1st years of life of FXS children, when brain is still remodeling, to allow proper formation of the synaptic network.

Hence, we propose that peptides/peptidomimetics could compensate the FMRP deficiency restoring the imbalance of protein synthesis and actin dynamics, suggesting a new and promising strategy for treating FXS.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

This perspective was conceived by DD and AR. DD and AR wrote the manuscript. Table and figure were prepared by AR. All authors contributed to the article and approved the submitted version.

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