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REVIEW ARTICLE

Drugs Development and Fragile X Syndrome Translational Success Story

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New medication development is a lengthy process with the ultimate goal to bring it to the market and to address unmet clinical needs. The data on the time and resources that a pharmaceutical company invests to develop a new medication is often unavailable or controversial. However, it is a known fact that such complex research takes at least ten years and hundreds of millions of US dollars. In 2010, the average cost for pharmaceutical companies to bring a new drug to the market was estimated to be approximately $1.8 billion [1]. On the other hand, this is 'a nine zeros-fairy-tale' for many people who believe that the drug research and development does not cost as much [2]. Unfortunately, more often than not, these complex research endeavours turn out unsuccessful. For example, results of pre-clinical studies conducted in 2006-2015 show that only 9.6 % of all these studies result in a new drug development [3]. Among them, compelling evidence-based data have emerged in the field of neurodevelopmental disorders. Specifically, Fragile X Syndrome (FXS), which is caused by a deficit of fragile X mental retardation protein (FMRP), takes the lead toward targeted treatments as multiple preclinical studies of drugs modifiers of underlying psychopathology in FXS were followed by multiple clinical trial But, let's start from the beginning...

Pre-clinical studies.

Every research and development (R&D) project that aims to develop a new drug goes through a "funnel" process as it searches for a new compound to become a new drug. Available statistics show that a pharmaceutical company explores 5.000 to 10.000 chemical compounds in the hopes that one of them will become a new drug. On average, after the first positive results from laboratory studies out of 5.000 to 10.000 tested compounds only about 250 chemicals are short-listed in the hope to develop them into medications. Moreover, only about a dozen out of the short-listed ones will be approved for clinical trials in humans after the studies have been completed in strictly controlled laboratory conditions [4].

Regardless, a pre-clinical study covers the interval between the drug development (the synthesis or discovery) of a new chemical compound, and its first application in humans. Sometimes these drugs include substances of natural origin such as plants, microorganisms, invertebrates, etc., or already known drugs whose efficacy needs improvement. On the other hand, sometimes we come across this drugs are *de novo* synthesised and designed. Nowadays, computer programmes are often used in drug design (e.g. computer-aided drug design or *in sillico* drug design). The majority of these pre-clinical studies are conducted in accordance with the guidelines of the International Council on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Guidelines) and Good Laboratory Practice (GLP) [5, 6].

At the beginning of a complex R&D process, there is very little information on the chemical compound that will be studied. Therefore, during a pre-clinical study, it is necessary to study characteristics of the drug: (i) the pharmacodynamics (how the drug affects an organism), (ii) the pharmacokinetics (how an organism affects the drug), (iii) drug absorption, distribution, metabolism and extraction (the so-called ADME), and (iv) its toxicology. Therefore, it is necessary to describe the pharmacological profile of the chemical substance at this stage in order to clearly determine its molecular mass, stability, lypophilicity, ionisation, permeability (using the PAMPA – parallel artificial membrane permeability assay, Caco-2 cell lines, drug permeability assay across the blood-brain barrier), safety of use, etc. Modern approach

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to drug development involves optimising (i) the drug affinity and selectivity intended to reduce its side effects, (ii) efficacy, (iii) bioavailability (the extent and rate at which the drug administered enters systemic circulation), and (iv) metabolism (extending the 'biological half-life,' a pharmacological parameter indicating how long the drug stays in the organism) [7, 8]. Historical data suggest that the synthesis of small molecules affecting a specific target (often referred to as a drug receptor) has been very successful. The story starts with Paul Ehrlich, who has searched for the 'magic bullet' drug [9]. It is also worth mentioning Scottish pharmacologist James Black, whose research has led to the development of beta-blockers and H-2 antagonist cimetidine and Akira Endoa, who developed statins [10, 11]. The search continues for new "targets" that have not yet been identified as drug targets. This primarily refers to newly discovered proteins or proteins whose function has been advanced through R&D [12]. However, these issues are far from being straightforward, which has been shown in the modern pharmacological concept, beyond the postulate set by Paul Ehrlich. Namely, the modern concept of having one drug with multiple targets: a *multi-target-directed ligand*. So far, this concept has been of particular significance in the R&D of drugs in the field of *neuroscience* [13]. Various compounds such as certain enzymes (kinase, metalloproteinase, etc.), cytokines and protein precursors are potential biomarkers. However, reliable and valid biomarkers have yet to be identified for the vast majority of these developing drugs [14]. The benefits of a new drug are usually determined by monitoring the biomarkers, which is important in the assessment of the drug's efficacy. In order to establish target engagement and demonstrate the drug's efficacy, such biomarkers must not only be highly sensitive and specific, but also easy to detect. Thus, identifying a biomarker that can indicate with certainty the mechanism of a drug is often challenging and lengthy process.

In practice, a pre-clinical study is also a multidisciplinary process that includes R&D from different scientific fields. As noted above, compelling evidence-based examples come from pre-clinical studies on a number of drugs for FXS, which is the most common cause of hereditary intellectual disability (ID) and a leading known single gene cause of autism spectrum disorder (ASD) [15-18]. The goal of these pre-clinical studies in FXS is to develop reliable outcome-measures and preferably biomarkers. As reviewed in Budimirovic and Subramanian [17], FXS is caused by silencing of the Fragile X Mental Retardation 1 (*FMR1)* gene, leading to a loss of production of its encoded protein: FMRP. More than 99% of the time, FXS results from an expansion of the CGG triplet repeat in the first exon of the (5'UTR) regulatory region of the *FMR1* gene on the X chromosome. FXS affects 1:2.5-4.000 males and 1:6.000 females across all racial and ethnic groups [17, 19]. FXS is a global neuropsychiatric disorder with abnormalities in signaling pathways coupled to multiple neurotransmitter receptors. FMRP belongs to the family of RNA binding proteins, whose four RNA binding domains can bind RNAs messenger as well as noncoding RNAs [20]. Specifically, FMRP forms a complex with the Cytoplasmic FMRP Interacting Protein 1 (CYFIP1) and the cap-binding scaffolding protein eIF4E, which prevents the formation of active translational initiation complexes, and represses (acts as a "brake") protein synthesis [21]. As a result, the loss of FMRP in FXS leads to "runaway" translation of important synaptic proteins, and subsequently disrupts many neuronal signaling pathways. For example, *up-regulated* are metabotropic glutamate receptor type 5 ($mGluR_5$) and mTOR signaling, and *down- regulated* are GABA and dopaminergic systems. Since FMRP and mGluR5 work in functional opposition, hallmark effects of *FMR1* silencing (no FMRP) are overactive glutamatergic signaling ('mGluR theory'), increased dendritic protein synthesis, and increased density of dendritic spines ("neuronal connections") [22-24]. Because FMRP is an important regulator of both basal and activity-dependent local neuronal protein synthesis and synaptic function, *FMR1* gene mutations can alter the course of brain development, cognition, and behavior throughout life [25].

To expand on the aforementioned, abnormal $mGluR_5$ function has been repeatedly demonstrated in the FX animal 'gold standard' models, namely fmr1-knock out (KO) mice [22]. Animal models of FXS and ASD suggest that glutamatergic dysfunction (overactive mGluR5s), and consequently an excitatory/inhibitory imbalance, represents a fundamental abnormality in both FXS and ASD [22, 26-31]. mGluR5s are G protein-coupled receptors coupling through Gαo/q that internalize for endocytosis and then reconstitute to appear on the cell surface [32,33]. Through downstream signaling, $mGluR_5$s activate the translation and the transcription of proteins necessary for synapse in the cortex, striatum, hippocampus, and amygdala [32].

Since FRMP normally acts as a negative regulator for translation ('brake') of protein synthesis in the brain of individuals with FXS, its deficit leads to excess protein synthesis [22, 34-36]. FMRP, the RNA binding protein, regulates the translation of many other proteins, and many of the RNA binding targets of FMRP converge with candidate genes for ASD (~1000 to date) [37]. Unfolding evidence also shows that the absence of FMRP in cortical neurogenesis [38] results in alterations of cortical neuronal differentiation and migration mediated at least in part via brain derived neurotrophic factor (BDNF) signaling. Importantly, alterations of BDNF/TrkB signaling caused by the absence of FMRP result in distinctive cellular and behavioral responses to fluoxetine in adult FXS mice [39], which requires further studies for identification of possible new treatment strategies. Furthermore, in the absence of FMRP, an increase in Rac1-GTPase-dependent NADPH-oxidase signaling leads to an excess of free radical production, which overtime produces oxidative stress

that is a crucial factor in disrupting neuron, astrocyte, and microglia communication [40, 41]. In addition to the potential therapeutic avenue of restoring excitatory/inhibitory balance in FXS, other molecular systems in FXS that carry such therapeutic potential are BDNF, [42] and secreted amyloid precursor protein alpha (sAPPα) [43]. BDNF is a protein that supports the maintenance of neurogenesis and synapses, and FMRP plays a role in BDNF-induced synaptic plasticity [44]. FMRP is known to repress the translation of APP RNA, and APPα in plasma is known to be elevated in FXS [45, 46]. Together, an excessive and poorly regulated *de novo* protein synthesis is pathogenic for FXS, and manifests in a wide range of neurobehavioral symptoms since the *FMR1* gene FM typically alters the course of brain development, cognition, and behavior throughout life [17]. Indeed, studies of humans with FXS, have consistently demonstrated a variety of neurobehavioral impairments, including neurocognitive (i.e., intellectual and attentional), and behavioral (i.e. social interaction disorders such as social anxiety and features of syndromic ASD, refers to ASD in FXS) [16, 18, 47, 48].

Before starting human subject research, it is necessary to obtain the toxicity data for the chemical substance studied, by conducting numerous pre-clinical *in vitro* and/or *in vivo* tests. A toxicology study means testing for the acute, sub-acute and chronic toxicity, mutagenicity, cancer risk factors, reproductive and local toxicity. Initially, it is critical to find out whether a substance is toxic to internal organs such as the heart, lungs, liver, digestive system and brain. Moreover, the effects on other organs are also examined (e.g. skin in case of a drug that targets a skin condition) [49]. In this type of research cell cultures and laboratory animals, primarily rodents and rabbits are used, while adhering to all ethical norms and the 3R rule (Replacement, Reduction, Refinement) [50]. In order to reduce the use of mammals in laboratory experimentation, alternatives (e.g. microscopic nematode C. elegans, zebrafish or common fruit fly (*Drosophila melanogaster*)) are used wherever possible. These alternative species have a relatively well-detailed genome that makes even some complex genetic research possible.

Translational medicine and ethics of drugs use.

The R&D advances of these pre-clinical lab drug studies are followed by a multidisciplinary approach known as *translational medicine,* which enables the '*transfer or translation*' of these results to the initial phase of clinical trials in humans. Importantly, the task of translational medicine is also to incorporate both pre-clinical advances with the clinical trials experience, including challenges, into scientific hypotheses. Therefore, we can say that translational medicine is a "two-way street" with concept that aims to increase efficacy of the new drug, using clinical experience to constantly 'direct' basic science research. Translational medicine makes it possible to fill the gap between our current knowledge and what we need for clinical practice, including reduction of the cost and duration of these clinical trials. In addition to its importance in the process of the new drug development, translational medicine is very important in the development of new diagnostic means (EUSTM, 2014).

Translational medicine has seen rapid development over the past fifteen years (although the term itself was introduced in the 1920s). It is based on a strong link between basic researchers and scientists in various clinical medical fields, whose aim is to prepare the best strategy to introduce a new therapeutic or preventative drug. The purpose of involving scientists from various scientific fields, including the non-medical ones, is to have a comprehensive approach to drug development. For instance, biomedical scientists and neuroscientists, as well as social scientists (psychologists, sociologists, anthropologists, linguists, economists, etc.) must be involved in the development of a drug for the nervous system. All aspects of the effects of a potential new drug must be taken into consideration, including potential side effects on the subjects' health. For example, a number of substances have cognitive effects although this is not their primary pharmacological effect [51]. In this case, translation means studying the effects of a drug on the primates' cognitive capacity (learning, memory, etc.), and using these results when designing clinical trials in humans.

The importance of translational medicine is clear in FXS. Studies in the KO animal model by Bear and colleagues (2004) have provided breakthrough insights into the pathophysiology of FXS that have led to novel therapeutic targets for its *core* deficits (e.g. the mGluR theory of fragile X). Many drugs, including mGluR5 antagonists and GABA-B agonists, can affect the levels of neuronal protein synthesis by targeting upstream signalling cascades that impinge on the mRNA translational machinery [22, 52-54]. On the other hand, commonly FDA-approved prescribed drugs in ASD (e.g. risperidone, aripiprazole) (RUPP, 2002) do not reverse the core features of the disorder, but instead only treat associated symptoms and behavioral problems associated with ASD (i.e., severe temper tantrums/irritability).

Clinical trials in humans.

The origin of the concept of clinical trials is unknown. In the Old Testament of the Bible, the Book of Daniel describes an experiment during which subjects were divided into two groups by the type of food they ate, over ten days. A similar procedure is described in the Canon of Medicine (Avicenna a.k.a Abu Ali Ibn Sina (980-1037)) where the efficacy of the medicines, i.e. the substances used in medicine at the time, was tested. The modern development of clinical trials started in 1930s, and the first randomised, double blind, placebo-con-

trolled study was conducted in 1946-1947 to examine the efficacy of streptomycin in pulmonary tuberculosis [55]. Although it is often said that clinical trials of new drugs (as well as vaccines, dietary supplements and medical devices) are 'experiments' on humans, they are conducted in *four phases*, under strictly controlled conditions, following the approval of all legal and/or regulatory authorities of the country in which the study is being conducted. Clinical trials are based on the cost-benefit analysis of a new drug, and their primary goal is to give well-founded data concerning the effects and safety of the new drug. It is essential that the benefits of using the new drug considerably outweigh the risks. Clinical trials differ by design, cost, and duration, depending on whether the study is conducted in one or more locations, in one or more countries, etc. The design and objectives of the study are described in detail in a clinical trial protocol. The protocol itself, and adherence thereto, ensures that all investigators involved execute the clinical study in the same way. Clinical trials may be sponsored by a government institution, a pharmaceutical company, a manufacturer of medical equipment, a biotechnology company, etc. Yet, to reiterate, only 10 % of all drugs that enter clinical trials actually get the regulatory approval for use in humans.

Clinical trial protocols require participants to provide a written informed consent document before entering into these trials. This document includes detailed information about how the research will be carried out, its duration, possible risks of participation, and potential benefits of participation. It also tells including that the subject that she/he may withdraw from the trial at any given moment. Clinical trials start with phase 1, in which a small number of usually healthy volunteers take part. This provides the information on the safety of the drug and its dose range, as well as the first information on its tolerance in humans. Overall, the key word in phase 1 of clinical trials is *safety*. This may be preceded by pilot trials on a small number of healthy volunteers that aim to confirm and expand the pre-clinical information on the pharmacodynamics and pharmacokinetics of the drug. In phase 2, which assesses the efficacy of the new drug but also keeps monitoring its safety, there are many more participants (100-300). Trials involving a greater number of subjects provide further information on the tolerance of a drug. Phase 3, which are often 'pivotal' effects size clinical trials, are usually multicentre trials that include up to a few thousand participants. The efficacy and safety of the drug continues to be monitored, and after the results of phase 3 are available, the drug is registered. Post-marketing trials (phase 4 trials) last as long as the drug is on the market, and provide further information on the drug, including its use in special groups of population, rare or unforeseen negative side effects, etc. After the drug has been registered, it is widely available on the market, which often provides information about tolerance issues that were not detected in previous phases. Some drugs have been withdrawn from the market due to serious unforeseen tolerance issues [56]. On the other side, it should be noted that participation in a cutting-edge clinical trial may be the patients' only hope that modern medicine can make available to them at that point.

'Translation' of targeted therapies in humans with FXS success story.

FXS has become a prototype neurodevelopmental disorder for developing neurobiologically targeted treatments. As such, FXS is one of the genetic diseases where translational medicine has been of particular significance. In fact, pre-clinical study breakthroughs have enabled clinical trials in humans with FXS more than with any other neurodevelopmental disorder, including the wide range of ASD. This is demonstrated in a multitude of advanced clinical trials whose aim is to introduce the drugs that can modify the core neurocognitive problems in FXS and possibly in ASD [15, 16, 57, 58]. FXS has well characterized genetics, there is advanced neurobiological knowledge about it, and an animal model is available. These advances, in conjunction with increasing work on psychopharmacology of preclinical breakthroughs such as arbaclofen, an GABA-B agonist, and mavoglurant (AFQ056), mGluR5 antagonist [52-54], has propelled this global neurodevelopmental disorder into the most translated among all neurodevelopmental disorders. Indeed, more than two dozen randomized, double-blind, placebo-controlled trials to target the core excitatory/inhibitory imbalance and other manifestations of FXS have been conducted in the past decade [57, 58]. Specifically, to date, a total of 22 controlled studies have been identified through a search of the literature and other sources; 19/22 (86%) have been registered on the National Institute of Health (NIH) *www.ClinicalTrials.gov* website as required by the Food and Drug Administration (FDA) Act of 2007 [57,58]. As expected from FXS neurobiology, the vast majority of these studies have targeted the core excitatory/inhibitory imbalance in the disorder, primarily through either $mGluR_5$ antagonists [(six studies on mavoglurant (AFQ056), basimglurant (RO4917523)] or gamma-aminobutyrics acid (GABA) agonists (three studies on arbaclofen, an agonist of GABA-B, and one study on ganaxolone, a GABA-A modulator), respectively. Importantly, the $mGluR_5$ antagonists studies represent ~1/3 of the total (6/22, 27%) and the above NIH/FDA-registered (6/19, 32%) trials [65, 66]. It is noteworthy that these clinical trials have only managed to focus on reversing social/behavioral symptoms that are part of the core FXS phenotype. That is to say, none of the aforementioned most recent clinical studies have addressed the core FXS plasticity deficit that would translate to changes in cognitive and learning measures.

While FXS became the prototype neurodevelopmental disorder for developing neurobiologically targeted treatments (i.e., treatments targeting neurobiological abnormalities resulting from the primary genetic defect),

limited data exists on the reliability and validity of most tools used to measure cognitive, behavioral and other problems in FXS in the above trials [59]. Furthermore, data on sensitivity to treatment of these tools is either limited or nonexistent. Thus, the absence of beneficial effects of the clinical trials likely reflects flaws in the original study design, including (i) problems with insensitive outcome measures for FXS, (ii) the need to measure outcomes across the spectrum of the FXS phenotype rather than a single behavior, (iii) the need to test the targeted treatments in younger patients with FXS, (iv) the need for longer exposure times to see change in a neurodevelopmental condition, and a lack of appropriate quantitative biological measures to determine behavioral and educaо tional improvement in the participants [59-65]. It is notevworthy that even a well-powered study by Berry-Kravis and colleagues (2016) that studied the $mGluR_5$ antagonist mavoglurant failed to meet the primary behavioral efficacy end point. Nevertheless, prior clinical trials constitute the foundations for additional work, including the development of more rigorous tools to measure outcomes such as reliable and valid biomarkers [58, 59, 66]. Indeed, there is a need to sort out response variability with biomarkers both to identify potential responders and to establish target engagement. Failed trials should also serve as 'lesa sons learned' when planning clinical trials in this area [57, 58]. Considering the progress of the studies in this field of medicine and their results, it is necessary to validate the available tools that measure outcomes in cognitive, language, and behavioral biomarkers in FXS patients as well as in individuals with ASD [59, 67]. In addition, a greater emphasis should be placed on properly identifying the end result that will determine the success of a therapy. The development and validation of tests measuring mental functions, identification of the end result, and identification of biomarkers that indicate the efficient of an FXS drug should be objective and directly connected with the quality of life of the patients [67]. All these translational challenges and appropriate designing of clinical trials for FXS are as important as determining the efficacy and safety of new drugs [57-59, 67, 68].

Conclusions.

New drugs development is a lengthy and costly process with roughly 10% of all pre-clinical studies resulting in a new drug development. The R&D advances in pre-clinical drug studies are followed by a multidisciplinary approach known as *translational medicine,* the 1-4 phases of clinical trials in humans. Over the last two decades, compelling evidence-based data have emerged in the field of neurodevelopmental disorders. Among them, FXS leads targeted treatments in drugs modifiers of underlying psychopathology, and at least part of ASD. To date, a total of 22 controlled studies have been identified through a search of the literature and other sources; 19/22 (86%) have been registered on the NIH *www.ClinicalTrials.gov* website, as required by the FDA Act of 2007. As expected from FXS neurobiology, the vast majority of these studies have targeted the core excitatory/inhibitory imbalance in the disorder primarily through either $mGluR_5$ antagonists or GABA agonists, respectively. It is noteworthy that thevse clinical trials have only managed to focus on reversing social/behavioral symptoms that are part of the core FXS phenotype. The core FXS plasticity deficit, which would translate to changes in cognitive and learning measures, has not been adequately studied yet in these clinical trials. Moreover, while FXS became the prototype neurodevelopmental disorder for developing neurobiologically targeted treatments, limited data exists on the reliability and validity of most tools used to measure cognitive, behavioral and other problems in FXS in the above trials. Considering the progress of the studies in this field of medicine and their results, it is necessary to validate the available tools that measure outcomes in cognitive, language, and behavioral biomarkers in FXS patients as well as in individuals with ASD. In addition, this effort ought to be directly connected with the quality of life of the patient toward a goal of personalized medicine.

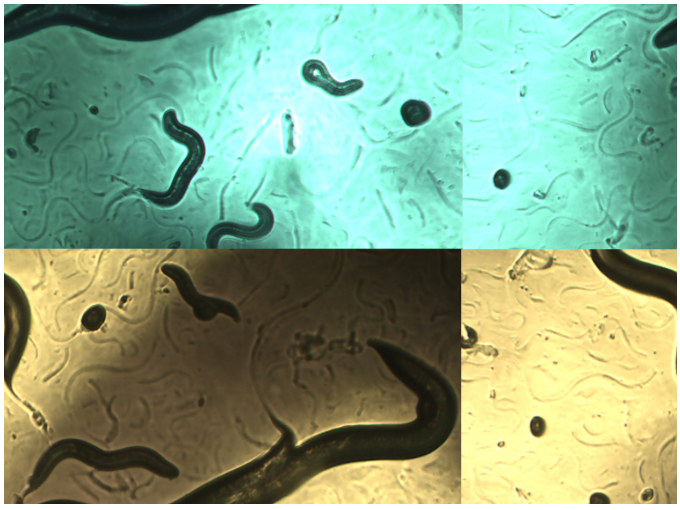

Figure 1. Using alternatives in pre-clinical trials: microscopic nematode *C. elegans* whose genetic map is known. Various genetically modified varieties are available. Since Dr Brenner's discovery (Nobel prize for 2002), the greatest number of Nobel prizes for physiology and medicine were awarded thanks to the research done on this nematode (Source: Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Belgrade; Head of Laboratory: Saša Trailović, PhD).

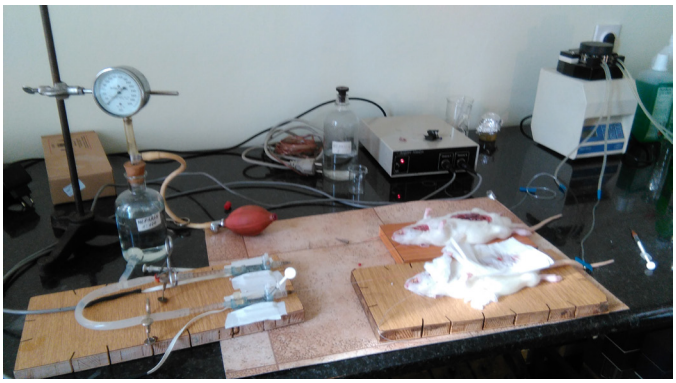

Figure 2. It is often impossible to avoid using laboratory animals (primarily laboratory mice and rats) in pre-clinical drug trials. Although laboratory animals are treated with adherance to all ethical standards, their use in experimentation is minimised. (Source: Laboratory for Ischemia/Reperfusion Injury in Kidney, Department of Pharmacology, Clinical Pharmacology and Toxicology, Faculty of Medicine, University of Belgrade, Head of Laboratory: Zoran Todorović, PhD).

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