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Significance statement

The HMG-CoA reductase inhibitors lovastatin and simvastatin have both been investigated in clinical trials designed to treat the cognitive deficits associated with neurodevelopmental disorders such as Neurofibromatosis type 1, Fragile X and autism. In a recent study, the therapeutic efficacy of lovastatin and simvastatin were compared in a Fragile X (*Fmr1*) mouse model. The authors concluded that lovastatin was superior to simvastatin in rescuing the *Fmr1* phenotypes, and cautioned against considering simvastatin as treatment for neurodevelopmental disorders. We discuss these findings in the context of published literature and argue that more support is needed for this potentially far-reaching conclusion. We further provide recommendations to improve the translation of pre-clinical studies of cognitive disorders into the clinical domain.

The potential use of statins for antagonizing RAS (rat sarcoma viral oncogene homolog) signaling was first recognized nearly three decades ago (Mendola and Backer, 1990; Sebti et al., 1991). Functional RAS requires post-translational farnesylation to become membrane bound and active. Since farnesyl (like cholesterol) is a product of the mevalonate synthesis pathway, its synthesis can be reduced by interfering with the rate-limiting enzyme, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase). Statins, designed as high-affinity HMG-CoA reductase inhibitors, are commonly prescribed for hypercholesterolemia. Over the past several decades, various types of statins have been extensively investigated as potential cancer therapeutics using cellular models, mouse studies, and human clinical trials (Gazzerro et al., 2012; Pisanti et al., 2014; Sopková et al., 2017). On the basis of these findings, Alcino Silva and colleagues explored whether statins might have efficacy in the treatment of RASopathies – a group of neurodevelopmental disorders resulting from mutations that lead to overactivation of the RAS/extracellular signal-regulated kinase (ERK) signaling pathway. Specifically, it has been shown that lovastatin can ameliorate the cognitive deficits in animal models of Neurofibromatosis type 1 (*Nf1* mice) and Noonan syndrome (*Ptpn11* (Protein Tyrosine Phosphatase Non-Receptor Type 11) mice) (Li et al., 2005; Lee et al., 2014), although it failed to rescue the deficits in a mouse model of Costello syndrome (*Hras* mice) (Schreiber et al., 2017).

To translate the mouse findings to the clinic, statins were tested in several randomized placebo-controlled trials aimed at improving cognitive function (Krab et al., 2008; Van Der Vaart et al., 2013; Bearden et al., 2016; Payne et al., 2016; Moazen-Zadeh et al., 2018; Stivaros et al., 2018). These trials used either the first commercially-available statin, lovastatin, or a second generation statin, simvastatin, that is highly similar in structure and pharmacokinetics to lovastatin (Neuvonen et al., 2008; Gazzerro et al., 2012). Notably, simvastatin has not been used in animal models of the RASopathies, but like lovastatin, simvastatin has been shown to decrease ERK signaling in cultured cells (Fürst et al., 2002; Guillén et al., 2004; Miura et al., 2004; Ghittoni et al., 2005; 2006; Khanzada et al., 2006; Ogunwobi and Beales, 2008; Sundararaj et al., 2008; Kang et al., 2009), as well as *in vivo*

(Chen et al., 2010; Lee et al., 2011; Takayama et al., 2011), including the brain (Ghosh et al., 2009). Simvastatin has a 2 to 4-fold increased potency against HMG-CoA reductase and a higher blood–brain barrier permeability compared to lovastatin (van Vliet et al., 1996; Gazzerro et al., 2012; Sierra et al., 2011; Fong et al. 2014). The comparative properties of simvastatin and lovastatin might suggest at minimum the non-inferiority of simvastatin versus lovastatin. Although both lovastatin (Mainberger et al., 2013; Bearden et al., 2016) and simvastatin (Stivaros et al., 2018) showed some potential benefits in smaller trials for NF1, 3 independent large randomized controlled trials of cognition and behavior in children with NF1 using a dose of 40mg/day of simvastatin (Krab et al., 2008; Van Der Vaart et al., 2013) or lovastatin (Payne et al., 2016) failed to show efficacy in the primary outcome measures, even when treatment was administered for 1 year (Van Der Vaart et al., 2013). Thus, no benefits (nor meaningful differences) have been observed between simvastatin and lovastatin in treating NF1-associated cognitive dysfunction. The only sufficiently powered trial that has suggested a benefit for statin treatment on behavior came from a recent study on children with ASD, in which simvastatin was used adjunctively, which yielded a significant improvement of irritability and hyperactivity, but not on 3 other scales of a behavioral checklist (Moazen-Zadeh et al., 2018).

In light of these mostly negative findings, it is crucial to try to understand why these clinical trials failed. For that, more research is required, and the study by Muscas and co-workers (Muscas et al., 2019) is a very important step in that direction. In this study, the authors compared lovastatin with simvastatin treatment in an animal model of Fragile X (*Fmr1* (Fragile X mental retardation) mice). Although ERK signaling in *Fmr1* mice is not increased under baseline conditions, it has been shown that the ERK pathway in these mice is hypersensitive and contributes to the excessive protein synthesis which is considered one of the core mechanisms underlying Fragile X syndrome pathophysiology (Osterweil et al., 2010). Moreover, lovastatin treatment rescues the ERK-dependent increased of protein synthesis as well as the sensitivity to audiogenic seizures of *Fmr1* animals (Osterweil et al., 2013). Given that simvastatin is a more potent inhibitor of HMG-CoA reductase than lovastatin, one would expect that simvastatin treatment would result in a better, or at least a similar rescue. However, in the recent study, Muscas and co-workers surprisingly concluded that lovastatin is superior over simvastatin in reducing ERK activation, as well as in its ability to rescue the downstream phenotypes of ERK activation: increased protein synthesis and sensitivity to audiogenic seizures. Therefore, the authors caution against the assumption that simvastatin is a suitable substitute for lovastatin with respect to the treatment of Fragile X or other neurodevelopmental disorders.

If correct, this conclusion would have far reaching implications. Given the increased potency of simvastatin to reduce HMG-CoA reductase, it would suggest that the previously demonstrated rescue of RASopathy phenotypes by statins is not mediated by attenuation of RAS farnesylation but rather through an unknown mechanism that is absent or less potent for simvastatin. This would have considerable impact in the design of potential future clinical trials for treatment of cognitive deficits in RAS related disorders. However, in reviewing the study of Muscas *et al* (2019), the question arises whether the study truly represents a side-by-side comparison that warrants such a strong conclusion. Most notable, there is no experiment in which lovastatin and simvastatin are compared at the same dose (and with the same vehicle). In addition, a statistical analysis that would enable a direct comparison of lovastatin and simvastatin is lacking.

Given the aforementioned large body of literature that shows that simvastatin can reduce RAS/ERK signaling in cultured cells as well as *in vivo*, the finding by Muscas and colleagues, that simvastatin (in contrast to lovastatin) fails to reduce ERK signaling in brain slices, is quite remarkable. However, it is important to note that the investigators used 50 µM lovastatin but a 100 to 500-fold lower dose of simvastatin (the maximum used simvastatin dose is 0.5 µM). Importantly, the authors previously showed that a lovastatin dose of 10 µM is not effective in this particular assay (Osterweil et al., 2013), hence, the failure of simvastatin to reduce ERK activation at doses far below that is not entirely surprising.

For the protein synthesis experiments (which is sensitive to increased ERK signaling), the investigators used again a much lower dose of simvastatin (10 to 500-fold lower) compared to lovastatin (the maximum used simvastatin dose is 5 µM). The lack of efficacy at such a low dose of simvastatin is not entirely surprising, as the authors previously showed that the lovastatin dose needs to exceed at least 10 µM to be effective in this assay (Osterweil et al, 2013). An elegant study by Tuckow et. al, (2011) showed that 10 µM simvastatin is indeed able to reduce protein synthesis in a mevalonate dependent way, which indicates that at this dose (and under these conditions) there is a clear HMG-CoA dependent effect of simvastatin on protein synthesis.

The most surprising finding of the study by Muscas and colleagues is the finding that simvastatin treatment at low dose actually worsened the *Fmr1* phenotype by further increasing protein synthesis rates. This effect was found to be independent of ERK signaling. This aspect of the study is not only a noteworthy finding, it is also a very worrisome finding with respect to Fragile X clinical trials, where the overarching goal is to use statins to reduce protein synthesis and thereby rescue the behavioral phenotypes (Çaku et al., 2014). For the follow-up of these trials it would be of great importance to know if a comparable (low) dose of lovastatin (below the dose needed to inhibit ERK) would have a similar negative effect on this phenotype, especially since the dose that can be safely used in clinical trials is much lower than the *in vivo* dose used in this study.

Whereas in the large, placebo controlled clinical trials lovastatin was used at the same dose as simvastatin, Muscas and colleagues used a 2 to 30-fold lower dose of simvastin than the dose used for lovastatin (100mg/kg) for their *in vivo* epilepsy experiments. Importantly, the authors previously showed that reducing the lovastatin dose to 30 mg/kg, only rescues the seizure phenotype of *Fmr1* mice in certain mouse strains (*i.e.* inbred C57BL/6; Osterweil et al., 2013), indicating that also for lovastatin a lower dose than 100mg/kg may not always be effective in this assay.

Beside these differences in dosing, it is questionable if the overall experimental design justifies the conclusion that lovastatin is superior over simvastatin to rescue the core phenotypes of *Fmr1* mice. If the ultimate goal of the study is to directly compare two drugs with each other, the drugs should not only be tested side-by-side as interleaved experiments, they should also directly be compared with each other using a statistical analysis that tests for a main effect of treatment, and if significant, followed by a post-hoc analysis to compare the drugs. That this can have a substantial effect on the conclusion, can be illustrated by reanalysis of the dichotomous audiogenic seizure data from the Muscas *et al* paper. Performing such analysis using a logistic regression model, reveals that there is a significant main effect of genotype ($\chi^2(4)=51$; $p<0.0001$), no effect of vehicle ($\chi^2(2)=0.3$; $p=0.9$) and no interaction of vehicle and genotype ($\chi^2(1)=0.2$; $p=0.7$). These are important

control measures since different concentrations of DMSO solvent were used for each drug and could potentially affect the outcome on seizures (Carletti et al., 2013). This analysis further shows a trend for a main effect of treatment ($\chi^2(6)=12$; p=0.07), but not for the interaction between genotype and treatment ($\chi^2(4)=4$; p=0.3). When performing a post-hoc Tukey's test, neither the *Fmr1*-lovastatin versus *Fmr1* 'low dose' of simvastatin (p= 0.96) nor the *Fmr1*-lovastatin versus *Fmr1*-'high dose' of simvastatin treatment (p>0.99) are significantly different from each other. Hence, despite the fact that the lovastatin dose was 2-30 fold higher than simvastatin dose, it does not seem to perform significantly better than simvastatin in this seizure assay.

So how *can* the lack of efficacy of both lovastatin and simvastatin in prior randomized clinical trials of neurodevelopmental disorders be explained, and what can we learn from pre-clinical studies such as the Muscas et al. (2019) study? We believe that two factors are very important to consider when translating findings in animal models to clinical trials in humans.

The first critical factor is the translation of dosing from mice to men. The dose in which a particular drug rescues a phenotype in animal model does not always translate into a clinically applicable and safe dose in humans. For instance, the study by Muscas et al used a lovastatin dose of 100 mg/kg (IP injection) for testing of audiogenic seizures in the Fragile X mouse model. This dosing regimen is much higher than needed to inhibit HMG-CoA reductase (Van de Steeg *et al.,* 2013), or the dose used for behavioral rescue in earlier studies of RASopathy mouse models (10 mg/kg, subcuteanous injection; Lee et al., 2014; Li et al., 2005; Schreiber et al., 2017). More importantly, it is approximately 100-fold higher than the equivalent dose used in the clinical trials when also considering bioavailability for oral versus IP injection (Zhu et al., 2011; Dose conversion calculated by FDA guideline 'Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers', www.fda.gov/media/72309/download). Hence, although the partial rescue of audiogenic seizures is of compelling scientific interest, it is important to realize that the direct translational value of such high doses is limited. And even though a rescue of behavioral deficits in *Fmr1*, *Nf1* and *Ptpn11* animal models has been observed using an oral dose that more closely reflects the dosing used in clinical trials (Li et al., 2005; Osterweil et al., 2013; Lee et al., 2014; Asiminas et al., 2019), it still cannot be excluded that the effective dose of statins in the mouse brain is different from the human brain, as even small species differences in blood brain permeability could eliminate the beneficial effect of statins (Hoshi *et al.,* 2013). This study by Muscas et al (2019) underscores the importance of looking at effective dosing ranges, and more detailed (*in vivo*) pharmacological studies in animal models should be performed to elucidate the dose-dependency of therapeutic benefit.

The second factor that may affect successful translation to patients is the timing of drug administration. Whereas most pre-clinical studies involved drug treatment of adult animal models of neurodevelopmental disorders, it is conceivable that this may not be effective in human patients and that treatment of patients should be started in young children to be maximally effective. Conversely, if a behavioral rescue is observed in young mice (*e.g.* the rescue of seizures in *Fmr1* mice was performed on Postnatal day (P)18-P29 mice, Osterweil et al., 2013; Muscas et al., 2019), it is important to investigate if such a rescue is still observed when the brain has fully matured. Interestingly, a recent study in a rat model of Fragile X syndrome demonstrated that adult *Fmr1* animals no longer exhibited cognitive deficits following brief lovastatin treatment at young age only (Asiminas et al.,

2019). Such studies should be further exploited to delineate the precise critical period for optimal treatment of neurodevelopmental disorders (*e.g.* see Silva-Santos et al., 2015).

Once these two critical parameters are known for both simvastatin and lovastatin, it may be warranted to consider new clinical trials of statins for treatment of cognition in neurodevelopmental disorders. Hopefully, when using the right conditions, statins will be as effective in humans as they were shown to be in multiple animal models.

Legend Figure 1.

To ensure optimal translation of animal experiments to cognitive clinical trials, it is important that the drug treatment used in animal studies resembles that of clinical trials with respect to equivalent dose (considering also interspecies differences in pharmacodynamics and pharmacokinetics of target tissue), route of administration and drug similarity. Moreover, it is important to take into account the timing of drug administration, as treatment of neurodevelopmental disorders may require intervention during a critical window of development.

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