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## EVEROLIMUS DOES NOT PREVENT LAFORA BODY FORMATION IN MURINE LAFORA DISEASE

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Healthy teenagers until the onset of Lafora disease (LD), patients may experience decade-long progressive and soon-intractable myoclonus, epilepsy, psychosis and dementia culminating in a vegetative state, and death in status epilepticus. The causative defects are loss-of-function mutations in the genes encoding the interacting enzymes laforin (EPM2A glycogen phosphatase) and malin (EPM2B ubiquitin E3 ligase). Through yet unclear mechanisms, loss of the laforin-malin function results in malstructured glycogen that aggregates and accumulates into Lafora bodies (LB), which induce both a defect in autophagy (which might otherwise possibly clear LB) and neuronal death.[1,2] Of major therapeutic importance, in LD mouse models reducing glycogen synthesis (through knockout of muscle/brain glycogen synthase [Gys1] or its activator protein PTG), even by only 50%, clears LB, restores autophagy, and rescues the LD neurologic phenotype.[2,3]

The mechanistic target of rapamycin (mTOR) is a homeostatic hub that promotes anabolism (including protein synthesis, cell growth, and proliferation) and downregulates autophagy. Rapamycin (sirolimus) and its analogs (e.g., everolimus) inhibit mTOR.[4] Recently, it was reported that rapamycin also downregulates glycogen synthesis in mouse models.[5] Dual glycogen synthesis reduction and autophagy enhancement (through mTOR inhibition) suggest this class of drugs as a perfect potential fit for an LD therapy. We tested whether everolimus would be therapeutic in the *Epm2a*$^{-/-}$ LD mouse model. We chose everolimus because it possesses improved pharmacokinetic, pharmacodynamic, and blood-brain barrier–crossing properties over sirolimus, is in clinical use in neurology (tuberous sclerosis), and is in clinical trials as a potential general antiepileptic drug.

In the LD mice, LB are not yet present at 1 month of age and are abundant by 3 months. We administered everolimus (30 mg/kg), or vehicle, to *Epm2a*$^{-/-}$ mice by gavage once daily from age 1 to 3 months. This achieved very high levels at 3 months, both in the periphery (309 ± 118 ng/mL in blood 24 hours after the last dose and 122 ± 20.6 ng/g in the muscle) and in the brain (186 ± 50.9 ng/g) (mean ± SD; e-Methods at Neurology.org/ng). There was, however, no difference between treated and untreated mice in LB (in the muscle or brain), glycogen content, Gys1 activity, or markers of autophagy (LC3 and p62) (figure).

Everolimus does not appear to be a potential therapy for LD. We did not study the LD behavioral phenotype, which occurs much later (~12 months) when LB are profuse, but this phenotype is predicted by and inextricably linked to LB and glycogen accumulation,[2,3] neither of which was reduced in our study. It is important that we confirmed that the dosage used achieved supramaximal everolimus exposure, with trough levels in the blood, muscle, and brain higher by 2-, 5-, and 30-fold, respectively, vs those reached by the fully immunosuppressive/antiproliferative dose of 5 mg/kg in mice.[6] These levels were also 20–60-fold higher than immunosuppressive levels in transplant patients.

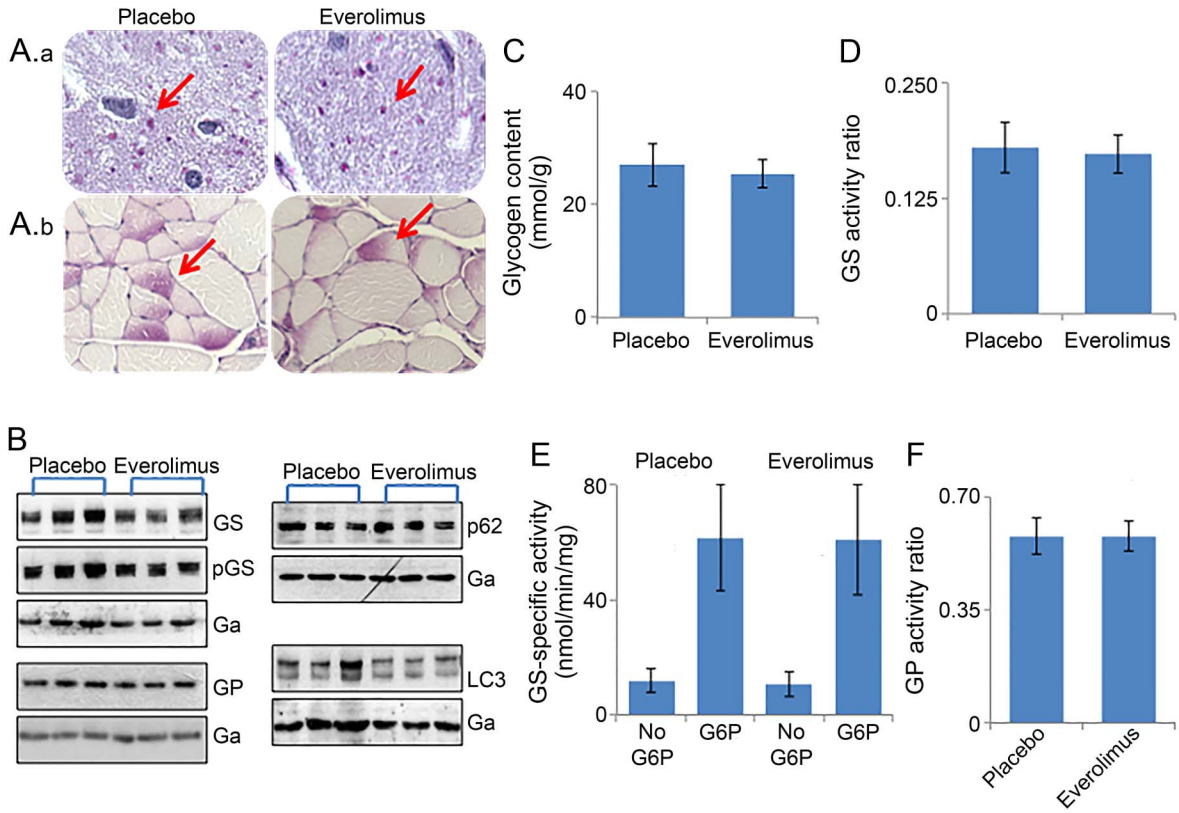
Everolimus did not have the effect on Gys1 activity reported for sirolimus. Why the 2 analogs might differ in their effects on this enzyme is unclear. The inhibitory effect of sirolimus on glycogen synthesis is surprising because mTOR increases overall cellular energy (adenosine triphosphate),[4] consistent with which both sirolimus and everolimus reduce glycolysis,[7] which should increase, not decrease, glycogen synthesis. Confirmation of the sirolimus effect on glycogen synthesis in new experiments, perhaps in LD mice, appears warranted. Certainly, lack of effect of everolimus on Gys1 and glycogen synthesis explains the lack of effect on LB, as the latter have only ever been possible to reduce through downregulation of glycogen synthesis.[2,3] Surprisingly, everolimus also did not affect autophagy (at least as assessed through the LC3 and p62 markers), suggesting that its effect on mTOR might be more restricted (to the anabolic arm of mTOR) than that of sirolimus. Everolimus and sirolimus have previously been shown to have certain different effects in the brain, e.g., again surprisingly, everolimus, but not sirolimus, increases mitochondrial energy generation, while both, as mentioned, decrease glycolysis.[7] Might the 2 compounds have sufficient differences for sirolimus to work in LB prevention where everolimus failed?

LD is arguably the severest epilepsy of adolescence. Opportunely, mere 50% glycogen synthesis downregulation rescues the disease in mouse models,[2,3] and

**Supplemental data at Neurology.org/ng**

**Figure** **Lafora bodies, glycogen content, glycogen-metabolizing enzyme activities, and autophagy markers are unaltered in 3-month-old laforin-deficient mice treated with everolimus from age 1 month**




(A) Representative brain cortical (A.a) and skeletal muscle (A.b) sections stained with periodic acid–Schiff–diastase for detection of Lafora bodies (arrows). (B) Western blots on skeletal muscle extracts with antibodies against glycogen synthase (GS), phosphorylated GS (pGS), and glycogen phosphorylase (GP), and autophagy markers p62 and LC3. Glyceraldehyde 3-phosphate dehydrogenase (Ga) is the loading control for each blot. (C) Skeletal muscle glycogen content in millimoles per gram of tissue. (D) GS activity ratio, i.e., GS activity with no added G6P (G6P is the potent allosteric activator of GS) divided by GS activity with 7.2 mM G6P (the latter resulting in maximal activation of GS). This ratio conveys how much of total potential existing GS activity in the tissue is actually active, i.e., the enzyme's activation state (ratio). (E) Components of the above ratio (i.e., separate activities with no G6P or with 7.2 mM G6P) in nanomoles per minute per milligram of protein. (F) GP (a glycogen-degrading enzyme) activity ratio (±3 mM of the GP allosteric activator adenosine monophosphate). n = 6 for all experiments (e-Methods).

this extent of reduction would be wholly safe in humans as evidenced by normal health in heterozygous parents of patients with glycogen storage disease type 0 (patients lacking glycogen synthase). Search is actively under way for inhibitors of glycogen synthase as a therapy for LD. Our results suggest that these do not include everolimus and do not support the off-label use of everolimus in LD. Repeat of a study such as ours with sirolimus remains worthwhile.

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# Everolimus does not prevent Lafora body formation in murine Lafora disease

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