TO THE EDITOR:

More to voxelotor than meets the eye?

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For 22 years, treatment of sickle cell disease (sickle cell anemia) was confined to a single approved therapy.1 Finally, in 2017, there began a wave of 3 new approvals,2 including the first small molecule inhibitor of hemoglobin (Hb) polymerization: voxelotor (GBT440).3-5 Considerable excitement about this addition to the therapeutic arsenal6 was followed by several recent concerns.7-9 However, a complete, rational assessment of voxelotor’s safety and efficacy is presently precluded by fundamental gaps in our understanding: one of which we describe here.

The principle on which the drug is based seems straightforward. When Hb delivers oxygen, its quaternary structure shifts from high-affinity R to low-affinity T, and it is only the latter structure that can polymerize.10 Thus, by “locking” the Hb molecule out of the T structure with voxelotor, the option for polymerization would be denied. However, as the higher-affinity R structure, by definition, clings more tightly to oxygen, it has been proposed to reduce functional oxygen delivery or to at least lower the tissue pO2.8 The ability of the drug to favor the R structure seems evident in oxygen dissociation curves (Figure 1A).3 When voxelotor is bound to all the Hb molecules in a sample, the curve’s normal sigmoidal shape, representing the interplay of binding curves for the high-affinity R structure and low-affinity T structure in mutual equilibrium, is replaced by what seems to be a single, high-affinity curve. Solutions only partially saturated by the drug displayed a superposition of the usual Hb binding curve and this high-affinity curve, according to its concentration.

As their name implies, the oxygen dissociation curves begin at atmospheric oxygen pressure, with the drug introduced exclusively to R-state Hb. What happens, though, if one begins under deoxygenated conditions introducing the drug to T-state Hb and then adds oxygen? This procedure has been done twice, with markedly different results. Dufu et al added the drug to cells sickled via deoxygenation and obtained the results shown in Figure 1B.11 It is clear that this is not the curve shown in Figure 1A: the p50 is significantly greater than either the p50 of the voxelotor-modified Hb or unmodified Hb, and the shapes are markedly different. An elevated p50 is immediately suggestive of a T-state; however, the observed value is not characteristic of a normal T state. The known responsiveness of T-state affinity to external perturbations12-14 offers a possible explanation for this behavior.

What about the other experiment? Henry et al introduced voxelotor to fully oxygenated samples measured during deoxygenation (as in Figure 1A), and, after a brief pause, reoxygenated7 samples. The process of deoxygenation and reoxygenation was repeated twice with lengthening pauses. However, none of the reoxygenation curves resembled those reported by Dufu et al. From the modeling of those experiments, Henry et al concluded that at very low pO2, the drug dissociated from the R structure upon deoxygenation and transitioned to T. This implies that an instrument that could accurately measure low pO2 would reveal a cooperative binding curve in the presence of a saturated voxelotor, which is unfortunately not observable using the method used for most contemporary oxyhemoglobin dissociation curve measurements, as shown in Figure 1A.

Must one of these experiments therefore be wrong? We believe there is a way to reconcile both results. Voxelotor binds strongly to one α chain and then weakly to the other via a single hydrogen bond.15
disrupting the formation of 2 salt bridges. These normally stabilize the T state by connecting the α terminus of one chain to α 141 on the other α chain. Elimination of α 141 is known to cause a 15-fold increase in oxygen affinity, which is in excellent agreement with the p50 changes with voxelotor as shown in Figure 1A. This suggests that the T state may be accomplished if only one contact point is released, and the drug remains held to the other, albeit more weakly. This would keep only one salt bridge from forming and impede the transition less forcefully. In this situation, reversion to the R state upon reoxygenation makes drug rebinding to the second contact point easier, such that a subsequent dissociation curve would more likely assume a canonical voxelotor-mediated shape, as reported by Henry et al. However, the fact that the drug may remain bound while the molecule switches to the T structure does not mean this is the only way in which it can bind to that structure. Introducing voxelotor with Hb in the T state ab initio could present alternative binding sites and thus produce distinct behavior. The drug binding to the T state must also reduce its cooperativity, as evidenced by the failure of the p50 to increase to its normal value. The combination of the stability of the structure and its lower cooperativity would have the effect of generating the noncooperative oxygen binding curve observed by Dufu et al.

This suggests that there may be more than one way to bind the drug to sites in the T state. The experiment by Dufu et al started with deoxygenated blood and allowed the drug to bind, whereas the experiment by Henry et al first bound the drug to oxygenated cells, which were subsequently deoxygenated. Although equilibrium dictates that no matter how the process begins, it should arrive at a unique final state, it is far from clear that equilibrium would have been reached in the experiments by Henry et al, as the authors themselves argue persuasively.

Being consumed orally, voxelotor is transported to the blood upon absorption by the intestinal villi, whose baseline low pO2—dubbed “physiological hypoxia”—ensures the drug will encounter a considerable fraction of already deoxygenated Hb species. This means that its entry into the microcirculation could result in binding to the T state in a fashion akin to the experiment by Dufu et al. In a recent preliminary data set for 9 pediatric patients, for whom voxelotor was added to hydroxyurea treatment, a variety of oxygen dissociation curves was evident, with some reflecting the noncooperative character of Figure 1B. This suggests that T-state binding is indeed relevant in vivo.

Although the reduced oxygen affinity of the curves in Figure 1B would facilitate oxygen offloading compared with those in Figure 1A, the ultimate clinical impact of drug binding to the T state depends on whether sickling is affected. The inhibition of sickling is possible, as it is well known that not all T structures polymerize equally. For example, deoxy Hb in the presence of the red cell inorganic phosphate, 2,3 diphosphoglyceric acid, has a lower solubility than in its absence, although both are T structures. In fact, this is consistent with the known link between higher levels of 2,3 diphosphoglyceric acid in patients with sickle cell and increased sickling and forms the basis for treatment strategies seeking to reduce levels of this allosteric modifier.

However, there are currently insufficient data to conclude whether voxelotor inhibits sickling in the T state. Using their novel sickling assay, Henry et al showed slowed sickling times, but it was not clear to what extent this was owing to the conversion of Hb to the R state. Dufu et al observed a reversal of sickling when they introduced the drug into deoxygenated cells, although the methodology used did not permit a firm conclusion on whether this was caused by voxelotor modification of the T state or delayed melting of the polymer at ~32 torr sample pO2. From the available clinical data, it is also not possible to determine whether this mode of action plays a major role; for example, although the mitigating effect of T-state binding could factor into the drug’s safety, interpretation is confounded by increases in Hb level and cell deformability. An experiment that would resolve this issue might entail first deoxygenating sickle Hb and then adding voxelotor before raising the temperature and assessing solubility. If solubility increases, it can be concluded that voxelotor inhibits sickling in the T state.

Voxelotor is now widely used in the clinic, without the complications once feared but with differing perspectives regarding its
A complete understanding of its interaction with the T structure might help to resolve such uncertainty and deserves prompt investigation. The implications of such knowledge might hold immense promise, if not to assuage theoretical concerns or optimize dosing for voxelotor, to lay the foundation for drugs that might act in a similar fashion. Indeed, numerous additional small molecule inhibitors of sickle Hb polymerization are already in development.\textsuperscript{26,27} We hope this letter will encourage exploration of T-state modulation to help advance these programs and ultimately improve the health of patients living with sickle cell disease.

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