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In Vivo Delivery of Morpholino Oligos as Therapeutics: What Barriers Still Exist?

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Perspective

Uniqueness is sometimes synonymous with loneliness. This happens to be particularly true with regard to the *in vivo* delivery technology of Morpholino antisense oligo, where Vivo-Morpholino [1,2], a conjugate with a *unique* dendrimeric octa-guanidine delivery moiety, is competing *lonely* with those containing a vast variety of cell-penetrating peptides [3,4,5] including mostly cationic and/or amphipathic peptides (this class of conjugates is referred herein as pep-Morpholino). While limited enhancement may be achievable to improve the efficacy and safety profile for Vivo-Morpholino, a massive area remains to be explored using pep-Morpholinos containing certain ideal peptides which may be highly effective and non-toxic for *in vivo* delivery of Morpholino oligos in living animals. As improvement and exploration are in progress, it may be also appropriate to consider what antisense technology truly needs and how we can move forward with the currently available achievement.

It has been rigorously confirmed that bare-Morpholino (the oligo itself without conjugation of any delivery moiety) shows robust safety profile in the tests either *in vitro* or in living animals [6]. However, as a class of macromolecule (molecular weight between 6,000 and 10,000 Daltons), bare-Morpholino, at any dosage, shows only marginal or often negligible efficacy *in vivo* owing to its poor permeability to the cells. Because of its low toxicity and low efficacy, bare-Morpholino has been used traditionally at high dosages. Consequently, the mindset of using a high dosage has been relayed for delivery-enabled Morpholinos *in vitro* or in animal studies.

Vivo-Morpholino or pep-Morpholino in a broad range of dosages shows the significant desired activity *in vivo*. In order to obtain quick and complete gene knockdown results, most biologists prefer to use the highest possible dosage. However, the accompanying toxicity when a delivery moiety is added becomes an issue even though it may be known that considerable amount of antisense agent has been delivered into cells with the advancement of *in vivo* delivery technology. Optimization of the dosage towards the lower end is a reasonable approach. So far only one report using significantly low dosage of Vivo-Morpholino for *in vivo* studies was published [7]. The dosage (a cocktail of 10 Vivo-Morpholinos, 0.6 mg/kg each) after repeated administration every 2 weeks for 18 weeks demonstrated good efficacy in the DMD (Duchenne Muscular Dystrophy) model mice without detection of obvious immune response and renal and hepatic toxicity at the end-point of the treatment. It is yet to be defined whether the efficacy resulted from the accumulation of each individual oligo's activity or the collection of multiple synergies. Nonetheless, the results indicated a positive trend that lowering the dosage can minimize the toxicity while still achieving efficient gene regulation activity.

Patience and persistence are therefore needed to use the low dose in long term to achieve effective results. However, it is hard to have everything in place in reality. Currently there is a clinical trial of a Morpholino-based drug, eteplirsen (AVI-4658) which Sarepta Therapeutics uses for treatment of DMD. The clinical study has been continuing with high dosage of the drug to treat 10 DMD boys for so far more than 3 years [8] and it seems that Sarepta is determined to keep the study ongoing indefinitely until a certain dramatic therapeutic endpoint is truly reached. Eteplirsen is a bare-Morpholino which may initially enter diseased "leaky" muscle cells to show some therapeutic benefit. However, until the rejuvenated muscles again degenerate and become leaky again, further entry of bare-Morpholino into those muscle cells are hampered by reduced leakiness of treated muscle tissue. These cycles of improvement and degeneration may be a factor compromising the definitive therapeutic outcome [5,9]. Great patience has been shown in the above ongoing clinical trial, but the drug under clinical investigation was unfortunately not structured to penetrate into cells *in vivo*. In a second case, a testing drug was a delivery-enabled Morpholino, but the mindset of using high dosage was kept unchanged. A few years ago, the same company initiated a pre-clinical trial using PPMO [5] (one kind of pep-Morpholino containing arginine-rich peptide) of the same high dosage as the bare-Morpholino and shortly thereafter the trial was terminated because of the unbearable toxic side effect. If the strategy could be accordingly adjusted to pursue lowering the dose regimen of Vivo-Morpholino or PPMO, Sarepta, with its long term effort, could have achieved their success in their clinical trials (not to mention how much can be saved economically for assembling those hugely expensive Morpholino oligos).

Encouraging results from recent improvement on Vivo-Morpholino have shown that the delivery efficacy can be substantially increased; allowing further to reduce the dosage, thus further to lower the toxicity. Besides the high efficacy and low toxicity that can be achieved by this optimization step, use of the currently available delivery-enabled Morpholinos or those improved variants in low dosage has other merits as well. First, it is extremely difficult, if not entirely impossible, to find a highly efficient delivery technology which does not have any toxic effect in any dose range. Even if such a delivery moiety is found, high dosage may still cause some undue problems. From what we have learned, the toxicity of Vivo-Morpholino is very likely due to acute hemolysis [10]. If it proves to be true, the toxicity can be easily reduced or diminished by simply using low dosage and/or dilute concentration [11]. Secondly, off-target problems [12] have been reported when a very high dose of bare-Morpholino was used

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for gene regulation in zebrafish. Conceivably, using low dosage of Vivo-Morpholino or pep-Morpholino can have the advantage of avoiding the off-target problem which we have not seen yet from the high dosage of bare-Morpholino in clinical trial since it is poorly permeable into cells, but will see, potentially down the road, from the high dosage of Vivo-Morpholino or pep-Morpholino if the toxicity problem is out of the account, or a non-toxic, highly efficient delivery-enabled Morpholino that is eventually discovered. Additionally, it is probably reasonable to place preference of Vivo-Morpholino over pep-Morpholino based on the structural characteristics. The former is completely composed from unnatural synthetic components, whereas the latter is assembled from natural amino acids which risk an immune response, thereby preventing repeated administrations for diseases requiring long-term treatment. Admittedly, there is one technical bottleneck for carrying out tedious low dose, long-term studies in the drug discovery and development. The practical hurdle in preclinical stage is to work along with uncooperative small mice whose tails are not so endurable for numerous needle treatment. Once in clinical study, human beings are definitely the friendliest animal in the world to communicate and collaborate.

Should we get over our common mindset barrier and begin to use Vivo-Morpholino in low dosage and keep away from those potential snags including afore-mentioned toxicity issue, off-target problem and immunogenicity, or stay along the high-dosage path until when we step on those "mines"? Low dosage and long term treatment may be the keys for successful application of delivery enabled Morpholino therapeutics. On the basis of the antisense mechanisms [13], unless a controlled release technology is involved [14,15], it is difficult to foresee that a single shot of high dose antisense drug can serve the purpose of curing a disease of which its aberrant genes are hard to be promptly and specifically bound in the dynamic genetic forest. Antisense is a gentle and serene therapy to treat genetic diseases, something like a tranquil fountain to nourish the earth, not a pouring storm to flood the ground.

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