Follow-up Q&A from PDHC Webinar Translation into the Clinic Initiative: UNDERSTANDING HOW PERMEATION ENHANCERS CAN DELIVER PEPTIDES ACROSS THE GUT WALL. GI PHYSIOLOGY 101 FOR PEPTIDE DRUG HUNTERS with Jerome Hochman, Ph.D. and David Brayden, Ph.D.

1. ***Question:*** **Regarding the mechanisms or hypotheses behind PE-assisted peptide permeation, there seems to be a general view that permeation occurs through tight junctions and/or directly across the cellular membrane. What are the key experimental data supporting these mechanisms, particularly the direct membrane permeation pathway? Are these gaps identifying these and other possible mechanisms?**

David: The evidence for flux via both pathways is from cells, mainly Caco-2. Labelled peptides can be imaged in the paracellular pathway - a bit like Jerome showed for PCC with lanthanum. There is also good evidence showing that C10 activates calmodulin and MLCK to control tight junction opening while reducing TEER. Since flux immediately follows, the paracellular path is implicated. This path is also partial to small hydrophilic molecules.

For the transcellular path, Stephen Buckley et al showed labelled semaglutide in gastric epithelial tissue, which supported that route. However most evidence for transcellular is indirect from coarse granular simulations and where PEs have been localised to mixed micelles - which can cross due to their lipophilic nature. You can prove that SNAC embeds in giant liposomes, depending on its monomeric state. Since the "quick-sand" paper suggests close association of sema with SNAC, a transcellular path must also be in play. However, evidence from Lilly in their recent paper on the oral GLP-1/GIP peptide also showed that SNAC altered ZO-1 expression in TJs in tissue, so this suggests that SNAC has multiple mechanisms.

Ask my former PhD student, John Gleeson, Senior Scientist at Rahway, Merck to figure it out!

Jerome: I agree with David's response about the two mechanisms. In the case of tight junction disruption by caprate and acylcarnitines, the disruption of tight junctions was demonstrated in vitro and in situ by the passage of electron dense and fluorescent polar markers into the paracellular space. Additionally Freeze fracture EM of Palmitoyl carnitine treated Caco-2 cells showed breaks in the tight junction strands (J Pharmacol Exp Ther. 1994 May;269(2):813-22). As David presented, the mechanism for this is probably through increasing intracellular Ca++ modulating the perijunctional actomyosin ring and activating protein kinases. However, in vivo differences in exposure levels and intact biological organization could result in subtle changes underlying the mechanism.

In the case of the transcellular pathway by SNAC, the evidence is less definitive and is to an extent a matter of exclusion (factors that are not altered by SNAC) rather than direct observations. MD simulations are compelling but require substantial supporting data that the simulations reflect a more complex cellular membrane. It would be interesting to look at the effects of SNAC on IEC monolayer labeled with fluorescent lipids and the transit of fluorescent peptides (in epithelial cells using super resolution fluorescence microscopy. This could potentially resolve passage across the apical membrane and transit through and out of the cell.

2. ***Question:*** **How much GI stability vs peptidases do you need to dial into your peptide to get a bioavailability >1%?**

David*:* I don't think a lot of peptidase inhibition is needed to get that 1% F - if very GI-labile semaglutide is anything to go by. Your own work on MEDI GLP-1 peptide suggests that built-in stability can add a few % which can be bumped further by the CDC enhancer. On the other hand, the MK0616 seems quite stable and still gives 1% with C10, so go figure.

Jerome: That is a difficult question to answer since there isn't a standard assay for peptidase activity nor scaling factors to translate from in vitro to in vivo. Since the readout for stability will depend on the assay conditions (I would screen in serum, trypsin, chymotrypsin, pepsin, and elastase and potentially brush border membrane vesicles) and select standard drug markers as benchmarks (octreotide and other oral somatostatin ligands relative to somatostatin, and GLP-1 versus semaglutide). In the case of GLP-1 DPP-iV susceptibility was dialed out and prolyl hydrolase activity can be an issue for proline containing peptides.

3. ***Question:*** **How important is ligand efficiency in the design of drugs administered with PE formulation?**

David: By that I take it you mean whether the peptide can still bind its receptor with high affinity following absorption. It's critical. High affinity and potency are a given for an oral programme.

Jerome: By ligand efficiency I assume you mean the binding affinity relative to the size of the molecule (non H atoms). The potency/affinity will always be a factor as higher potency means you can get a pharmacological effect at lower systemic concentrations. However, the impact of molecular size on absorption will be reduced with PE. That being said, permeability is only one factor that molecular size and non-H atoms can influence. If your target is intracellular passage across the plasma membrane will be influenced by size and polar constituents. Susceptibility to some xenobiotic metabolism may also be higher with greater non-H atoms in the structure, and the volume of distribution could be lower (thus shorter systemic half life) for compounds with low ligand efficiency. Given these considerations I would anticipate that LE impact should be lower with PEs but without specific concerns or clear trends between ligand efficiency and PK/PD, I would not weigh ligand efficiency heavily in candidate selection.

4. ***Question:*** **Interesting MoA studies on SNAC inserting into the membrane- I can see how this works at the apical surface. How do PE's affect exit at the basal surface - if at all? Is there data on SNAC distributing to the basal surface and facilitating transport, or do peptides rely on slow passive permeability to get into the bloodstream?**

David*:* There is imaging showing SNAC distribution in epithelia, but to my knowledge, nobody has studied how it emerges on the basolateral side, likely detached from payload. However, we know that SNAC permeates extensively and quickly and in vivo it is entirely absorbed within a

few mins. The "quicksand" paper suggests that the intracellular pathway is complex even if passive.

We know very little about the exit process. Assumptions are made that SNAC exits easily as it is a small molecule with high permeability. For the peptide, very little research on its exit, except that it must do according to transepithelial fluxes. No doubt it has the same problems getting out as it had getting in, but we don't know whether it is still associated with the PE (unlikely?)

Jerome: I agree with David on this topic. The exit from cells of SNAC enabling drug uptake is an open question that should be answered. One possibility is that entrance into the cell is enough to commit a portion of the drug to exit the basolateral membrane (i.e. the fate of the peptide once it gets in the cell is exit the apical membrane, exit the basolateral membrane, or be metabolized/ compartmentalized in the cell). This process would be highly inefficient, but the bioavailability in the SNAC enhanced formulation is pretty low. If this was the case the upper limit to the extent of drug absorption with SNAC would be low, but with a potent drug it could be sufficient.

5. ***Question:*** **From your research, have you compared C8 vs C10 PEs? If so, which showed better performance?**

David: In our hands C10 is better than C8. See: https://pubmed.ncbi.nlm.nih.gov/25460147/ C8 can still be good in complex formulations like Mycappsa.

Jerome: David is the expert on this, but I think it is worth assessing both in initial preclinical animal studies since performance could be dependent on both the drug and the biological conditions (e.g. potency relative to tolerability, food effects, drug solubility considerations).

6. ***Question:*** **Would it be better for adsorption of peptides when your peptide can stay together with PE? Will they diffuse through tight junction at the same time?**

David: We don't know the answer to this. Most literature suggests dissociation once TJs are opened, no evidence for parallel flux together, not surprising as the PEs tend to permeate really well in their own right. The transcellular story is more complex as suggested by the quick-sand paper.

Jerome: I agree with David on this question. I don't know of any studies that addressed this but my gut feeling is that the larger size of the PE/peptide complex would restrict passage through the tight junction. It would be interesting for both tight junction disruption and the quicksand model effect to quantify transport of both the PE and the peptide across monolayers. If they go across as complexes, permeability of the PE should be equal to or greater than that of the peptide.

7. ***Question:*** **You have mentioned that PEs (C10 and SNAC) work better in intestinal environment than in the stomach then why are the marketed products with PEs not enteric coated?**

David*:* Mycappsa capsules are enteric-coated, but the others are not- as you rightly state.

My view is that there is more to this than the Fagan paper suggests. In gastric pH, C10 and SNAC are more oily and less soluble than in their salt form (upper GI). However, since they both buffer the pH around the IR table to pH 5 and 6, this should help their release profile while blocking pepsin - but Fagan's profiles do not agree. Then again, in vitro release profiles in FaGGIF may not map to in vivo. I don't think we understand this very well.

Jerome: I'm not sure the complete story out yet. For Enlicitide the initial phase 1 study was without enteric coating, but they mentioned that a comparison of enteric coated with non-coated was pending but I have not seen those results published. I suspect that a more complete picture will be available after Enlicitide receives final approval.

8. ***Question:*** **It was mentioned that the effectiveness of permeation enhancers depends on the peptide and experimental system. From a drug discovery perspective, what physicochemical properties of a peptide would lead you to pursue an oral formulation with a permeation enhancer rather than investing in an alternative delivery strategy early in development?**

David*:* The macrocycle programmes of Merck, J & J and Chugai have addressed this with computer simulations, AI, and high throughput in vitro experimentation. I would be looking to have a really potent peptide with MW below 3-4 kDa, in-built stability and ideally a long half life, perhaps a good albumin binder. Chugai's LUNA 18 of 1200 MW was so permeable it did not need a PE, but needed a solubiliser. The best scenario is to get 1- F without a PE if possible and J & J's anti-IL-23 also achieved this.

Jerome: I think the pursuit of oral versus other forms of administration may be more dependent on other factors than physicochemical properties (physicochemical properties can be adjusted through chemical approaches) but therapeutic properties, and safety margin, marketing considerations may be more important. In the case of insulin drugs subcutaneous administration combined with subtle sequence alterations enabled introduction of fast release/short exposure time for prandial glucose control to long release (degludec) for basal glucose control. Lipidation/acylation strategies can extend exposure of some peptides to weekly or even monthly regiments. David has also published compelling results on sublingual delivery. From my perspective oral is an obvious choice for ROA but not necessarily the best for all indications (however perceptions are hard to change).

9. ***Question:*** **Can you comment on whether you think mucus layer effects to a significant extent drug+enhancer delivery to the membrane, and how?**

***David**:* Yes, mucus is important and we do not model it very well in co-cultures. Its presence is a much bigger detriment for nanoparticles which get caught up in it. Mucolytics like NAC have been used in PE systems, but they don't seem to have a dramatic boosting effect on absorption. Recently, Hanne Neilsen's lab showed that SNAC changes the viscosity of mucus, so this may contribute to its efficacy as a PE. See: Physical and barrier changes in gastrointestinal mucus induced by the permeation enhancer sodium 8-[(2-hydroxybenzoyl)amino]octanoate (SNAC) - PubMed

10. ***Question:*** **Could you please comment on the potential use of focused ultrasound to enhance permeation? Read recently about explorations of its use in enhancing permeability of the blood brain barrier. Here is a reference that may be of interest https://www.nature.com/articles/s44172-026-00597-5**

David: Ultrasound has been used by the Gio Traverso group at MIT to boost peptide and RNA absorption: see: https://pubmed.ncbi.nlm.nih.gov/34403749/

There are a number of companies working in that space: Baywind Bio, SuonoBio, however, the toxicology and regulatory path is uncertain. All are in the preclinical phase.

The BBB work is intriguing as it can be used to get nanoparticles across for cancer.

Jerome: I'm afraid my knowledge on this topic is very limited, but I would be concerned about whether the cost would be restrictive for most peptide drugs. It would be interesting though to look at applications toward local delivery (e.g. solid tumors) and some of the more expensive medically intense therapeutics such as gene therapy and gene editing and CAR-T cell production.

11. ***Question:*** **Will SNAC become a "generic" PE?**

David: Well I guess it is, since it is now on the FDA inactive ingredients list, has GRAS status and is used as an excipient. It is used in dietary supplements and in an oral B12 under (weak) medical food guidelines. Lots of companies supply it, and some at GMP grade.

12. ***Question:*** **Do you think that the smaller size of peptide may be better for oral delivery?**

David: Yes, absolutely, the current macrocycles are in the 2K MW area. Tons of work shows lower absorption of large peptides and zero for proteins.

***Jerome**:* Smaller size will definitely be an advantage from a permeability standpoint, but it has to be balanced with potency and systemic PK. One of the features of peptide drugs is the incredibly high potency against targets with fairly flat binding surfaces. This is achieved by engaging a large surface area as opposed to a narrow pocket. Therefore reducing the size may rapidly come at the expense of potency. A couple of papers by Tom Tucker showed optimization of PCSK9 peptide including reducing the size of the mRNA display lead using

information from peptide-target co-crystal structures (J. Med. Chem. 2021, 64, 22, 16770–16800 & J. Med. Chem. 2020, 63, 22, 13796–13824)