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Traveling the Vitamin B_{12} Pathway: Oral Delivery of Protein and Peptide Drugs

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Oral routes of administration for therapeutic peptides and proteins face two major barriers: proteolytic degradation in the stomach and an inadequate absorption mechanism for polypeptides within the intestinal lumen. As a result, peptide-based therapeutics are administered by injection, a painful process associated with lower patient compliance. The development of a means of overcoming these two major obstacles and enabling the successful delivery of peptide therapeutics by the oral route of administration has therefore been the target of extensive scientific endeavor. This Minireview focuses on oral peptide/protein delivery by the dietary uptake pathway for vitamin B_{12}. Recent progress in this field includes the delivery of erythropoietin, granulocyte-colony-stimulating factor, luteinizing-hormone-releasing hormone, and insulin.

1. Introduction

Various factors combine to make the oral administration of peptides and proteins a major goal in drug delivery.[1] Transport, permeability, gastrointestinal stability, and the size of protein molecules are all factors that contribute to this challenge. Ways of overcoming these difficulties have been investigated extensively by a variety of approaches. This Minireview focuses on a unique uptake system that has received increased attention over the past decade, namely, that of vitamin B_{12} (cobalamin). Vitamin B_{12} (abbreviated B_{12}) is an essential nutrient cofactor for all animals, including humans. B_{12} is a complex molecule consisting of a nucleotide moiety and a planar porphyrin-like corrin ring, which contains a central cobalt(III) atom (see Figure 3). It is synthesized by bacteria and enters mammals through an intricate food chain, or is produced by microorganisms present in animal intestine or rumen. In humans, dietary intake of meat, liver, fish, eggs, and milk is the main source of B_{12}. The average adult ingests 5–30 μg of vitamin B_{12} per day, approximately 2–3 mg of which is typically stored by the body in a healthy adult (with a permanent liver reserve of about 1 mg). The existence of an enterohepatic recirculation pathway (1.4 μg per day from bile salts) coupled with body stores explains why B_{12} deficiency does not appear for several years after B_{12} absorption has been interrupted.

The use of the intricate dietary uptake pathway of B_{12} to deliver pharmaceuticals has received attention since the 1970s; however, advances during the past decade have rekindled interest in this pathway. The use of B_{12} for oral drug delivery is fundamentally dependent on the ability to adapt B_{12} as an effective drug-delivery vehicle, which is in turn highly dependent on the mechanism by which B_{12} is both protected and absorbed from the gastrointestinal tract. We provide a brief discussion of this mechanism herein; more detailed insight into this pathway can be found in reference [2].

B_{12} is released from food by the action of peptic enzymes and the acidic environment of the gastrointestinal system. It is then bound and transported by two glycoproteins, intrinsic factor and haptocorrin. Haptocorrin is secreted by salivary glands and released additionally by the gastric mucosa.

Salivary haptocorrin (HC; also known as transcobalamin I (TCI) or R binder) has a high affinity for B_{12} under acidic conditions (pH < 3). By binding to B_{12} under such conditions, HC protects B_{12} from acid hydrolysis. It also reduces scavenging by intestinal fauna. The HC:B_{12} complex travels from the stomach to the duodenum, where the increased pH value decreases the affinity of HC for B_{12}. Pancreatic enzymes digest HC to release B_{12},[36] which then binds to the second of the two gastric transport glycoproteins, intrinsic factor (IF; Figure 1).

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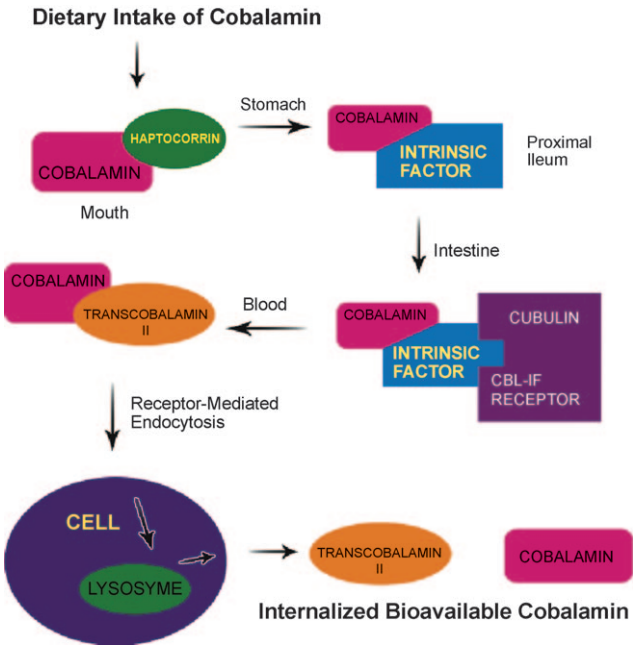

Figure 1. Dietary uptake pathway for vitamin B_{12}.

IF is secreted from the gastric mucosa and the pancreas. It facilitates transport across the intestinal enterocyte, which occurs by receptor-mediated endocytosis at the apically expressed IF–B_{12} receptor (cubulin). Following transcytosis, and between 2.5 and 4 h after initial ingestion, B_{12} appears in blood plasma bound to transcobalamin II (TCII; Figure 2). B_{12} is then cellularly internalized at the TCII:B_{12} receptor by endocytosis and released by the degradation of TCII by lysozyme.

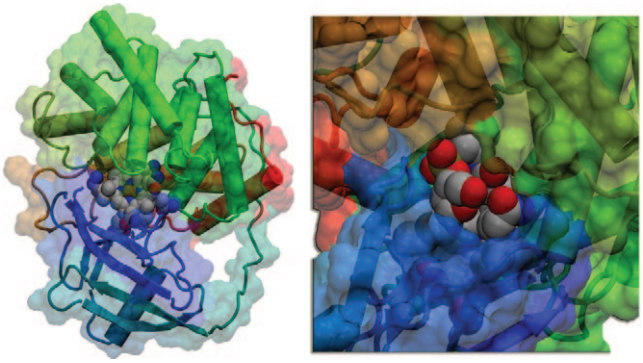

Figure 2. Holo-TCII with bound vitamin B_{12}. A solvent-accessible area of B_{12} around the ribose group is shown on the right. Images created by Dr. Damian Allis.

2. Chemical Aspects

An understanding of the binding between B_{12} and each of the various binding/carrier proteins is crucial, as a potential therapeutic should ideally be bound to B_{12} in such a way that the extremely high affinity (IF, TC, HC: $K_d \approx 5$ fM) of the various B_{12}-transport proteins remains unchanged for the modified B_{12} complex. The mechanism of discrimination between B_{12} and B_{12} analogues and the specific B_{12}-binding proteins has recently been investigated.[3]

Critically important to the successful use of the B_{12} pathway is thus the need to couple B_{12} with the peptide/protein such that neither molecule obstructs the other. The vitamin must still be recognized by the series of proteins involved in its uptake, and the peptide/protein must be able to interact with its receptor/target to induce the desired effect. Therefore, on both molecules, specific sites that are known, or postulated, not to be important for recognition and activity must be chosen for conjugation.

B_{12} and the peptide/protein can be coupled together directly or held at a distance from one another by "spacer" units; alternatively, carriers containing, but not conjugated to, the desired peptide/protein can be conjugated to B_{12}. The conjugation of peptides/proteins to B_{12} by these approaches has been successful at three major sites: 1) at peripheral propionamide units on the corrin ring (there are three such units; however, interference with IF uptake is only avoided at the ε position), 2) through the 5′-hydroxy group of the ribose unit of the α "tail" of B_{12}, and 3) to the phosphate unit, also in the α "tail" (Figure 3).

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Timothy Fairchild completed his PhD at the University of Western Australia under the guidance of Prof. Paul A. Fournier in the field of glycogen metabolism. After holding dual appointments at Edith Cowan University and the University of Notre Dame Australia, he moved to Syracuse University as Professor of Exercise Physiology. He is currently director of the Healthworks Laboratory. His research is focused on therapeutic approaches to the treatment of diabetes and obesity.

Robert Doyle was born in Dublin, Ireland in 1976. He received his BA in natural sciences in 1998 from Trinity College, Dublin and completed his PhD in chemistry there in 2002 under the guidance of Dr. Paul E. Kruger. After a year at the Australian National University, he joined the research group of Prof. Ann M. Valentine at Yale University as a postdoctoral associate. He moved to Syracuse University as an assistant professor in 2005. His research is focused on the roles of metals in biology and medicine and targeted drug delivery for the treatment of diabetes and obesity.

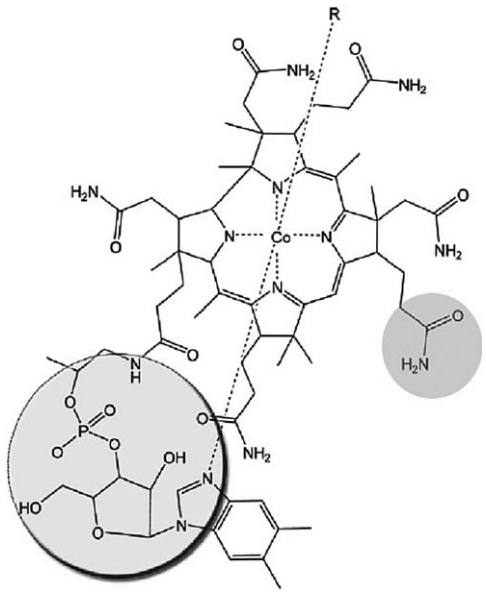

Figure 3. Conjugation at the highlighted sites in B_{12} does not interfere critically with the affinity of transport proteins for the complex. R = OH (hydroxocobalamin), CN (cyanocobalamin), Ado (5′-deoxyadenosylcobalamin).

The site can be selected on the basis of ease of chemical synthesis or the type of chemical bond or spacer desired. Successful uptake has been proven for each of the three positions. The ε-propionamide is converted into the carboxylic acid by heating at reflux in acid, and the desired ε-monocarboxylic acid B_{12} derivative is isolated by column chromatography. Various common agents, such as DCC, EDAC, CDI, CDT, and SPDP, have been used for the coupling step (see Table 1). A fourth site of conjugation that has been utilized for the synthesis of small molecules for oral delivery through the B_{12} pathway is the β axial site at the cobalt atom of B_{12}. This approach typically involves the reduction of Co^{III} to strongly nucleophilic Co^{I} (with sodium borohydride or Zn/HCl) under strictly oxygen-free conditions, and subsequent treatment with an organic halide. The organometallic Co–C bond formed is highly unstable and sensitive to light. Given the rather extreme reaction conditions and the instability of the resulting conjugates, this route has not been explored from the perspective of peptide/protein delivery. For interesting examples of the coupling of small molecules to the cobalt center and their transport in vivo, the reader is referred to the detailed studies of Alberto and co-workers[4] and Grissom and co-workers.[5]

Finally, Olesen et al.[6] used a fifth site, on the deoxyadenosyl group, for coupling with bovine serum albumin. In this case, 5′-deoxyadenosylcobalamin was used as the B_{12} source. This reaction was low yielding and produced, like direct conjugation to the cobalt atom, a highly light-sensitive product.

In general, conjugation at the β-ligand position of the cobalt atom has been avoided. Typically, the strongly binding cyano group has been used to "cap" this position during conjugation at peripheral sites.

2.1. B_{12}–Peptide/Protein Conjugates

The first B_{12}–protein conjugate was reported in 1971 (see Table 1).[6] In this study, Olesen et al. built bioconjugates of B_{12} with bovine serum albumin (through coupling to the phosphate moiety of B_{12} in the presence of EDAC by using hydroxocobalamin as the starting material) and with succinylated γG-globulin (by using 5′-deoxyadenosylcobalamin as the starting material, which resulted in conjugation to the amine group of the deoxyadenosyl unit). Olesen et al. did not investigate these conjugates for drug delivery but because in 1970 great difficulties remained in the isolation of B_{12}-binding proteins, such as IF and TCII. They reasoned that the immobilization of these conjugates on cellulose solid supports may provide a route for the isolation of uptake proteins from serum by affinity chromatography and demonstrated successfully that such an approach could be used to isolate B_{12} proteins from human gastric juice and serum.

The production of B_{12} bioconjugates for radioimmunoassay purposes was a further development in the use of such systems in the clinical setting. This area was developed throughout the 1970s by the research groups of Woldring,[7–9] Niswender,[10] and Ahrenstedt.[11] Their approach involved the conjugation of B_{12} to human serum albumin (HSA) to produce antigens to which antibodies could be raised. However, the approach did not include oral delivery but rather subcutaneous injection. B_{12} was typically conjugated to HSA by using carbodiimide-based coupling agents in the presence or absence of *N*-hydroxysuccinimide. Injection with B_{12}–HSA compounds induced an antibody response in rabbits.

Although these examples of early B_{12}–protein conjugates have led to further research in the field of B_{12}–protein-based immunoassays (see, for example, recent patents by Bio-Rad Laboratories[12,13]), the field of B_{12}–protein conjugation for oral drug delivery really began with the seminal studies of Russell-Jones and co-workers in the 1990s.[35] Publications in 1995 and 1996 describing the synthesis and oral delivery of granulocyte-colony-stimulating factor (G-CSF) and erythropoietin (EPO; see Table 1)[14,15] with demonstrated in vivo activity led the way. G-CSF is a protein factor that stimulates the production of white blood cells in the body. G-CSF is used by Amgen to make the injectable drugs neupogen (filgrastim) and neulasta (pegfilgrastim), which are used to keep the white-blood-cell count of cancer patient at a normal level during chemotherapy. EPO stimulates the maturation of erythroid progenitor cells into mature erythrocytes. It is applied in the treatment of anemia in kidney-dialysis patients. As is the case with all therapeutics mentioned herein, they must be administered parenterally. The publications presented methods for the conjugation of G-CSF and EPO to ε-monocarboxylic acid modified B_{12}. The use of spacers of different lengths enabled the formation of bioconjugates that maintained significant affinity for intrinsic factor and delivered up to 85% of the parenterally administered protein in investigations in vivo (see Table 1).

Russell-Jones et al. also synthesized a luteinizing-hormone-releasing hormone (LHRH) and LHRH antagonists conjugated to B_{12}.[16] LHRH regulates the synthesis and

Table 1: Key examples of B_{12}–protein/peptide bioconjugates.[a]

Protein/Peptide	Size [kDa]	Conjugation site	Linker[b] (coupling agent)	Activity	Year	Ref.
Directly conjugated:						
BS albumin	66	phosphate	phosphate–amine (EDAC)	ND[c]	1971	[6]
γG-globulin	150	phosphate	phosphate–amine (EDAC)	ND	1971	[6]
HS albumin	66	ε-monocarboxy	GABA (EDAC)	antibody response	1974 1978 1979	[11]
IFN-con	22	5′-ribose hydroxy	glutaroyl (CDI)	24–28% activity[d]	1994	[34]
G-CSF	19.6	ε-monocarboxy	disulfide (SPDP)	61–66% activity[e]	1995	[14]
			amide (EDAC)	29–85% activity[e]		
			hydrazide (EDAC)	ND, 100% activity[e]		
EPO	34	ε-monocarboxy	amide (EDAC)	ND, 34% activity[e]	1995	[14]
			hydrazide (EDAC) (DSS)	17–22% activity[e]		
ANTIDE-1	1.6	ε-monocarboxy	EGS (EDAC)	ND	1995	[16]
			amide (EDAC)	30% IF recognition		
			disulfide (SPDP)	65% IF recognition		
			sterically hindered thiol (SMPT)	54% IF recognition		
			thioester (NHS ester of iodoacetic acid)	81% IF recognition		
			transglutamase-cleavable tetrapeptide (EDAC)	60% IF recognition		
ANTIDE-3	1.6	ε-monocarboxy	EGS (EDAC)	ND	1995	[16]
			amide (EDAC)	ND		
			disulfide (2-iminothiolane)	ND		
			sterically hindered thiol (SMPT)	37% IF recognition		
			thioester (NHS ester of iodoacetic acid)	65% IF recognition		
			transglutamase-cleavable tetrapeptide (EDAC)	48% IF recognition		
LHRH	1.2	ε-monocarboxy	amide (DCC/NHS)	45% absorbed	2000	[33]
DP3	0.9	ε-monocarboxy	amide (EDAC)	23% absorbed	2000	[33]
			hexyl (EDAC)	42% absorbed		
insulin	5.7	5′-ribose hydroxy	amide (CDI, CDT)	26% drop in plasma glucose	2007	[30]
Delivery of encapsulated insulin:						
B_{12}-coated dextran nanoparticles	5.7	ε-monocarboxy	amide (CDI)	70–75% drop in plasma glucose	2007	[31]
B_{12}-coated dextran nanoparticles	*5.7*	ε-monocarboxy	amide (CDI)	70–75% drop in plasma glucose	2007	[32]

[a] Abbreviations: BS = bovine serum, HS = human serum, IFN-con = consensus interferon, G-CSF = granulocyte-colony-stimulating factor, EPO = erythropoietin, ANTIDE = *N*-Ac-D-Nal(2), D-Phe(pCl), D-Pa1(3), Ser, Lys(Nic), D-Lys(Nic), Leu, Lys(*i*Pr), Pro, D-Ala-NH_2, LHRH = luteinizing-hormone-releasing hormone, DP3 = octapeptide (Glu-Ala-Ser-Ala-Ser-Tyr-Ser-Ala), GABA = γ-aminobutyric acid, EDAC = 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, CDI = 1,1′-carbonyldiimidazole, SPDP = *N*-succinimidyl-3-(2-pyridyldithio)propionate, DSS = disuccinimidylsuberate, SMPT = 4-[(succinimidyloxy)carbonyl]-α-methyl-α-(2-pyridyldithio)toluene, NHS = *N*-hydroxysuccinimide, DCC = *N*,*N*′-dicyclohexylcarbodiimide, CDT = 1,1′-carbonyldi(1,2,4-triazole), EGS = ethylene glycol bis(succinimidyl succinate). [b] The linker was chosen for the greatest yield and/or activity. [c] ND = not determined. [d] Activity relative to that of native IFN-con. [e] Activity relative to that of unconjugated G-CSF or EPO.

release of pituitary gonadotropins. Many analogues of LHRH have been developed that can treat gonadotropin-dependent disorders. Generally, the amount of therapeutic LHRH needed for oral delivery is in excess of 100–1000 times the amount used for parenteral delivery. Therefore, clinical LHRH antagonists are currently administered by injection or as a nasal spray.[16] One such analogue is ANTIDE (*N*-Ac-D-Nal-(2), D-Phe(pCl), D-Pal(3), Ser, Lys (Nic), D-Lys(Nic), Leu, Lys(*i*Pr), Pro, D-Ala-NH_2), which potently inhibits ovulation and produced a chemical castration effect in male rats and monkeys. Russell-Jones et al.[16] conjugated positions 6 (in ANTIDE-1) and 8 (in ANTIDE-3) to the ε-monocarboxy position of B_{12}. A series of noncleavable and cleavable linkers were investigated, as was the length of individual spacer units. ANTIDE-1 and ANTIDE-3 were attached to (2-aminoethyl)amido ε-B_{12} through various linkers. Noncleavable linkers were constructed by using anilido, ethylene glycol bis(succinimidyl succinate) (EGS), or disuccinimidyl suberate spacers (see Table 1). The conjugates showed in vitro activity comparable to that of native ANTIDE; however, their in vivo activity was greatly decreased, most likely as a result of steric effects due to the direct conjugation of the peptide to the vitamin or rapid clearance by the B_{12}-binding proteins. Bioconjugates with cleavable linkers containing disulfide bonds were slightly less potent in vitro than those with anilido and EGS linkages; however, spacers with disulfide bonds showed markedly increased activity in vivo (see Table 1). Spacers with γ-glutamyl-ε-lysine bonds, which can be cleaved by serum transglutaminases, were also used. The resulting conjugates exhibited reasonable in vitro activity, but showed greatly reduced in vivo activity. In IF-binding assays, all conjugates of ε-B_{12} exhibited comparable activity to that of unmodified B_{12}. Poor in vivo activity was most likely due to the steric effects associated with the direct conjugation of the peptide to the vitamin or to rapid clearance by the B_{12}-binding proteins.

In more recent studies described in Section 3, Russell-Jones and co-workers and Petrus et al. demonstrated successful in vivo applications for the delivery of insulin.

3. Biological Aspects

The use of B_{12} to deliver therapeutic peptides has gained much recent attention as a result of both its potential to aid absorption from the gastrointestinal tract and its potential to increase plasma-residency time.[17] However, the use of B_{12} as a drug-delivery vehicle is limited by the quantity of B_{12} that may be absorbed from the intestinal lumen with a given dose. It remains unknown whether B_{12} absorption varies with total body stores of B_{12}; however, the fraction absorbed decreases as the oral dose is increased.[18] Nonetheless, the total absorption of B_{12} increases with increasing intake.

Studies to measure the absorption of B_{12} rely on whole-body counting of radiolabeled B_{12}, the counting of radiolabeled B_{12} in the stool, or both. An advantage of the use of animal models in the study of B_{12} metabolism is the ability to extract tissues and thereby quantify the distribution of the label in the tissue. Early reports, however,[19] made it appear that the B_{12} pathway was a phenomenon that was unique in humans and too dissimilar in rats to be comparable. These early studies on the absorption of radioactively tagged B_{12} in the rat had to be conducted with large doses, as the specific activity of the material was too low to enable accurate counting at lower doses. With the availability of labeled B_{12} with higher specific activity, however, it is possible to use very much smaller test doses and still retain high accuracy. Indeed, the rat model has now been shown to be a very useful for the evaluation of B_{12} absorption and excretion. The rat demonstrates similar responses to those of humans, even when human or porcine IF is coadministered in the gastrectomized rat. The initial findings in the rat[20] demonstrated a mean absorption of 52.3% when a dose of 1–10 ng was administered, whereas all animals absorbed less than 10% of doses of 1–2 μg. This result was confirmed by Moertel et al.,[19] who used doses of 0.01–0.545 μg and calculated the mean percentage of the dose absorbed to be between 53.4% (for the lowest dose) and 8.0% (for the highest dose).

Adams et al.[21] measured the fractional absorption of radiolabeled B_{12} in humans and reported that nearly 50% was retained at a dose of 1 μg, 20% at a dose of 5 μg, and just over 5% at a dose of 25 μg. A second dose of B_{12} given 4–6 h later was absorbed equally well;[22] however, this length of time between doses may not be required, as the average recycling time of cubilin is only 30 min.[23] When large doses of crystalline B_{12} are ingested, up to approximately 1% of the dose may be absorbed by mass action, even in the absence of IF.[24] However, this mass action is unlikely to occur when relatively small B_{12} is conjugated to a much larger therapeutic peptide.

To assess the feasibility of utilizing B_{12} as a carrier for peptides, it is important to relate the observed values of B_{12} absorption to the requirements of peptide delivery. Owing to very poor absorption across the intestinal cell lining and the harsh environment of the gastrointestinal tract, the oral bioavailability of peptide drugs is typically less than 1–2%.[25–27] An early study in humans with insulin demonstrated that approximately 0.5% of active insulin may be absorbed when introduced directly in a large quantity (100 units per kilogram of body weight) into the upper jejunum of the digestive tract.[28] Given that this mode of delivery bypassed the proteolytic environment of the stomach and that this study was conducted in a patient who had undergone total pancreatectomy, it would be safe to assume that this value of 0.5% is close to the upper limit for the oral delivery of nonprotected insulin. In order for a delivery system to be truly beneficial, it must raise the efficiency of absorption significantly above this base level of 0.5% (for insulin). Since the efficiency of B_{12} absorption is between 5 and 55%, the use of B_{12} to enhance the uptake of peptides is certainly feasible. However, as this level of efficiency is only viable at the upper threshold of absorption (with doses of approximately 20–25 μg in humans), a peptide that is only present in low doses would serve as a suitable candidate for this pathway. Alternatively, it may be possible to increase the load (or cargo) of each individual B_{12} molecule, so that a single B_{12} molecule carries multiple peptides conjugated either directly or indirectly. Multiple dosing of B_{12} conjugates may also be an important future avenue of investigation.

4. Advances

Since IF binding is critical for the success of the B_{12}-uptake pathway, the affinity of modified B_{12} towards IF must first be explored. McEwan et al.[29] synthesized 12 biologically active derivatives of vitamin B_{12} with spacers attached to the 5′-hydroxy group of the ribose unit. The potential of these derivatives to act as delivery agents for proteins, nanospheres, or immunogens by using the vitamin B_{12} uptake system was evaluated by determining their affinity for intrinsic factor (IF) and non-IF. The ribose 5′-carbamate derivatives showed similar affinity for intrinsic factor to that of the ε-monocarboxylic acid of B_{12}. The affinity for non-IF was similar to that of unmodified B_{12} or even higher for some of the smaller derivatives. Nanoparticles derivatized with B_{12} 5′-carbamate adipic dihydrazide were transported into Caco-2 cells with significantly higher efficiency than unmodified particles.[29]

Following these findings with IF, Russell-Jones and co-workers investigated through radiolabeling with ^{57}Co and ^{125}I the extent to which B_{12}-coupled peptides were absorbed.[33] A series of experiments were carried out to investigate the binding of the B_{12}–peptide bioconjugates to IF, the binding of IF–B_{12}–peptide complexes to specific cellular receptors (the IFCR) on the surface of Caco-2 monolayers, and the transcytosis of B_{12}–peptide conjugates across endothelial barriers. They also attempted to confirm their results in vivo by using rat models. All B_{12}–peptide bioconjugates bound to IF and were recognized by IFCR receptors on Caco-2 monolayers. The binding was saturable and could be inhibited by the addition of a 20-fold excess of B_{12}–IF. In vivo studies in the rat showed absorption of 53, 45, 42, and 23% of the applied radioactivity for B_{12}, B_{12}–LHRH, B_{12}–Hex-DP3, and B_{12}–DP3, respectively. Upon the addition of a more than 10^5-fold

excess of B_{12}, the absorption decreased to less than 4%. The tissue distribution of the conjugates was similar to that observed with regular B_{12}.

Petrus et al.[30] recently utilized the B_{12}-uptake pathway for the oral delivery of insulin conjugated directly to the 5′O position of the ribose tail of B_{12}. The lysine residue located at position 29 of the B chain of bovine insulin was conjugated through coupling in the presence of CDT (Figure 4). The product was purified by ion-exchange chromatography and identified as a monodisperse species following ultracentrifugation. Lys29 was chosen as the conjugation position for ease of synthesis, as the other two lysine residues of insulin can be selectively protected, and because Lys29 is known to be involved in oligomerization but not activity. Spectrophotometric binding studies showed that the B_{12}–insulin bioconjugate was still recognized actively by intrinsic factor.[30] To examine the in vivo efficacy of the B_{12}–insulin conjugate, blood from a streptozotocin-induced diabetic rat model was sampled prior to and subsequent to oral administration of the B_{12}–insulin conjugate over a 5 h period, and the results were compared to the blood glucose response following administration of a solution with an equimolar amount of free insulin. The measurement of fasting (>4 h) blood glucose levels prior to the administration of compounds confirmed hyperglycaemic levels (15.6 ± 0.8 mmol L^{-1}) and thus indicated an insulin-deficient state. The administration of the B_{12}–insulin conjugate led to a 4.7-fold greater decrease in the area under the blood-glucose curve ($p = 0.056$) than the administration of free insulin.[30] To identify whether the corresponding change in the concentration of glucose in the blood was mediated by a B_{12}-dependent uptake pathway, the blood glucose response to the B_{12}–insulin conjugate administered in the presence of a 10^5-fold excess of "free" B_{12} was investigated. The blood glucose response in this instance was significantly less pronounced, which indicates that "free" B_{12} saturated the uptake pathway and hindered the delivery of the B_{12}–insulin conjugate.[30]

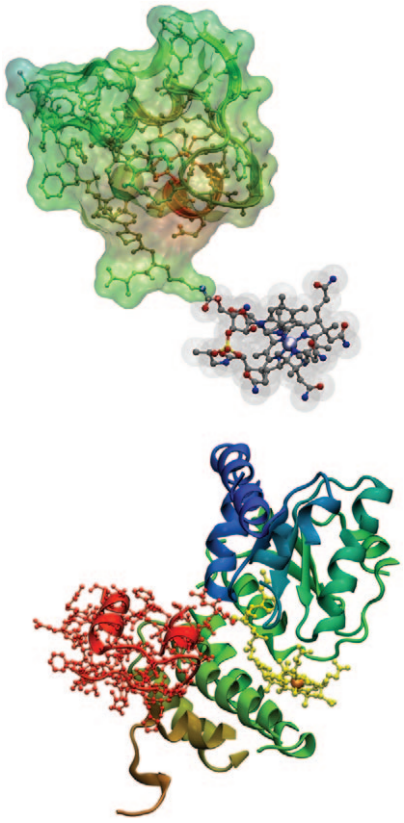

Figure 4. Top: B_{12}–insulin conjugate connected between the lysine residue of the insulin B strand (Lys29) and the hydroxy group of the B_{12} ribose unit. Bottom: The same conjugate bound to TC II; insulin is in red, B_{12} is in yellow.

Although the experiments of Petrus et al. are important, the absolute drop in the blood glucose level (by approximately 30% of the initial value) does not meet the requirements for clinical practice (ca. 75%). As the investigators administered an oral dose that exceeded the upper limit of absorption by the B_{12}-uptake pathway, it is expected that this drop in the blood-glucose concentration by about 30% of the initial value represents an upper limit for insulin delivery by the B_{12}-uptake pathway when an insulin/B_{12} conjugation ratio of 1:1 is adopted.

To avoid this potential upper limit, Jain, Russell-Jones, and co-workers[31,32] recently examined the use of B_{12} nanospheres for the delivery of insulin. The B_{12} nanospheres were prepared by modifying the surface of nanoparticles (NPs) with succinic anhydride and then conjugated with amino-substituted B_{12} derivatives through a carbamate linkage. The pharmacological availability of 70 K nanoparticles (NPs) containing 2, 3, and 4% (w/w) insulin was 1.1-, 1.9-, and 2.6-fold higher than that of NPs without B_{12}. This result is consistent with the hypothesis that the uptake of the NPs was mediated by B_{12}. Following oral administration of these carriers (20 IU kg^{-1}), glucose concentrations in the plasma reached a nadir after 5 h of 70–75% of baseline values. The decrease in the blood glucose level was less pronounced, but the concentration remained stable for 54 h. The animals in this study were fasted for just 1 h prior to administration; therefore, the baseline values may have been elevated, as food intake over the previous 4 h was not monitored or restricted. Furthermore, problems with formulation, such as varied protein dispersity and inconsistency in the amount of protein encapsulated, are associated with this approach. Nevertheless, this study demonstrated that the use of B_{12} nanospheres can lead to clinically relevant decreases in glucose levels. The results have now led to the development of the drug oradel (Apollo Life Sciences), which will soon enter phase I clinical trials.

5. Future Directions

The synthesis and in vivo activity of B_{12} nanospheres has significant potential for oral peptide/protein delivery. However, complexities associated with nanoparticle design must still be overcome, including production costs, possible complications associated with nanoparticle toxicity, and inconsistencies in the quantity of the peptide delivered, as well as formulation/polydispersity issues. However, for certain proteins, including those for which the B_{12} pathway does not

enable the delivery of a clinically relevant dose, this approach becomes a potentially viable route.

The direct conjugation of peptides/proteins to B_{12} would be the ideal utilization of this delivery mechanism. We feel that such an approach will be developed along two lines: first, the use of this pathway for the delivery of peptides/proteins that are only observed at very low serum concentrations, and second, the attachment of a chain of peptides to the B_{12} molecule by using cleavable linkers. The optimal chain length will need to be assessed, as it would be expected that the level of protection by B_{12} and its uptake proteins may be diminished with increasing chain length. Early studies that we carried out in collaboration with Shimadzu Scientific Instruments (Maryland, US) indicated that B_{12} alone does not prevent proteases, such as trypsin, from digesting insulin. Consequently, we employed biodegradable polymer systems to aid in the protection of the protein. By designing the system in such a way that the polymer is also conjugated to the B_{12}–protein conjugate and envelopes the exposed areas of the protein, greater protection against the proteolytic environment of the stomach can be achieved.[38]

Although the oral route is the most convenient method of drug administration, advances in technologies for oral peptide/protein delivery have not, to date, lived up to expectations. However, new delivery strategies associated with even small improvements in drug delivery could lead to significant improvements in patient compliance and clinical outcomes. Furthermore, the successful oral delivery of proteins would enable the development of orally administrable vaccines, the use of which would lead to a greatly increased immune response at a major site of pathogen entry, namely, the gastrointestinal mucosa. The development of an oral delivery mechanism for vaccines and other therapeutic peptides would be a significant medical contribution to the developing world. This possibility is a compelling incentive to further pursue the B_{12} dietary uptake pathway for oral drug delivery.

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