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REVIEW ARTICLE

Noninvasive delivery systems for peptides and proteins in osteoporosis therapy: a retroperspective

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Abstract

Objective: The aim of this review is to provide the reader general and inspiring prospects in various attempts to make noninvasive delivery systems of calcitonin and teriparatide feasible and as convenient as possible. *Background*: Calcitonin and teriparatide play an important role in both calcium homeostasis and bone remodelling. Currently calcitonin is available as a subcutaneous injection and as a nasal spray whereas teriparatide is administered subcutaneously. In the past few years, an increasing number of articles about drug delivery systems for calcitonin and teriparatide have been published. These delivery systems have been developed to overcome the inherent barriers for the uptake across the diverse membranes on the various routes for protein and peptide delivery. *Results*: Co-administration of permeation enhancers, mucoadhesive agents, viscosity modifying agents, multifunctional polymers, protease inhibitors as well as encapsulation and chemical modification are utilized in order to improve calcitonin and teriparatide absorption after oral, nasal, pulmonal, or buccal administration. *Conclusion*: The majority of research groups have been working on the development of formulations based on the encapsulation of molecules in biodegradable and biocompatible polymeric nanoparticles. However these observations are based on data obtained under different experimental conditions. Hence, it is difficult to compare the obtained results in order to draw general conclusions about the most promising characteristics required for oral and nasal formulations for these peptides.

Key words: *Calcitonin; teriparatide; drug delivery; permeation enhancer ; mucoadhesive agent*

Introduction

Osteoporosis is defined as a 'systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture'[1]. Its causes are multifarious: heredity as well as different lifestyle factors such as physical inactivity, use of tobacco, and nutrition habits are of importance[2]. Osteoporotic fracture, which is the major health consequence of this condition, may occur at any skeletal site. The primary sites are the spine, hip (proximal femur), humerus, and distal forearm[3]. Postmenopausal women are at greatest risk of developing osteoporosis because of the accelerated loss in bone mass associated with menopause. One out of three postmenopausal women and one out of five men over the age of 50 years will experience osteoporotic fractures[4]. For example, in 2003, 7.8 million Germans (6.5 million women) were affected by osteoporosis. Of these 4.3% experienced at least one clinical fracture. Only 21.7% were treated with an anti-osteoporosis drug. The total direct costs attributed to osteoporosis amounted to €5.4 billion[5]. This confirms that osteoporosis is not only a major impact on peoples' health and quality of life but also an enormous economic burden on health-care systems in Europe and worldwide. The report of the European Commission (1998) estimates an increase in the incidence of hip fractures in Germany until the year 2040, from 117,000 hip fractures in the year 2000 to 240,000 hip fractures[5]. As fractures are not only the main cost drivers but also

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the reason for the increased mortality rate in osteoporosis patients[6], it is essential to identify individuals at high risk and take preventive measures to reduce the costs associated with osteoporosis.

The state-of-the-art osteoporosis therapy includes lifestyle modifications on the one hand and pharmacotherapy on the other hand. Lifestyle modifications contain avoidance of smoking and drinking alcohol, exercises, and intake of calcium. Furthermore hip-protector pads reduce hip fractures in elderly people[7]. Pharmacotherapy consists of antiresorptive agents that decrease bone turnover. Besides calcitonin and teriparatide that will be discussed more in detail in this review, bisphosphonates are another possibility. Bisphosphonates are compounds that specifically bind to the hydroxyapatite crystals on bone surfaces and inhibit osteoclast function. They were shown to be effective in decreasing vertebral fractures among postmenopausal women who were at high risk for such fractures[8]. Furthermore, selective estrogen-receptor modulators such as Raloxifene are a valuable treatment for both preventing and treating postmenopausal osteoporosis. Raloxifene offers additional extra skeletal benefits. It is particularly beneficial for people at risk for vertebral fracture if they are also at increased risk of coronary artery disease or breast cancer. Raloxifene can also be used in combination with aminobisphosphonates for patients at risk of hip fractures[9]. Another alternative is the hormone replacement therapy. Hormone replacement therapy is recommended primarily for menopausal and vasomotor symptoms. Patients at increased risk of breast cancer, heart disease, stroke, or thromboembolic events should be cautioned against use of hormone replacement therapy[10].

However, new strategies remain to be identified and further optimization of well-established methods is needed to face the increasing requirements in the near future. This review examines the many attempts made to develop alternative, more convenient systems for calcium concentration-modifying peptide delivery and concentrates on the success in developing potential noninvasive technologies and devices and on major new milestones in modern delivery for the effective treatment of osteoporosis.

Calcitonin is a polypeptide hormone consisting of 32 amino acids. It is involved in the regulation of bone turnover[11]. It is known to be of basic importance in the treatment of osteoporosis, Paget's disease, hypocalcemia, and similar pathological conditions. CT, which is available as salmon, eel, and human calcitonin, inhibits osteoclast activity and rapidly decreases bone resorption. It has been found that sCT and eCT are more potent in mammals, especially in humans, than the actual human or other mammalian CT. The reason for this phenomenon is still not fully understood[12]. The major physiological role of CT is to control the calcium concentration as well as its metabolism in the body. Its major responsibility is to reduce the amount of calcium in the bloodstream. For this purpose, it increases the rate of calcium clearance from the kidney. It also reduces the amount of calcium excreted by the bone by inhibiting the osteoclast activity. Finally, it decreases the amount of calcium that could be absorbed from the small intestine[13]. Its production is inhibited when the calcium concentration is decreased beyond normal levels and the parathyroid hormone (PTH) is then secreted. PTH is the physiological antagonist to CT. In conjunction, these two hormones act to maintain a normal concentration of calcium in the bloodstream. CT is available as an injection (50–100 IU daily) or nasal spray (200 IU daily). Results from a single controlled clinical trial indicate that CT may decrease osteoporotic vertebral fractures by 35% but does not reduce the risk of nonspine fractures. The disadvantages of treatment with CT are that the suppression of disease activity does not persist after withdrawal of therapy and the development of drug resistance. While it does not affect other organs or systems in the body, injectable CT may cause an allergic reaction and unpleasant side effects including flushing of the face and hands, urinary frequency, nausea, and a skin rash. Nasal CT is easy to administer and can prevent bone loss[14], but its long-term efficacy in decreasing the rate of fractures has not been adequately evaluated. The primary side effects with nasal CT are a runny nose, headache, back pain, and nosebleed (epitasis). However, because of its analgesic effects, CT is recommended for patients with painful vertebral fractures[15] and may be a good option for pregnant women on chronic therapy, because the use of bisphosphonates is contraindicated for them[16,17].

Teriparatide is approved for the treatment of osteoporosis in postmenopausal women and in men who are at high risk for a fracture[18]. Teriparatide is a part of PTH with a molecular weight of 4117.72 g/mol and consists of the amino acid sequence 1–34 of the complete molecule that contains amino acid sequence 1–84 with a molecular weight of 9000 Da. This medication rebuilds bone and significantly increases bone mineral density, especially in the spine. Teriparatide may be an option for patients who continue to lose bone mass during treatment with other osteoporosis medications. Teriparatide is self-administered as a daily injection from a preloaded pen containing a 1-month supply of medication. It can be taken for a maximum of 2 years[19]. At the end of 2 years, to retain the benefits of treatment with teriparatide, most experts recommend that patients start an antiresorptive medication. Side effects include leg cramps and dizziness. In animal studies, very high doses of teriparatide given for a long period of time increased the incidence of rat osteosarcoma. Although common in rats, this type of tumor is extremely rare in adult humans.

For this reason, the FDA approved its use for up to 2 years only[20]. Teriparatide should not be used in people who may be at increased risk for this tumor. This includes patients with Paget's disease, children with growing bone, persons with unexplained serum alkaline phosphatase elevations, and those who have had radiation treatment involving the skeleton. It also should not be given to people with metabolic bone diseases such as hyperparathyroidism and those who have had cancer metastases to bone[21]. Eli Lilly has launched a product in the injectable form. In addition, Daiichi and West Pharmaceuticals are working on nasal formulations of PTH products.

Oral administration

The efficiency of transfer of orally administered drugs from the site of administration to the site of action may be limited by several factors. These include the release of the drug from the administered formulation and the changing conditions that the drug has to pass on its way to the target receptor[22]. The primary problems encountered with orally delivered protein pharmaceuticals include the poor intrinsic permeability across the intestinal epithelium[23], susceptibility to enzymatic degradation[24], rapid post-absorptive clearance[25], and chemical instability[26]. The oral bioavailability of CT and teriparatide is limited because of enzymatic degradation and limitations to their permeability. Sinko et al.[27] evaluated the regional difference of oral absorption of sCT and administered sCT into intestinal ports at low volume (25 mg/5 mL/dog) and with a fast infusion rate (12 mL/min). Absorption of sCT from the ileum was better than from other regions studied (Figure 1). Interestingly, the bioavailability of unformulated sCT from ileum (0.064 ± 0.004%) was higher than from duodenum (0.039 ± 0.017%) and colon (0.021 ± 0.004%). However, results display that the oral bioavailability of calcitonin is extremely limited and variable[27]. The oral administration of teriparatide did not lead to any elevation in circulating blood PTH levels[28]. Nevertheless, the development of an oral formulation of CT and teriparatide seems to be feasible as CT is an excellent candidate for the development of alternate delivery systems (DDS) because of its size and wide therapeutic index[29] with a preference toward the oral delivery route. As the hepatic metabolism of CT is minimal, the rate-limiting step to successful oral administration seems to be the delivery into the port vein[30]. However, whereas the permeation-enhancing effect for compounds smaller than 30Å in diameter seems to be less important, it becomes essential for compounds like calcitonin displaying a molecular size above the presumptive 'cutoff' of the absorption membrane[31].

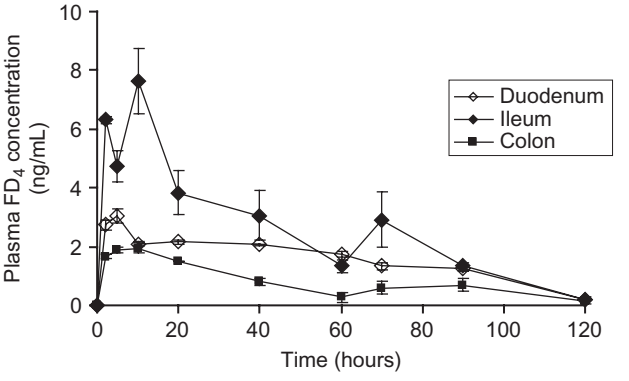

Figure 1. Plasma concentration (mean ± SEM) of sCT versus time curves after bolus duodenal, ileal, and colonal administration of 25 mg/5 mL/dog in IVAP dogs (n = 5 and 6). Figure adapted from Sinko et al.[27]

Another requirement for its oral delivery is intestinal stabilization because sCT and teriparatide are a substrate for the pancreatic serine protease trypsin[18,32]. Four general approaches—coadministration of auxiliary agents, encapsulation, administration of emulsions, and chemical modification—are currently being considered for improving calcitonin absorption in the gastrointestinal (GI) tract, and these are described in detail below[33].

Coadministration of additives

Permeation enhancers and protease inhibitors in peptide drug formulations have been investigated for many years[34]. As reported previously, luminally secreted serine proteases seem to be mainly responsible for digestion of sCT including trypsin[32], α-chymotrypsin[35], and elastase[36]. In addition, Werle et al.[18] evaluated the stability of teriparatide toward GI proteases[18]. Trypsin, chymotrypsin as well as pepsin have been identified to degrade teriparatide extensively. Elastase also degrades teriparatide to a certain but comparatively less degree. Membrane-bound peptidases have equally been shown to cause degradation of teriparatide. Furthermore, aminopeptidase N was found to be involved in this degradation process[18]. To overcome the enzymatic barrier Shah and Khan[37] investigated protective effects of various ovomucoid species against sCT metabolism by serine proteases. sCT solutions were incubated at 37°C with trypsin, α-chymotrypsin, or elastase in 50 mM Tris buffer (pH 8.0) containing or lacking different concentrations of tOVM, dOVM, or cOVM and aprotinin. Within this study, ovomucoids were shown to protect sCT metabolism by serine proteases depending on the species from which they were isolated. tOVM and dOVM were found to be superior to cOVM for protection against all three enzymes by inhibiting all three proteases in contrast to cOVM that was ineffective against chymotrypsin[37].

The pH value of the environment is crucial to the activity of GI digestive enzymes. Therefore Lee et al.[38] investigated the relationship between the modulation of intestinal pH and the oral absorption properties of sCT. As the pH stability of pancreatic trypsin (human) is optimal at pH 5–6 and decreases to approximately 45% of the activity remained at pH 4[39], Lee et al. enhanced the uptake of sCT through the addition of CA. An enteric formulation containing sCT and CA was given orally to dogs. As expected, the intestinal pH was significantly affected by the amount of CA in the formulations. By increasing the amount of CA in the formulation, the oral absorption of sCT increased gradually. These results indicate that the oral absorption of sCT is directly related to the stabilization of sCT by the reduction of the intestinal pH[38]. In a further publication, Sinko et al.[27] developed two different capsule formulations containing sCT, CA, CL (DDS 1), or sodium taurodeoxycholate (TDC; DDS 2). Both capsules were coated with hydroxypropylmethylcellulose phthalate to retard release in the stomach. Capsules were administered orally to dogs and it was observed that formulations containing TDC or LC had a significantly higher effective permeability as compared to their respective controls. Bioavailability enhancement ranged from 98% to 337% for all treatments and regions studied. When compared to sCT alone (without CA), bioavailability enhancement ranged from 1220% to 3070% for DDS1 or DDS2, suggesting that two mechanisms of enhancement, a reduction in the proteolytic degradation in the GI lumen and a permeation enhancement, account for the higher sCT absorption (Figure 2)[27].

In many previous studies on protein and peptide delivery, the permeation-enhancing effects of various surfactants have been demonstrated. For the oral administration of sCT, Takatsuka et al.[40] have investigated the applicability of simultaneous use of mucolytic agent *N*-acetyl acetate with nonionic surfactant TX-100 to peroral delivery of sCT. Synergistic enhancement of intestinal sCT absorption was observed when *N*-acetyl acetate was coadministered with TX-100 and was comparable with or even higher than those of classical absorption enhancers. The combination of CA with taurocholate also provided a clear enhancement but significantly lower than that of NAC+TX-100. The combination of CA and taurodeoxycholate showed a remarkable enhancement of absorption, with a bioavailability comparable to that obtained with NAC+TX-100. Regarding oral delivery of biologically active PTH, Leone-Bay et al.[41] administered an aqueous solution containing PTH and the delivery agent *N*-[8-(2-hydroxy-4-methoxy)benzoyl]amino cyprylic acid (4-MOAC) to monkeys. The relative bioavailability of oral PTH was 2.1% relative to subcutaneous administration. The biological activity of the orally delivered PTH/4-MOAC was further evaluated in a rat model of osteoporosis. These studies demonstrated that the bone formation following oral PTH/4-MOAC administration was comparable to that formation following PTH injections. The 4-MOAC-mediated absorption of PTH is hypothesized to be the result of a noncovalent interaction between 4-MOAC and PTH[38]. Accessorily, Ogiso et al. prepared an oral emulsion, which was prepared by coating the w/o/w emulsion with 0.1% carbopol and a strong mucoadhesion of the emulsion to the GI mucosa could be observed. A capsule containing eCT, taurocholate, and lyophilized carbopol administered orally gave a sustained but comparatively small calcium-lowering effect[42]. However, although surfactants showed good intestinal absorption enhancement properties, they were reported to cause an intestinal mucosal damage[43–45].

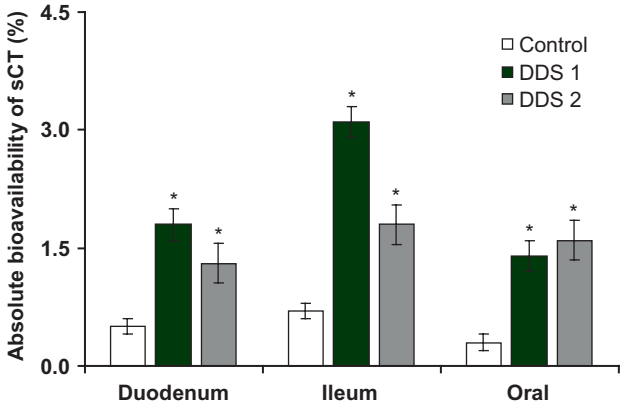

Figure 2. Absolute bioavailability (mean ± SD) of sCT formulations after duodenal and ileal administration as a solution in IVAP dogs (n = 5 and 6), and oral administration as an enteric capsule in normal dogs (n = 4). *Indicates the significant difference from control by $P < 0.05$. Figure adapted from Sinko et al.[27]

In contrast, chitosan (CS) has proven to be a useful excipient in various DDS as it exhibits favorable features such as nontoxicity, high cohesive, and mucoadhesive properties and can therefore be utilized as carrier matrix for the noninvasive administration of (poly)peptides. However, chitosan does not exhibit enzyme inhibitory properties[46], and the coadministration of enzyme inhibitors leads to various toxic side effects[47]. This problem could be solved by the covalent immobilization of inhibitors to polymers used as drug carrier matrices, such as chitosan or the utilization of thiolated polymers, so-called thiomers. The immobilization of thiol bearing moieties on the polymeric backbone of polymers such as chitosan or poly(acrylic) acid[48–50] leads to such thiomers. Because thiomers were introduced in the pharmaceutical arena, they demonstrated various merits in numerous publications regarding mucoadhesive[51], permeation-enhancing[52], and enzyme inhibitory[53] properties. Guggi and Bernkop-Schnürch[52] covalently attached Bowman-Birk inhibitor (BBI) and elastase inhibitor, elastinal, to the polymer chitosan. The mixture of the two chitosan

derivates displayed a strong protective effect for CT toward trypsin, chymotrypsin, and elastase. In a further study, Guggi et al. synthesized a novel chitosan-pepstatin A derivate and evaluated its excellent inhibitory efficacy toward pepsin that leads to an enhanced oral uptake of sCT[54]. Assured by these results, Guggi et al. developed an oral sCT delivery system consisting of Ch-TBA, sCT, mannitol, chitosan-BBI conjugate, and chitosan-elastinal conjugate. This mixture was compressed to tablets and was administered orally to rats. Results indicated a decrease in the calcium level of the dosed animals in comparison to control tablets being based on unmodified chitosan (Figure 3)[55]. The addition of glutathione to the tablets led to a further improvement in the oral bioavailability of sCT. The mechanism being responsible for this permeation-enhancing effect seems to be based on the thiol groups of the polymer. These groups inhibit protein tyrosine phosphatase, being involved in the opening process of the tight junctions, through a glutathione-mediated mechanism[56]. Results from an in vivo study confirmed this theory as tablets consisting of sCT, Ch-TBA, chitosan-pepstatin A, and glutathione led to a decrease in the calcium plasma level of 10% for at least 12 hours (Figure 4)[54].

Encapsulation of anti-osteoporotic peptides

Delivery systems are designed to protect an incorporated drug from degradation and to provide a controlled release. Particulate drug carriers that have been investigated for this purpose are o/w emulsions, liposomes,

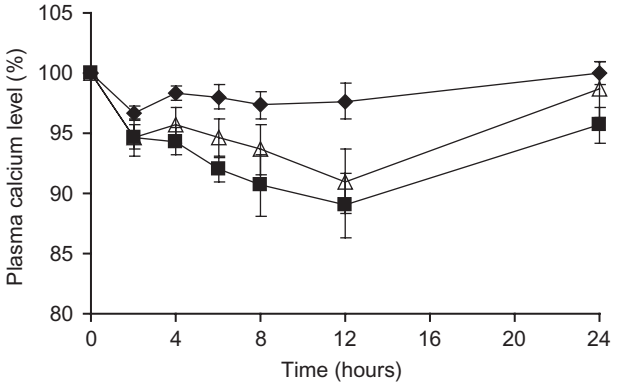

Figure 3. Decrease in calcium level in plasma as a biological response for the salmon calcitonin bioavailability in fasted rats after oral administration of test formulation A (containing 50 μg sCT, 1340 μg Ch-TBA conjugate, 200 μg Ch-BBI conjugate, 200 μg Ch–elastinal conjugate, 10 μg glutathione, and 200 μg mannitol) (■), test formulation B (containing 50 μg sCT, 1350 μg Ch–TBA conjugate, 200 μg Ch–BBI conjugate, 200 μg Ch–elastinal conjugate, and 200 μg mannitol) (△) and control B (containing 50 μg sCT and 1950 μg chitosan) (◆). Indicated values are the mean results from six rats (test formulation A) and from four rats (test formulation B and control B) ± SD. Figure adapted from Guggi and Bernkop-Schnürch[36].

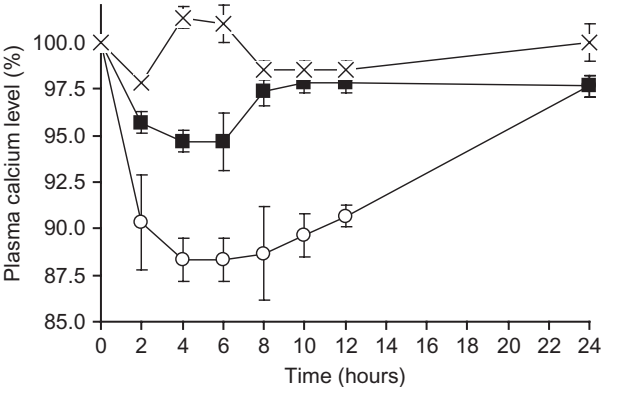

Figure 4. Decrease in calcium plasma level as a biological response for the salmon calcitonin bioavailability in fasted rats after oral administration of dosage form A (containing 50 μg sCT, 3450 μg Ch-TBA conjugate, 1000 μg Ch–pepstatin A conjugate, and 500 μg glutathione) (o) of dosage form B (containing 50 μg sCT, 3950 μg chitosan, and 1000 μg Ch–pepstatin A conjugate) (■) and of dosage form C (containing 50 μg sCT and 4950 μg chitosan) (X). Indicated values are the mean results from five rats ± SD. Figure adapted from Guggi and Bernkop-Schnürch[36].

microparticles, and nanoparticles based on synthetic polymers or natural macromolecules[57]. Liposomes are well-established and intensively investigated particulate carrier systems that have been successfully employed for the controlled release and site-specific drug delivery. Liposomes consist of one or more phospholipid bilayers separated by internal aqueous compartments[58].

Song et al. prepared proliposomes containing sCT and TDC, designated as TDC proliposomes. Because of the formation of lipophilic ion pair complexes, the entrapment efficacy of sCT into liposomes increased and was 2.8-fold greater than control proliposomes. In addition, liposomes from the TDC proliposomes were much smaller compared to control proliposomes. These changes from TDC proliposomes increased permeability, entrapment efficacy, and vesicle size might influence the intestinal absorption of sCT[59]. In contrast, Thirawong et al. prepared self-assembling PLNs by a simple mixing of cationic liposomes with pectin solution to improve intestinal absorption of eCT. The surface charges were shifted from positive to negative after mixing with pectin, and average particle size of PLNs was significantly larger than that of initial cationic liposomes. The eCT-loaded PLNs demonstrated a strong pharmacological action over the eCT solution and eCT-loaded liposomes, in which an enhanced and prolonged reduction in plasma calcium concentration of rats was observed. This was attributed to the ability of pectin to adhere to the mucous layer and to prolong the retention time on the intestinal mucosa[60]. In another study, Takeuchi et al. compared different mucoadhesive liposomes. Mucoadhesive liposomes were prepared by

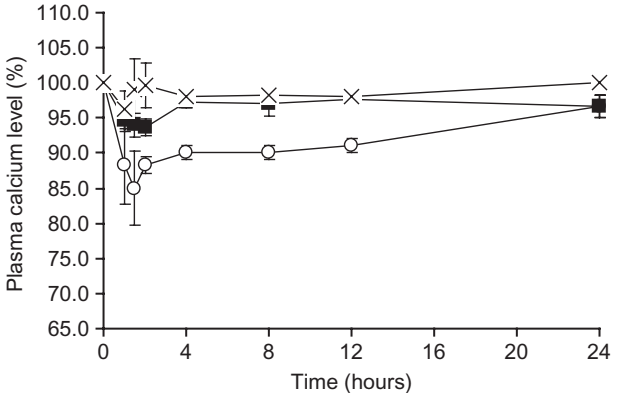

Figure 5. Change in blood calcium concentration after administration of calcitonin incorporated into negatively charged Non-Lip (■) or CS-Lip (o) or calcitonin in a solution (X). Dose of calcitonin was 500 IU/kg rat in each case. Indicated values are the mean results from five rats ± SD. Figure adapted from Takeuchi et al.[61]

coating multilamellar CP-Lip, as well as CS-Lip. The mucoadhesive properties of these two resultant polymer-coated liposomes and the positively or negatively charged noncoated liposomes were evaluated on rat intestine. The rank order was Cs-Lip ≥ CP-Lip > positively charged Non-Lip > negatively charged Non-Lip. In addition, administration of CP-Lip and CS-Lip containing calcitonin showed an enhanced and prolonged reduction in blood calcium concentration (Figure 5)[61]. Furthermore, Lamprecht et al. prepared a pH-sensitive colon-specific microparticulate DDS by a double w/o/w emulsion method using Eudragit P-4851F. Encapsulated CT proved a controlled release and leads to a distinct increase of the pharmaceutical effect compared to free CT. The relative pharmacological effect became most intense after 8–12 hours based on the selective pH-dependent delivery. Microparticles consisting of Eudragit P-4851F displayed a fourfold increase of the area above the curve of calcium blood level compared to levels reached after CT solution[62].

Another strategy to circumvent the GI degradation of peptides and proteins seems to be the application of solid lipid NPs[63]. Garcia-Fuentes developed new drug nanocarriers that consist of lipid NP with a hydrophilic coating (either PEG or CS)[60]. Afterwards, they investigated the behavior of PEG- and CS-coated lipid NPs in terms of their utility for oral peptide administration[64,65]. After demonstrating the stability of these systems in simulated GI media and the ability to release sCT[64], the next goals included the study of interactions between these surface-modified lipid NP and Caco-2 cells and to evaluate the potential of these nanostructures as oral delivery systems for sCT. Results indicate that lipid NPs coated with PEG or chitosan were able to enter the Caco-2 cell monolayers. Moreover, chitosan-coated NPs were able to enhance the oral absorption of sCT leading to a prolonged hypocalcemic response, whereas PEG-coated NPs were unsuccessful at increasing the absorption of the peptide[65].

Colloidal carriers such as polymeric NP and nanocapsules are a promising way to improve the oral bioavailability of peptides and proteins. The proposed mechanisms explaining the efficacy of these nanocarriers are the great surface interaction of the nanocarrier with the absorptive epithelium, which can be further enforced by the use of bioadhesive materials, and the protective effect of the carrier for the associated peptide. Actually, the effectiveness of nanocarriers at improving the absorption of labile macromolecules depends strongly on their polymer composition. For example, one alternative to circumvent the GI degradation is the absorption enhancement by nanoparticles composed of novel graft copolymers having a hydrophobic polystyrene backbone and hydrophilic polyvinyl branches. These NPs were prepared by the free radical copolymerization between hydrophilic macromonomers terminating in vinylbenzyl groups and styrene. Their surfaces were covered with hydrophilic polymer chains and their diversity was controlled by designing and synthesizing different functional macromonomers[65]. NPs having pH-sensitive anionic poly(methacrylic acid), cationic poly(vinylamine) (PVAm), thermosensitive nonionic poly(*N*-isopropylacrylamide) (PNIPAAm), and highly water-soluble nonionic poly(vinylacetamide) (PNVA) on their surfaces[66]. Sakuma et al.[67] reported that the absorption of sCT through the GI tract was enhanced by these NPs. This absorption enhancement effect was affected by the macromonomer structure, and sCT was significantly enhanced by NP having PNIPAAm on their surfaces. Sakuma et al. proposed the hypothesis that these NPs have not only the property of bioadhesion to the GI mucosa but also that of stabilizing peptide and protein drugs in the GI tract. However, the hypocalcemic effect after oral administration of the mixture of sCT and PNIPAAm NP was only retained for 4 hours and was independent on the nanoparticle size and molecular weight of the macromonomers. These results point out that the absorbed amount of sCT was not sufficient for inducing the substantial pharmacological activity of sCT. Sakuma et al. found two ways of further improvement of this low absorption of sCT. One way is to optimize the administration schedule[68]. When the dose of the mixture was bisected and one-half was given orally 40 minutes after the other half, the sCT-induced hypocalcemic effect was markedly enhanced by PNIPAAm NP. Sakuma et al. proposed that there must be an interaction between the hydrophilic mucous layer and hydrophilic NPs just after administration. After a while these NPs become hydrophilic at body temperature because a phase transition of the branches on the NP surface occurs at 35°C. It is likely that these hydrophobic

NPs subsequently desorb from the hydrophilic mucous layer to a certain extent[63]. The other way to improve sCT absorption is the optimization of the chemical structure of NP. When styrene was polymerized with two different kinds of macromonomers, polystyrene NPs have two macromonomer chains on their surface[69]. The enhancement effect of sCT absorption by PNIPAAm NP was increased significantly by introducing cationic PVAm chains to the surfaces. The reduction of the blood calcium concentration was increased about threefold. On the other hand, the introduction of nonionic PNVA chains completely eliminated the absorption-enhancing function of PNIPAAm NPs[69]. In addition, Sakuma et al.[70] ascertained that PNIPAAm and PVAm NPs completely protected sCT against degradation by pepsin. In another study, Yoo et al. formulated sCT into biodegradable NP using sCT–oleate complexes. sCT amphiphile complexes were formed by hydrophobic ion pairing using three different kinds of amphiphilic molecules of fatty acids, phospholipid, and surfactant, namely, sodium oleate, DMPG, and sodium deoxycholate. Hydrophobic ion pairing for proteins and peptides has been utilized for the enhancement of protein partitioning in a nonpolar phase. sCT NPs were readily taken up by Caco-2 cells, and sCT was transported across the Caco-2 monolayer in vitro. In vivo experiments showed that sCT was orally absorbed[71]. Prego et al. prepared sCT-loaded chitosan NPs by the solvent displacement technique. Chitosan NPs presented a positive surface charge and an efficient encapsulation of sCT. Following oral administration to rats, NPs exhibited the ability to reduce calcemia levels[72]. In a continuative study, Prego et al. explored the possibilities to further improve the surface properties of chitosan nanocapsules in terms of their interaction with the GI environment. For this purpose, chitosan NPs were coated with PEG as PEG improves the biocompatibility of chitosan and the stability in biological fluids. As a consequence, PEG facilitates the transport of bioactive macromolecules across the intestinal mucosa[73]. The pegylation of chitosan NPs resulted in an improvement in the in vitro stability of the nanocarriers and also in an increase in the cellular viability of the Caco-2 cells. Moreover, it could be demonstrated that these new nanocapsules are very efficient at increasing the intestinal absorption of sCT[73].

As mentioned above thiomers seem to be a promising tool for the delivery of hydrophilic macromolecules. Recently, NPs consisting of thiomers were developed and they displayed some notable features. Because of the covalent crosslinking via disulfide bonds, thiomer particles display a greater stability than the corresponding ionically crosslinked particles[74]. Because of the presence of thiol groups on NPs, their mucoadhesive properties are more than twofold improved and, in addition, drugs can be incorporated easily in such particles[75]. As oral formulations of insulin are currently in development, it is likely that similar formulations of CT or teriparatide will be developed if the significant hurdles of oral peptide delivery are overcome. For example, Deutel et al. prepared a novel protein–thiomer nanoparticulate delivery system. Protein-loaded NPs were obtained by the formation of hydrogen bonds between poly(vinylpyrrolidone) and poly(acrylic acid) (PAA)-Cys or PAA with the model-protein insulin. Serum insulin concentrations and reduction of blood sugar values were determined after oral administration of nanoparticles formulated as enteric-coated tablets and suspensions. Results displayed a low-serum insulin concentration and pharmacological efficacy in terms of blood sugar reduction after oral administration of coated tablets. Additionally, nanoparticulate suspensions led to significant serum insulin concentration[76].

Administration of calcitonin-containing emulsions

Baluom et al. examined the possibility of increasing the oral bioavailability of sCT by incorporating the drug in a new type of powdered SME and checked whether the incorporation of Carbopol 940 into the SME could further improve sCT absorption. After intracolonic administration, the SME formulations yielded a significant reduction in plasma calcium level as compared to administration of sCT in normal saline. In the case of MA-SME, the reduction was more profound and was 1.5-fold prolonged in the colon and an increase in the absolute bioavailability of sCT of 14.7% could be determined[77]. These results were in good accordance with another study by Bai et al., in which the in vitro efficacy of PAA polymers in inhibiting degradation of sCT was determined[78]. The extensive inhibition by these polymers seems to correlate with the ability to acidify the incubation medium based on the carboxylic acid groups. It is expected that as Carbopol polymers dissolve in the intestinal lumen, they release protons, creating a local acidic environment in which proteolytic activities will be inhibited[79].

Chemical modification

Lipidization of a polypeptide appears to be a reasonable approach for developing oral delivery systems, because there are examples of natural peptides with high lipophilicity, such as cyclosporine A, that can be absorbed in the GI tract[18]. Wang et al. developed a method of conjugation in aqueous solution and can regenerate the original active polypeptides in the tissue or the blood. This method is called reversible aqueous lipidization (REAL). Generally, REAL-modified peptides exhibit an increased stability, plasma half-life time, and epithelial

absorption[80]. Wang et al.[80] lipidized sCT by using this REAL technology. The conjugation was based on the formation of two new disulfide bonds connecting *N*-palmitoyl cysteine, the lipid moiety, with dithiothreitol-generated free thiol groups in sCT. Because of the structural similarities of the newly formed disulfide bond to the naturally occurring cysteine–cysteine bond in endogenous peptides and proteins, it is anticipated to undergo reduction and regenerate the native peptide in a biological system. Thus, the biological properties of the native peptide would be preserved and possibly enhanced[81]. REAL-sCT was given orally and was injected subcutaneously in mice, and the plasma calcium level was monitored. Results indicate that the oral absorption of sCT in rats was minimal. On the other hand, peptide absorption was significantly enhanced upon lipidization. Lipidized sCT exhibit improved pharmacokinetic and pharmacodynamic behaviors and a 19-fold higher area under the curve (AUC) after oral administration in comparison to oral sCT[81].

Nasal delivery

The mucosa of the nasal cavity offers a large surface area with extensive blood supply that makes it an interesting target for DDS. Moreover, the nasal route allows us to circumvent the hepatic first-pass metabolism. However, the uptake of polar high-molecular-weight drugs, such as peptides like CT and PTH, is limited. Generally, proteins and peptides display a relative bioavailability of approximately 1%. Nevertheless, a nasal dosage form for CT (Miacalcin® Novartis) has been introduced to the market in Europe and the United States in the 1990s. Miacalcin® is an aqueous solution, which is sprayed into the nasal cavity. For this commercial dosage form, bioavailabilities between 3% and 7% have been stated[82]. For oral PTH (1–34), no nasal dosage form has been introduced to the market thus far. Relative nasal bioavailabilities compared to subcutaneous injections of 12.1% (without any additives) and 17.6% (with 1% bovine serum albumin added) on average have been determined in rats by Agu et al.[83].

In recent years, several approaches have been made to improve the nasal bioavailability of CT. The major obstacles for CT as for every other peptide on its way from the nasal cavity to the systemic bloodstream are limited paracellular transport across the epithelial membrane because of tight junctions and the rapid mucociliar clearance of administered formulations from the nasal cavity. For nonmucoadhesive formulations, a half-life of clearance of approximately 15 minutes has been shown[84]. A further barrier can be enzymatic degradation. Within the nasal lumen and the epithelial barrier, endo- and exopeptidases have been identified. However, in contrast to oral drug delivery, enzymatic degradation is regarded as a minor impact on nasal delivery. For example, it could be shown that only neglectable rates of nasal insulin doses were degraded by enzymes from the nasal cavity[85]. For these reasons, most of the approaches to overcome the mentioned barriers include permeation enhancers, viscosity-modifying and mucoadhesive agents, and multifunctional polymers that combine most of the mentioned features. Furthermore, several authors concentrated on the advantageous properties of dry powder formulations compared to liquid formulations.

Absorption enhancement

To improve the nasal uptake of sCT, numerous permeation enhancers have been investigated. These include bile salts, dihydrofusinate, sodium tauro-24,25-dihydrofusidate as well as other surfactants. Furthermore, cyclodextrines, acylcarnitines, sucrose cocoate, and chitosan have been investigated concerning their permeation-enhancing capacities for sCT.

In the early stages of research on nasal calcitonin delivery, different surfactants have been investigated as permeation enhancers. Lee et al. could determine a 14-fold improved serum level of sCT in human volunteers with a 0.5% sodium tauro-24,25-dihydrofusidate containing solution compared to a formulation without any enhancers[86]. Pontiroli et al. utilized sodium glycocholate and dihydrofusinate as surfactants for nasal CT delivery in human volunteers. Furthermore, dihydrofusinate was tested in spray and powder formulations. They could demonstrate a significantly improved bioavailability compared to CT without surfactants[87]. Within the different surfactant formulations, no significant difference could be shown. In another study, the permeation-enhancing capacities of acylcarnitines for nasal sCT delivery were investigated. Kagatani et al.[83] found that lauroylcarnitine chloride displays the best improvement rate in bioavailability at 0.1%. Despite their outstanding absorption-enhancing features, surfactants never became a realistic tool for transmucosal delivery system because of their membrane irritation and cytotoxic effects. For example, Pontiroli states irritations in volunteers for sodium glycocholate[88].

Hence, other possible permeation enhancers such as cyclodextrins and chitosan got into broader focus. As for many other peptide drugs, cyclodextrins have been used for CT delivery. Schipper et al. demonstrated the absorption-enhancing effect of methylated β-cyclodextrins. An addition of 3% dimethylated β-cyclodextrins has been shown to be most effective when coadministered with an aqueous sCT solution. A decrease of serum calcium of 23.1% on average was achieved, which means an almost eightfold improvement over

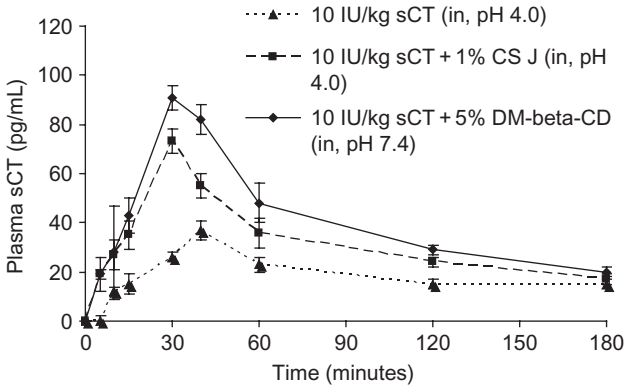

Figure 6. Plasma sCT concentrations after nasal (in) administration of sCT (10 IU/kg) with and without enhancers. Data = mean ± SD. Figure adapted from Sinswat et al.[90]

control[89]. Sinswat et al. compared the enhancing effect of β-cyclodextrins and different types of chitosans on nasal absorption of sCT. This workgroup used CS J and CS G. Thereby, CS J showed an improved pharmacological effect with decreasing pH (pH 4.0). CS G showed its maximum effect at pH 6.0. Both types showed concentration-dependent effects between 0.25% and 1%. Addition of 1% CS J showed a twofold increased uptake of sCT compared to sCT alone and has been shown to be superior to an addition of 5% dimethyl-β-cyclodextrin[90]. Plasma concentrations are shown in Figure 6. At present, chitosan and its derivatives appear to be the less irritant alternative over cyclodextrins. Ahsan et al. have introduced two relatively unknown additives as absorption enhancers into CT delivery, alkylglycosides like TDM[91] and sucrose cocoate[92]. In the above-mentioned study, 0.125% and 0.25% TDM caused a four- and sevenfold increase in AUC of the plasma calcitonin–time curve compared to calcitonin alone, respectively. SL-40 is a mixture of fatty acid sucrose esters that can be obtained from esterification of coconut oil with sucrose. It has widely been used as an excipient in cosmetics and features an HLB value of 15. Ahsan et al. studied the effect of 0.5% sucrose cocoate in aqueous solutions on the nasal absorption of CT. Within this study, it was found that 0.5% SL-40 improves CT uptake significantly and by these means leads to a significantly reduced serum calcium level. TDM and SL-40 show structural similarities and might cause their effects by intercalation with the lipid bilayer because of its chemical structure or by loosening tight junctions. Matsuyama et al. developed different formulations using NAC as a mucolytic agent to enhance the nasal absorption of sCT. A combination of NAC and a surfactant-type penetration enhancer was examined to optimize nasal absorption with minimized damage to the mucosal membrane. NAC is supposed to lower the viscosity of the mucus and to facilitate the access to the membrane for the surfactant. Laureth-25 was used as nonionic surfactant. By adding 5% NAC, an approximately twofold increase in bioavailability could be observed, 5% laureth-25 alone showed an 1.7-fold increase while the combination of both leads to an 2.8-fold increase. Moreover, they compared their result to the effectivity of 1% sodium glycocholate and concluded that their dosage form was equally effective. To justify this approach, local toxicity of NAC and laureth-25 was assessed by measuring hemolytic activity and leakage of phospholipids. Laureth-25 did not show hemolytic activity in concentrations up to 5%, and the combination of both additives did not show a significantly different phospholipid release from the membrane compared to pure saline[93].

Dry powder formulations

Insulin solutions with DM-β-CD as a permeation enhancer showed an improved bioavailability in rats whereas similar formulations did not show significant effects in humans. Schipper et al.[94] could demonstrate that a lyophilized powder formulation with these components was capable of improving the bioavailability of insulin in rabbits significantly, whereas a liquid formulation did not show a significant increase in bioavailability. The knowledge that, in certain cases, powder formulations display superior effectivity led to several studies that dealt with powdery formulations. Ishikawa et al. were investigating the influence of $CaCO_3$ as an insoluble carrier on the systemic bioavailability of eCT after nasal administration in rats. For this purpose, radiolabeled eCT was added to $CaCO_3$ and mixed. Finally, the powder mixture was dried in vacuum. Liquid formulations consisted of radiolabeled eCT in normal saline. The nasal absorption was tested in vivo in rats while the in vitro nasal transport was investigated in horizontal diffusion chambers on nasal mucosa from rabbits. An almost twofold increased bioavailability could be obtained with the powder formulation over liquid formulations. Furthermore, a more rapid absorption across the membrane was observed, as well as an advanced hypocalcemic effect. Within permeation studies across nasal mucosa from rabbit, no significant difference was shown. They concluded that the improvement is based on a prolonged residence time on the nasal mucosa[95]. According to these results, Matsuyama et al. developed powder formulations with different amounts of ethylcellulose as a filler excipient and NAC as a mucolytic agent, respectively. Formulations were tested in rats and beagle dogs. The bioavailability was compared to a powder formulation containing sodium glycocholate and to liquid formulations with or without NAC. Within this study, the addition of NAC to a liquid formulation led to an almost twofold improved

bioavailability in rats. Nevertheless, among their noninvasive formulations, the powder formulation containing NAC featured the highest relative bioavailability (30.0 ± 8.6%) and a fourfold improvement compared to the sCT solution. Moreover, the NAC containing powder formulation showed similar properties in beagle dogs. In addition, they determined the local toxicity of the formulations by pathological studies and the Draize test in rabbits. It is suggested that these formulations cause little or no irritations to the nasal mucosa[95]. Thereupon, Matsuyama et al.[96] investigated the influence of filler substances in powder formulations containing NAC on sCT absorption. Within this study, they compared ethylcellulose, lactose, HCO, talc, $CaCO_3$, crystalline cellulose, and microcelac (a spray-dried mixture of crystalline cellulose and lactose 25:75) regarding their suitability for a nasal peptide delivery system. Formulations were prepared by dissolving sCT in ethanol/water (3:7). Subsequently, these solutions were added to a portion of the filler substance. Finally, the wet powder mixture was dried in vacuum and filled up with the rest of the filler. NAC or sodium glycocholate were admixed as permeation enhancers to make up the final dosage form. In case of the lactose formulation, no ethanol/water solution was used to prevent lactose from dissolving. Consequently, lactose formulations were prepared by simple mixing. All the different formulations were tested in rats to assess their bioavailability. When they compared highly water-soluble lactose to insoluble ethylcellulose, they could demonstrate that the water-insoluble compound led to a threefold higher bioavailability. Furthermore, a correlation between water absorbability of the filler and bioavailability could be shown. Therefore, the rank order of bioavailabilities is given by microcelac < crystalline cellulose < calcium carbonate < talc < HCO < ethylcellulose. With the ethylcellulose formulation, a bioavailability of 30.0 ± 8.6 could be obtained. Within additional experiments it was investigated whether the ethylcellulose formulation is also suitable for other peptides. For the anti-osteoporotic peptide PTH (1–34), a bioavailability of 28.2% was achieved, which means a 3.3-fold increase compared to a saline solution of PTH (1–34). Plasma PTH concentrations after nasal administration of different formulations are shown in Figure 7. Prolonged residence time in the nasal cavity, enzyme inhibition because of acidification by NAC and reduced viscosity of the mucous layer are discussed as reasons for the improved bioavailabilities[97].

Microparticles for nasal calcitonin delivery

In recent years, many particulate formulations for oral delivery of CT have been developed, whereas only very few research works have been published on particulate

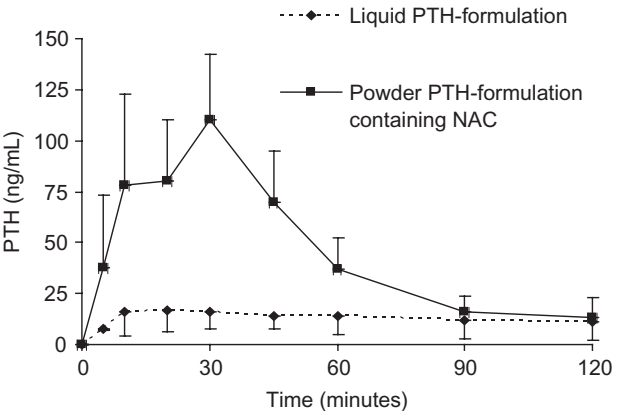

Figure 7. Plasma PTH concentrations after nasal administration of PTH in solution and in a powder formulation containing NAC. Data = mean ± SD. Figure adapted from Matsuyama et al.[93]

CT delivery to the nasal mucosa. Morimoto et al. have prepared gelatine microspheres by dropping aqueous gelatine solutions into olive oil while stirring and subsequent crosslinking with glutaraldehyde. CT was incorporated into the particles by impregnating them with aqueous sCT solutions. Microspheres from positively and negatively charged gelatine were examined. The practical relevance was evaluated by administering microsphere suspensions in PBS of pH 7 and was followed by assessment of the hypocalcemic effect in withdrawn plasma samples. Both types of gelatine displayed a significantly greater hypocalcemic effect. However, microspheres from positively charged gelatine were somewhat superior to negative ones. Moreover, the influence of particle size on the pharmacokinetic effect was investigated, but no significant difference in plasma calcium decrease by particles between 3 and 100 µm could be demonstrated[98].

According to the lack of published work in this field, micro- and nanoparticles still feature an interesting and promising approach in formulation sciences for the nasal delivery of CT and PTH.

Alternative routes for anti-osteoporotic peptide delivery

The pulmonary route provides a huge surface area for rapid drug absorption. Moreover, the clearance from the lower lung is a slow process, so that even drugs with comparably small absorption rates can be absorbed in effective quantities over time periods of 10–12 hours[99]. At least since the approval of inhalative insulin (Exubera) by Pfizer it is well known that the lungs are also permeable to peptides, which makes them an interesting target for anti-osteoporotic peptide delivery, as well. Nevertheless, the lungs feature a

diffusion barrier as well as an enzymatic barrier that have to be overcome by pharmaceutical technologies. According to the size of the peptide, the first or the latter have a greater impact on the pulmonal peptide delivery[100].

As sCT is a relatively small peptide, enzymatic degradation within the lungs limits its pulmonary bioavailability. Kobayashi et al. investigated the effectiveness of several protease inhibitors on pulmonary sCT absorption in rats. For that purpose, sCT solutions containing inhibitors were administered to rats. Plasma calcium levels were determined to quantify the effect. Additionally, enzymatic degradation of sCT in lung homogenates was investigated. Among the used protease inhibitors, bacitracin, chymostatin, and carboxypeptidase inhibitor achieved the best results, which could be backed up by in vitro degradation studies[101]. In a follow-up study, Kobayashi et al. examined different absorption enhancers concerning their properties to enhance sCT absorption in the lungs in dry powder and liquid formulations. Without absorption enhancers, sCT absorption was almost in the same range with both types of formulations. Absorption enhancers displayed greater effectivity within the dry powder formulations with DM-β-CD as an exception. The best absorption-enhancing effect could be observed for oleic acid, citric acid, and lecithin. However, as mentioned earlier, the usage of fatty acids and surfactants as absorption enhancers has to be observed critically because of local toxicity and irritable effects. Chitosan (CS) is known to be a useful absorption-enhancing agent and has recently been investigated regarding its capacities to improve the pulmonary uptake of sCT[102]. Yamamoto et al. utilized it to modify the surface of polylactide/glycolid particles loaded with eCT. Because of this modification, the zeta potential of their particles turned from positive to negative, whereas the size of approximately 650 nm did not vary significantly. A further effect of the modification was found to be a significantly reduced elimination time. This effect can be explained by the outstanding mucoadhesive properties. When they tested the pharmacological properties of these particles, it could be demonstrated that the hypocalcemic effect of the CS-modified particles was significantly greater than that of an eCT solution as well as the unmodified polylactide/glycolid particles. These observations suggest that chitosan further improved the absorption of eCT[103]. Patton et al. investigated the pulmonary bioavailability of PTH by intratracheal instillation and achieved results of approximately 40%[104]. Based on this knowledge, the pulmonary route became more interesting for the PTH therapy of osteoporosis. PTH 1–34 was administered via the pulmonary route in different powder formulations to rats by Condrons et al. Powder formulations consisted of lactose (or trehalose), human serum albumin, and dipalmitoylphosphatidylcholin (DPPC). Two formulations were investigated regarding their pharmacokinetic parameters. Formulation A consisted of 1% PTH, 30% albumin, 10% lactose, and 60% DPPC and formulation B consisted of 10% PTH, 30% lactose, and 60% DPPC. The bioavailabilities of formulations A and B equaled 21 ± 3 and 34 ± 5, respectively. Histological examination of the lung did not reveal harmful effects after a single dose, and measurement of biochemical markers did not show an acute inflammatory response[105].

Despite the fact that the lungs have been shown to be a good target for the delivery of macromolecules, there are still numerous challenges to achieve systemic bioavailability of anti-osteoporotic peptides via this route.

Another potential route for calcitonin delivery is the transport across the buccal mucosa. The buccal mucosa offers the advantages of a relatively low enzymatic activity and comparably high tolerance toward permeation enhancers. A decrease in serum calcium level after buccal administration of CT was first mentioned in the 1980s. Alur et al. utilized the gum of Hakea gibbosa (hakea) to sustain the release from buccal CT tablet. Moreover, this gum was thought to increase the mucoadhesiveness of the mentioned tablet. Hence, the developed tablets were evaluated in an in vivo study in rabbits. This formulation led to a rapid increase in plasma sCT and significant decrease in plasma calcium levels. An apparent bioavailability of 37 ± 6 was demonstrated. Surprisingly, a higher concentration led to a lower bioavailability[106]. Cui and Mumper developed calcitonin-loaded mucoadhesive bilayer thin-film composites (TFCs) including Noveon/Eudragit S-100 in a ratio of 3:1. Subsequently, TFCs were coated with a tragacanth containing wax. sCT was postloaded by dripping the drug solution onto the mucoadhesive side. TFCs were evaluated in a successive in vivo study in rats. As in previous studies on buccal peptide delivery, a rapidly increasing sCT concentration could be demonstrated as well as a decrease in plasma calcium concentrations. Surprisingly, the sCT concentrations remained on a comparably high level for more than 7 hours, whereas the calcium level reached the initial state after approximately 5 hours. A bioavailability of 43.8 ± 10.9% was reported[107].

Conclusion

There has been a long history of research directed toward the development of alternative routes for peptide and protein delivery instead of an injectable dosage form. Needle phobia and stress are the major

reasons for the investigation of all promising routes for peptide and protein delivery, ranging from oral to rectal, with a wide variety of devices and delivery systems.

Although a nasal dosage form for CT already exists, the oral route is the choicest route for drug administration. Therefore, the development of formulations for the oral delivery of CT makes up the major focus in current research. Limited paracellular transport across the epithelial membrane because of tight junctions in combination with enzymatic degradation are the major obstacles for the oral route. For these reasons, many of the approaches to overcome the mentioned barriers include permeation enhancers, enzyme inhibitors, and mucoadhesive agents, as well as multifunctional polymers that combine most of the mentioned features. In recent years, many groups have been working on the development of oral formulations for peptides and proteins, based on the encapsulation of molecules in biodegradable and biocompatible polymeric nanoparticles. Numerous nanoparticulate formulations have been developed for oral delivery applications and they all succeed in protecting the encapsulated CT in combination with the smaller size, which offers several advantages over other forms. When compared to single-unit preparations, nanoparticulate dosage forms distribute more uniformly in the GI tract, resulting in more uniform drug absorption and a reduced risk of local irritation[108].

To combat osteoporosis on the nasal route several approaches have been investigated. Concerning permeation enhancement, only cyclodextrins and chitosan play an important role because of the comparably low toxicity. Moreover, it is known that powder formulations that include an insoluble filler substance are more effective than liquid formulations. Within the study of Matsuyama et al., it could be shown that ethylcellulose (in combination with the mucolytic agent NAC) was most effective as a filler substance[97]. Currently, a lack of scientific publications on particulate peptide delivery systems for the nasal route is obvious. As micro- and nanoparticles have been shown to be effective on the oral route, the development of particulate delivery systems must be regarded as an important and challenging task.

Although numerous articles have been published up to now about delivery of sCT, these observations are based on data obtained under different experimental conditions. Hence, it is difficult to compare the obtained results to draw general conclusions about the most promising characteristics required for oral and nasal formulations for these peptides.

Declaration of interest

The authors report no conflicts of interest.

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