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The lectin–cell interaction and its implications to intestinal lectin-mediated drug delivery

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Abstract

Based on the fact that oligosaccharides encode biological information, the biorecognition between lectinised drug delivery systems and glycosylated structures in the intestine can be exploited for improved peroral therapy. Basic research revealed that some lectins can mediate mucoadhesion, cytoadhesion, and cytoinvasion of drugs. Entering the vesicular pathway by receptor mediated endocytosis, part of the conjugated drug is accumulated within the lysosomes. Additionally, part of the drug is supposed to be transported across the epithelium. Moreover, factors probably adversely influencing feasibility of the concept such as toxicity, immunogenicity, and intestinal stability of plant lectins are discussed. As exemplified by lectin-grafted prodrug and carrier systems, this strategy is expected to improve absorption and probably bioavailability of poorly absorbable drugs, peptides and proteins as well as therapeutic DNA.

Keywords: Bioadhesion; Lectin immunogenicity; Lectin toxicity; Lysosome; Microparticle; Mucoadhesion; Nanoparticle; Prodrug; Wheat germ agglutinin

Contents

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1. Introduction

At the beginning of the previous century, Paul Ehrlich attended C.M. von Weber's opera "Der Freischütz" and he was greatly impressed by the magic bullets. Max, a huntsman, fired these magic bullets in any direction and they yet reached their goal. Subsequently, Paul Ehrlich hit the idea of magic bullets as ideal drug delivery systems, which preferably recognise the affected tissue and release the drug at the intended site of action similar to guided missiles. Hundred years later, however, pharmaceutical technologists are still hunting for appropriate drug delivery systems to overcome the hurdles towards peroral drug delivery in order to guarantee comfortable administration of poorly absorbable, yet established drugs. Nowadays, in the era of genomics and proteomics, these challenges are extended to drug molecules with large spatial extension, poor lipophilicity and high susceptibility to inactivation.

In the opera, the magic bullets were targeted by the devil. In reality, fortunately, the drug delivery system is decorated with biorecognitive ligands, which possess high affinity to receptors at or close to the desired site of action. For that purpose, besides others, immunology offers the specific antibody–antigen interaction [1], and glycobiology provides the more or less specific interaction between carbohydrates and sugar binding proteins called lectins. Following on from the research of H.J. Gabius's group in Munich, it becomes more and more evident that the lectin-binding to certain oligosaccharides is not a freak of nature, but it represents another hardware for encoding biological information [2]. The importance of the so-called "sugar code" became manifest in the observation that certain oligosaccharide sequences mediate cell–cell interactions and cell-routing. In a similar way it is expected that certain information on a drug delivery system, encoded by either oligosaccharides or lectins, can trigger transport and accumulation of the payload in a certain region of the body.

1.1. Basic concepts of glycotargeting

In practice, two strategies of glycotargeting are pursued, relying on use of either the oligosaccharide moiety or the lectin as a component of the drug delivery system.

In the first approach, oligosaccharides or neoglycoconjugates form part of the drug delivery system. Because of the carbohydrate-tag, the drug delivery system can be recognised, bound, and internalised by endogenous lectins at the cell surface [3]. The first mammalian endogenous lectin, the hepatic asialoglycoprotein receptor, was discovered and isolated by Ashwell and Harford [4]. After de-sialylation, and thereby exposing terminal galactose residues, fetuin is cleared by liver cells to maintain proper serum concentrations of glycoproteins. In a similar way, delivery systems containing asialofetuin, galactose, or *N*-acetyl-galactosamine were bound and endocytosed, homing their payload in these cells [5]. As the non-carbohydrate part of the neoglycoconjugates marginally influenced the uptake, the payload of these delivery systems was increased by use of carbohydrate-modified HPMA or liposomes ([6–9], see also contribution of T. Minko in this issue). But this strategy is not limited to the liver or the colon. Also macrophages and mouse brain were shown to be targeted by mannosylated liposomes [10,11].

The second approach towards glycotargeting relies on inversion of the former concept. The drug delivery

system is decorated with lectins of certain carbohydrate specificity so that it can interact with glycosylated surfaces. Most of the absorptive epithelia are covered with a mucous layer and the skeletal, highly viscous structure of the mucus is built up from mucin molecules representing highly glycosylated proteins. At least at the first sight, the mucus represents the only target for lectin-mediated drug delivery systems. Within living memory, microorgansims benefit from the sugar code to adhere to the gut mucosa. These bacteria, constituting the normal microflora or the pathogenic status, utilise lectin–sugar interactions for their adhesion to sometimes well-defined regions of the gut mucosa. The interaction is mediated by carbohydrate-binding, threadlike proteins on their surface, the so-called fimbriae or pili. This attachment often represents an essential early step in pathogenicity of some microbial species since the absorption of enterotoxins is facilitated due to this intimate contact. In an effort to produce less immunogenic fimbriae for drug delivery purposes, *Escherichia coli* K99 fimbriae were truncated by DNA-technology and investigated for utility in bioadhesive drug delivery systems [12].

In contrast to classical mucoadhesion, which relies on non-specific interpenetration of polymer chains and mucus, the lectin–sugar interaction may represent a step forward towards epithelial drug delivery [13,14]. Provided that the lectin-grafted drug delivery system can penetrate the mucus layer, the carbohydrate layer surrounding each mammalian cell represents a second target. The so-called glycocalyx is built up of oligosaccharide moieties of proteoglycans, glycoproteins, and glycolipids anchored in the lipid bilayer of the cell membrane. Not only the glycocalyx of intestinal epithelial cells, but also that of the oral cavity as well as the airway epithelium represents potential sites for lectin-supported drug absorption (see contribution of J. Smart in this issue).

In an approach towards pulmonary delivery, lectinised liposomes (130–170 nm in diameter) were screened for binding to alveolar type II epithelial cells [15]. As compared to plain liposomes, the binding to A549 cells increased 6–11-fold upon surface modification with wheat germ agglutinin (WGA), Concanavalin A (ConA) or soybean agglutinin. The binding was not affected by a synthetic lung surfactant and no cytotoxic effect of the free lectins or the lectinised liposomes was observed. Upon incubation with primary cultured human alveolar epithelial cells, which exhibit barrier functions, the WGA-liposomes were not only bound but also taken up into the cells. In search for non-viral vectors for gene therapy of cystic fibrosis and as a basis for lectin-mediated gene transfer, 32 lectins were screened for binding and uptake into living human airway epithelium [16]. Whereas ConA was internalised within 1 h, the lectins from *Erythrina cristagalli* and *Glycine max*, peanut lectin, and Jacalin were taken up into the epithelium within 4 h. The endocytosis of WGA was minimal even after 4 h. Irrespective of the specificity of the lectin–carbohydrate interaction, the internalised lectins exhibited a non-selective binding pattern on the epithelium. Only peanut lectin bound to subpopulations of ciliated and non-ciliated cells.

This contribution focuses on the latter concept of glycotargeting, which relies on use of plant lectins for intestinal targeting. The interaction of plant lectins with glycosylated elements at different levels of the small intestine and the implications for drug delivery are discussed in more detail. After addressing some issues influencing feasibility of the concept, an outlook on lectin-mediated drug delivery to the small intestine is given, and exemplified by different lectinised formulations.

1.2. Objectives of peroral lectin-mediated drug delivery

For the patient, the gastrointestinal route is the most convenient and attractive method for systemic delivery of drugs. It affords ease of administration, high acceptability, compliance, and painless administration. For the health care system, the cost of peroral therapy is much lower than for parenteral therapy. From a technological point of view, however, it is one of the most challenging routes of administration. The gastrointestine offers a wide area for absorption of nutritients, electrolytes, and fluids but vice versa also protects humans from pathogens and harmful luminal contents. To fulfill this latter task, numerous barriers are present which counteract successful drug delivery. The interplay between digestive fluids and peristalsis dilutes and accelerates the transit of drugs and their delivery systems leading to poor absorption. The acidity of the gastric juice, luminal enzymes, and brush border hydrolases can degrade drugs. The

viscous mucus overlying the epithelium can limit access to the absorptive cell layer. Finally, the epithelial cells forming a tight layer via junctional complexes can impede absorption and restrict access to the systemic circulation. All in all, this can result in poor bioavailability.

Nevertheless, the intestine offers a large area, which is highly glycosylated. Stealing a glance at the lectin-mediated symbiosis between Rhizobia and legume roots, which makes nitrogen available for the plant, might be an impetus for use of this strategy to improve bioavailability of drugs by lectin-mediated adherence of drug delivery systems on intestinal surfaces. Successful mucosal absorption of drugs requires formulations, which prolong the residence time at the site of absorption and provide for an intimate contact to the absorptive tissue. That way, the concentration gradient of the drug between the lumen and the enterocytes is increased and absorption of drugs is facilitated by passive diffusion.

According to the presence of two distinct layers in the intestinal mucosa being rich in oligosaccharides, the targets of lectin-mediated drug delivery are the mucus layer and the glycocalyx of the underlying cells.

2. Mucoadhesive characteristics of plant lectins

The mammalian gatrointestinal tract is lined with a continuous layer of mucus which lubricates food masses, facilitates movement of the chyme, and protects the underlying tissue from gastric juice, proteases, pathogenic microorganisms as well as mechanical trauma. In humans, the thickness of mucus layer is 50–500 μm in the stomach and 50–150 μm in the colon. At the mean, the mucus forms a layer of 192 μm in thickness [17]. The gel-forming components of the mucus are glycoproteins of molecular weight higher than 2×10^6 Da, the so-called mucins. These glycoproteins from intestinal mucus contain 77.5% carbohydrate comprising *N*-acetyl-galactosamine, *N*-acetyl-glucosamine, galactose, fucose and sialic acid at a molar ratio of 1.0:0.6:0.7:0.3:0.5 as referred to the dry weight [18]. The mucins contribute only 0.5–4.0% by weight to the wet gastrointestinal mucus [19]. Besides water, other constituents of the mucus are salts, sloughed epithelial cells and bacteria.

The high viscosity makes the mucus a barrier against diffusion of drugs and delivery systems. As already confirmed by mucoadhesive formulations on the market, however, the fixation of the drug delivery system close to the absorption site improves the bioavailability of active agents.

In search for an experimental setup to examine both binding rate and specificity of the mucus–lectin interaction, the binding of fluorescent labelled analogues of plant lectins with different carbohydrate specificity to pig gastric mucin coated microplates was investigated [20]. Even though the visco-elastic properties of the gastointestinal mucus were not mimicked by this assay, the qualitative composition of pig gastic mucin reflects that of the human one. At neutral pH, the lectin-binding capacity of mucin followed the order WGA (sialic acid and *N*-acetyl-D-glucosamine) ≫ *Ulex europaeus* isoagglutinin I (alpha-L-fucose) ≫ lentil lectin (alpha mannose) = potato lectin (*N*-acetyl-D-glucosamine)>peanut lectin (galactosamine)>*Dolichos biflorus* agglutinin (*N*-acetyl-galactosamine). This ranking rather reflects the steric accessibility of the mucus proteins than the molar composition of mucins. As sialic acids often operate as chain terminators at the linear or branched glycoproteins, the WGA-binding was highest. Similarly, fucosyl residues are rather freely accessible since they form the end of lateral oligosaccharide chains. Although *N*-acetyl-galactosamine is most abundant, it is shielded from any interaction due to *O*-glycosidic linkage to the hydroxyls of serine and threonine at the protein backbone.

The mucin–lectin interaction is characterised by pH-dependence, specificity, and reversibility [20]. At pH 2.0 only 15% WGA, and at pH 6.0–7.0, 60–70% WGA were bound to pig gastric mucin as compared to the maximum binding rate at pH 5.0. Thus, it is expected that premature adhesion of lectin-grafted drug delivery systems to the gastric mucus will counteract adsorption to the intestinal mucus only to a minor extent. Additionally, the staining of fixed specimens of human gastric tissue with WGA revealed that superficial microvilli were labelled, whereas the extracellular mucus was negative [21].

The specificity of the mucin–WGA interaction was assessed by competitive assays. For displacement of 50% mucus bound lectin, a 72-fold molar excess of the corresponding carbohydrate was necessary [20]. Fur-

thermore, specificity of mucoadhesion was retained when tomato lectin (sialic acid, chitin), asparagus pea lectin (alpha-L-fucose), and *Mycoplasma gallisepticum* lectin (sialic acid) were conjugated to polystyrene latices [22].

Although the mucin interaction with latex conjugates seemed to be irreversible, the WGA–mucin interaction was found to be fully reversible and independent from time. That way, dissociation from mucins and association with analogous oligosaccharides, present in the mucus or at the glycocalyx of absorptive enterocytes, is facilitated. Additionally, competitive assays with Caco-2 monolayers and constant amounts of gastric mucin revealed that the WGA-combining sites of mucin are saturable. Basically, this initial saturation of mucins with lectins leads to bioadhesion and represents the first step in the course of lectin-mediated drug delivery.

In an approach towards mucoadhesive lectin-mediated drug delivery, Irache et al. prepared polystyrene latex conjugates with tomato lectin, asparagus pea lectin, and *M. gallisepticum* lectin by covalent coupling with carbodiimide or glutaraldehyde [22,23]. The mucoadhesive phenomena were characterised by size exclusion chromatography. According to the Scatchard plots of the mucin–latex conjugates, only one type of ligand–receptor interaction per lectin was involved in the mucin binding of the lectin-grafted nanoparticles. Consequently, at least one carbohydrate-binding domain of the lectin was preserved, despite of covalent immobilisation. The interaction of the lectinised particles with gastric mucin was three times higher than that of the controls represented by plain latex or bovine serum albumin (BSA)-grafted particles. The mucin-binding capacity of the colloidal formulations was 8.5–11.5 μg/μg lectin.

All in all, it is expected that the strong and specific interaction between mucin and lectin-decorated formulations will result in anchoring of the drug delivery system at the site of absorption (Fig. 1). In vivo, the transit time through the small intestine has been measured by use of a perspex capsule filled with technetium-99m labelled resin. Whereas the transit through the duodenum was too fast to be accurately measured, the capsule moved through the jejunum and ileum at a velocity of 4.2–5.6 cm/min [24]. This corresponds to a mean intestinal transit time of 110–140 min. Thus, the fixation or at least retardation of mucoadhesive drug delivery systems in the small intestine will shorten the diffusional pathway, which results in an increasing concentration gradient of the drug between the lumen and the absorptive epitheli-

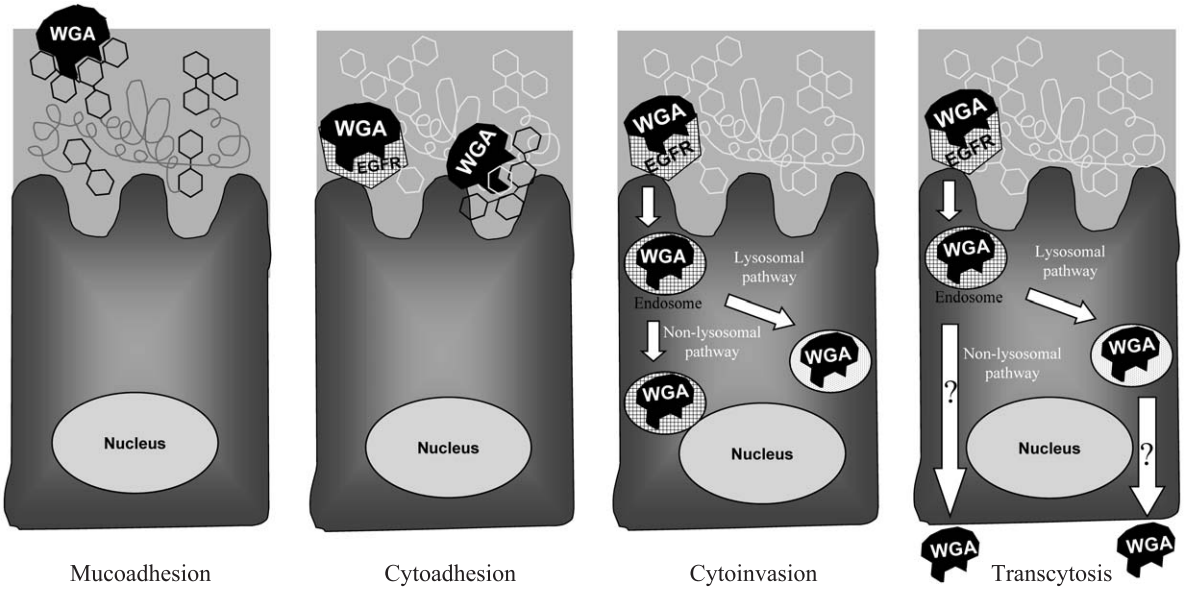

Fig. 1. Possible pathways for lectin-mediated drug delivery to enterocytes as exemplified by WGA.

um. Additionally, the intimate contact of the drug reservoir to the enterocytes as well as the protective effect of the matrix can reduce the degradation of drugs by luminal enzymes. That way the absorption rate is increased, probably resulting in improved bioavailability.

These findings and mechanisms strongly underline the utility of lectin-mediated drug delivery systems as mucoadhesive formulations as already postulated for classical mucoadhesives of the first generation. But there is a difference: the bioadhesion of mucoadhesive polymers relies on non-specific mechanisms, whereas lectin-mediated mucoadhesion relies on specific, key–lock like biorecognition. As non-specific interactions are susceptible to changes in pH and to interactions with food digesta, which probably reduce the mucoadhesive effect, specific mucoadhesiva of the second generation seem to be preferable. Another issue to be considered, is the rather rapid renewal of the mucus which restricts the contact time. A study with oral mucoadhesives revealed that the turnover time of the intestinal mucus layer, i.e. from production to digestion, is in the order of 90–240 min [25,26]. This rather rapid renewal also results in formation of shed-off mucus in the luminal content. Altogether, the contact time is reduced and at least part of the drug delivery system is eliminated prior to contact to the epithelium. As these limitations result from given physiological processes, not only the mucoadhesives of the first but also those of the second generation are expected to suffer from this source of premature inactivation as well.

3. Interaction of lectins with intestinal cells

Regardless of these constraints, some new perspectives were included into the concept of bioadhesion leading to the ‘‘bioadhesives of the second generation’’ [27]. First, these specific bioadhesive molecules should target the drug delivery system to specific regions within the gastrointestine where drug absorption is preferred. As the glycocalyx exhibits site dependent, regional variations in glycoconjugate composition, lectins should interact with the glycocalyx of certain cell-species in the intestine [28]. Additionally, the composition of the glycocalyx is altered upon malignant transformation of cells, opening new perspectives for lectin-mediated cancer therapy [29]. Second, the new generation of bioadhesives should extend the time scale for fixation. Whereas mucoadhesion is limited to a few hours, the migration of cells from crypts to the villous tips takes 2–3 days. Thus, fixation of the drug delivery system directly to the cell surface, in case of glycotargeting to the glycocalyx by aid of lectins, should enhance absorption of drugs. Third, the specific binding to the apical cell membrane should trigger vesiculation processes such as endo- or transcytosis. Whereas pinocytosis occurs spontaneously and a small amount of extracellular fluid, possibly containing drug molecules, enters the cell, receptor-mediated endocytosis can include the accumulation of high numbers of ligand–receptor complexes at the invagination of the apical cell membrane. Nevertheless, the transport capacity seems to be limited and may be only sufficient for drugs of high potency.

Another prerequisite for proper function of bioadhesive formulations of the second generation is the free access to the apical cell membrane. The diffusion through the scaffold of the intestinal mucus implies that the spatial extension of the drug delivery system will play a key role, which favours soluble prodrugs or colloidal formulations. Apart from that there is evidence that the mucus gel may not represent a continuous layer [30]. Furthermore, the intestinal mucus layer was found to be more permeable than the gastric one [31].

3.1. Glycosylation map of the small intestine

In an effort to explore whether plant lectins are suitable targeting agents for selective drug delivery to different parts of the gut, the group of A. Pusztai in Aberdeen, Scotland, did some pioneering work by elaboration of a glycosylation map of the gut [32]. To get a general survey of lectin-reactivity in vitro, different specimens of the gut from mice and rats were histologically examined after incubation with fluorescein-labelled plant lectins. The reactivity in vivo was confirmed by immunohistochemical detection of the lectin interaction after gavage of rats. In terms of the intestinal compartment, the studies in rats demonstrated that *N*-acetyl-glucosamine binding lectins such as WGA and tomato lectin avidly bind to the epithelial surface throughout the entire small intestine

[33]. In contrast, lectin binding to sialic acid—containing oligosaccharides was variable and slight. Whereas the binding of the lectin from the bark of *Maackia amurensis* was moderate to luminal membranes, strong binding to goblet cells and intestinal mucin was observed [34]. The reactivity of another sialic acid recognising lectin, the elderberry lectin, with epithelial cells, goblet cells, and goblet mucin was negligible. Fucose-specific lectins such as the *U. europaeus* isoagglutinin 1 bound almost exclusively to mouse M-cells and transcytosis was observed [35]. Although there is a high species variation in glycosylation of intestinal M-cells of mice, rats, and humans, these findings open new perspectives for vaccination purposes. The lectin from snowdrops and other mannose-specific lectins exhibited only slight binding to jejunal epithelial cells and moderate binding to M-cells [36]. At the first sight, lectins specific for complex glycosyl side chains such as *Phaseolus vulgaris* agglutinin from red kidney beans and the lectin from Robinia pseudoacacia seemed to be superior for intestinal targeting. They strongly and reversibly bound to villous and crypt epithelia as well as M-cells followed by high rates of endocytosis and transcytosis [34,36]. As these lectins exhibited no interaction with goblet cells and goblet mucin but uptake via both epithelial and M-cells, they offer two routes of entry into circulation without premature inactivation by goblet mucin. But it is likely that the toxicity of at least red kidney bean lectin will strongly restrict its utility for drug delivery.

3.2. Mechanisms of the lectin–cell interaction

Histology affords an overview about the distribution of lectin binding sites throughout the small intestine and gives only slight hints towards cellular processing of lectins with different carbohydrate specificity. In order to get a deeper insight into the underlying mechanisms and the mode of lectin–cell interaction, it is necessary to have a look at the cellular level.

As maintenance of primary cultures of mucosal tissue under cell culture conditions for prolonged time is rather difficult and poorly reproducible, experiments with single cells and monolayers of established cell lines are preferable. Caco-2 cells form confluent and polarised monolayers under cell culture conditions and differentiate towards mature enterocytes. Due to the morphological and functional resemblance to human intestinal cells, the Caco-2 model is well-established in pharmaceutical technology to study binding, uptake, and transport of drugs despite lacking mucus production [37,38].

3.2.1. Binding of lectins to the cell membrane

To estimate the lectin-binding capacity and vice versa to investigate the glycosylation pattern of human enterocyte-like cell lines (Caco-2, HT-29, HCT-8), prostate cancer cells (Du-145), and bladder carcinoma cells (5637), the interaction of a panel of lectins with different carbohydrate specificity was characterised by flow cytometry and fluorimetry [39,40]. In common with histological studies, all cell lines investigated exhibited highest binding capacity for WGA from *Triticum vulgare*. Out of this reason the interaction between Caco-2 cells and WGA will be described in more detail.

The WGA not only binds to malignant cells, but also to human colonocytes. According to saturation analysis, 25 pmol WGA adhered to 5×10^4 Caco-2 cells [41]. In comparison, the WGA-binding capacity of human colonocytes was fifteen times lower but still offering a high number of adhesion sites for lectinised formulations [42].

The WGA-binding to enterocyte-like Caco-2 single cells and monolayers is highly specific. Upon preincubation of the cells with tunicamycin, which inhibits the *N*-acetyl-glycosyl-transferase, the amount of cell-surface bound lectin decreased to about a third. As non-specific protein–cell interactions contributed to less than 1%, the cell-binding was attributed to presence of *N*-glycosylated oliosaccharides on the outer leaflet of the cell membrane. According to competitive assays with the complementary carbohydrate *N*,*N*′ ,*N*″-triacetylchitotriose, the number of lectin binding sites primary differs between malignant and healthy cells but secondary is also variable between malignant cell lines of different histological origin. Furthermore, the competitive assays revealed similar affinity of the interaction suggesting a common lectin receptor binding pattern. These findings also confirm the triacetylchitotriose binding motif of WGA indicating presence of *N*-acetylglucosamine containing structures at the cell coat. Moreover, WGA also binds to sialic acid moieties [43]. The

specific binding is based on the similarity in configuration of this sugar to *N*-acetylglucosamine at positions C-2 (acetamido-group) and C-3 (hydroxyl-group) of the pyranose ring.

Recent research on WGA-binding to isolated epidermal growth factor (EGF)-receptor and biomimetic membranes prepared from Caco-2 cells confirmed that the EGF-receptor is involved in carbohydrate specific cytoadhesion of WGA [44]. To avoid any interference with cellular uptake, the WGA-binding to the EGF-receptor was examined by means of artificial cell membranes containing Caco-2 derived EGF-receptor and silver nanoparticles for enhanced detectability. The EGF-receptor–lectin interaction was saturable and reached its equilibrium within 1 min. Competitive inhibition and displacement studies with the complementary triacetylchitotriose revealed that about 90% of the WGA–Caco-2 membrane interaction were mediated by *N*-acetyl-D-glucosamine or sialic acid containing oligosaccharides. According to F-WGA binding to the blotted receptor, part of lectin binding is mediated by the EGF-receptor but still other unknown components of the glycocalyx are involved. The carbohydrate specificity of WGA-binding to the EGF-receptor suggests that glycosylated structures of the receptor represent the lectin binding sites. The extracellular region of the EGF-receptor contains 12 potential sites for *N*-glycosylation in four domains [45]. Of the four domains, domain III was identified to play a critical role in ligand binding involving oligosaccharide side chains linked to Asn-328, Asn-337, Asn-389, and Asn-420 [46–48]. When the corresponding Asn-residues were replaced by Gln, the Asn-420-linked sugar chain was found to be essential for binding of EGF as well as controllable dimerisation of the occupied receptor [49]. Because characterisation of the carbohydrate side chains on human EGF-receptor revealed that the multiantennary carbohydrates contain sialylated motifs (up to tetrasialo), the WGA-binding site on the EGF-receptor seems to involve the Asn-420 oligosaccharide chain [50].

The pivotal role of the EGF-receptor in lectin binding opens two attractive areas for WGA-mediated drug delivery: First, the EGF-receptor is overexpressed in a high number of tumours including those of the liver, the breast, the lung, and the bladder. Thus, prodrugs or drug delivery systems containing WGA are expected to be appropriate for glycotargeting of anticancer drugs. Secondly, EGF-receptor containing cells exist in most healthy tissues of the body at a density of $2 \times 10^4 - 2 \times 10^5$ receptor molecules per cell [51]. Consequently, the cytoadhesive WGA opens a receptor mediated pathway for improved absorption of poorly available drugs (Fig. 1). Whereas mucoadhesives deliver their payload at the mucus, lectinised formulations can target drugs directly to the cell surface of absorptive enterocytes. Provided that the lectin-functionalised drug delivery system penetrates the mucus, the specific interaction with oligosaccharide moieties of the EGF-receptor and other, yet unknown components of the glycocalyx will further shorten the diffusional pathway of the drug. The high local concentration of the drug close to the absorptive barrier will increase the absorption rate and reduce the influence of degradative luminal enzymes.

3.2.2. Uptake of lectins into the cell

Apart from cytoadhesion, there is also evidence for cytoinvasive properties of WGA. As determined by ^{125}I-EGF binding and uptake, Caco-2 cells possess a single EGF-binding site and about 7×10^3 EGF-receptors per cell [52]. Thus, the enterocyte-like Caco-2 monolayers represent an appropriate artificial tissue to characterise the uptake process. Confocal laser scanning microscopy of Caco-2 single cells after incubation with fluorescein-labelled WGA and computer assisted quantitative evaluation of the images acquired revealed a temperature dependent behaviour of lectin distribution [41]. Upon incubation at 4 °C, the lectin was localised at the membrane section of the cells pointing to cytoadhesion exclusively. But raising the temperature to 37 °C led to distribution of most of the lectin mainly into the submembrane domain within 30 min. Finally, all the lectin was detected in the cytoplasmic section after incubation at 37 °C for 4 h. The uptake into the cytoplasm was also observed in well differentiated Caco-2 monolayers (Fig. 2) [53]. Confocal analysis of fluorescein-WGA preincubated cells that were counter stained for their actin cytoskeleton using TRITC phalloidin demonstrated membrane binding at 4 °C. Although the confluent and differentiated enteroycte-like cells exhibited an irregular apical surface due to protruding actin filaments, the vertical cross section clearly displayed intracellular accumulation after incubation at 37 °C.

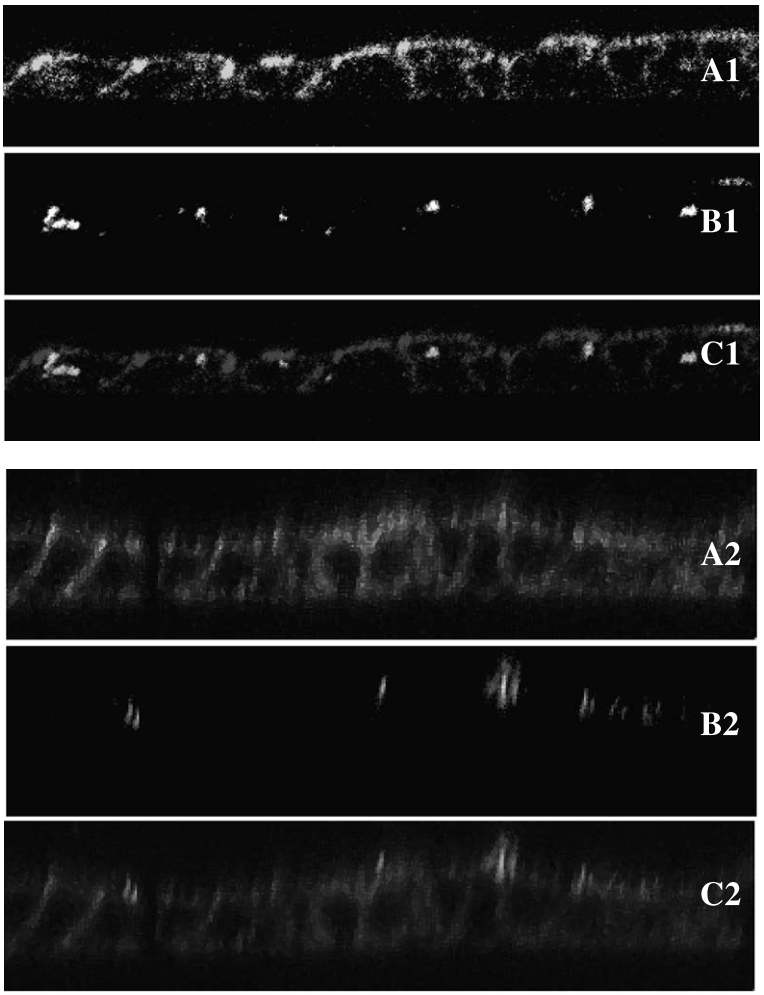

Fig. 2. Co-localisation analysis of WGA and actin filaments after incubation with Caco-2 monolayers at 37 °C (A1–C1) and 4 °C (A2–C2). After staining the actin filaments with TRITC-phalloidin (A), and detection of the WGA by its fluorescein label (B), the images acquired were overlaid (C), vertical cross section.

As opposed to qualitative confirmation of cytoinvasion, it is more skilful to directly quantify the extent of WGA taken up into the cytoplasm. The problems encountered derive from shielding of the fluorescein label by the cell membrane and from the pH-dependent quantum yield of the label which influenced the detection of the lectin. An assay protocol that is independent from these parameters relies on use of fluorescein-labelled avidin to detect membrane-bound biotinylated WGA. As the high molecular avidin neither binds to nor permeates the cell membrane, the amount of cell surface bound lectin is detected exclusively. According to a pulse/chase protocol, the lectin-binding sites on the apical surface of artificial intestinal tissue were saturated within 5 min at 4 °C (Fig. 3). Furthermore, the membrane bound fluorescence intensity was constant throughout the incubation time excluding dissociation of initially membrane bound lectin. However, upon chase incubation at 37 °C, the membrane bound fluorescence intensity immediately started to decrease by time. Since the cell-bound lectin was not released into the supernatant, the difference of monolayer-associated fluorescence intensity between 4 and 37 °C was attributed to uptake.

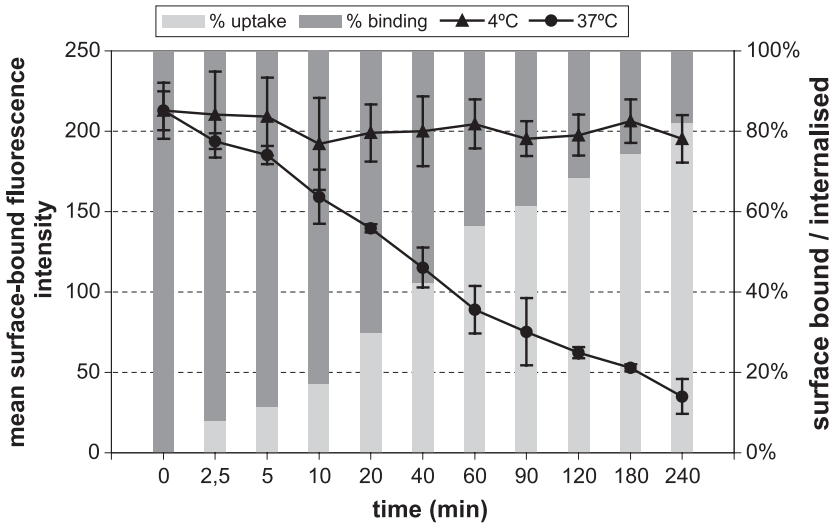

Fig. 3. Mean cell-membrane bound fluorescence intensity of the biotin–WGA–fluorescein avidin complex after incubation with Caco-2 monolayers at 4 and 37 °C. The difference in cell-bound fluorescence intensity between 4 and 37 °C was calculated as the amount of internalised lectin. The percentage of binding and uptake refers to incubation at 37 °C. Reproduced with permission from [53].

Initially, the uptake of membrane bound WGA was rapid as indicated by 50% uptake within about 50 min. With increasing incubation time the uptake rate decreased, but finally amounted to more than 80% of initially surface bound lectin within 4 h.

Whereas binding to the cell surface was constant and independent from time, the uptake process required elevated temperature and increased with time. As disposal of energy and enhanced fluidity of the cell membrane play a key role in internalisation of the lectin, active and energy consuming transport processes are main characteristics of lectin-mediated cytoinvasion. Regarding the identification of the EGF-receptor as a binding site for WGA, the uptake of the lectin can be attributed to receptor-mediated endocytosis. Although enrichment of WGA in coated pit regions was not confirmed until now, there is high similarity of binding and uptake of fluorescent-labelled EGF and WGA in Caco-2 and A-431 cells [54].

In terms of drug delivery, the cytoinvasion of WGA and probably other lectins will open a receptor-mediated pathway to overcome the formidable barrier function of the epithelium after peroral administration of drugs (Fig. 1). Besides the lectin-approach, recent research demonstrated efforts to utilise receptor-mediated endo- and transcytosis for improved absorption of drugs. The endocytosis of folic acid-conjugates turned out to be an encouraging strategy to target tumours overexpressing the folate receptor [55]. Another concept relying on vitamin absorption is the utilisation of the vitamin B12 receptor for endocytosis of conjugated drugs [56]. Problems such as the interference with absorption of nutrients and the limited transport capacity might be solved by use of nanoparticles grafted with vitamin B12 [57]. Exploiting the pathway of iron absorption via the transferrin receptor represents an additional strategy to enhance the absorption of proteins, especially insulin, and DNA [58]. As the transferrin receptor is essentially localised at the basolateral membrane of Caco-2 cells and enterocytes, co-administration of GTPase inhibitors is required to enrich sufficient numbers of the receptor at the apical membrane [59,60]. All in all the receptor mediated uptake of drugs, including the lectin-approach targeting glycosylated extracellular domains of membrane spanning receptors, is a promising strategy for enhanced absorption of drugs. But a lot of work is still necessary to figure out the advantages and disadvantages in vivo.

3.2.3. Intracellular trafficking of WGA

As a prerequisite for vesicular transport, the occupied EGF-receptor is accumulated in coated pits followed by formation of coated vesicles. In an effort

to clarify, whether clathrin or caveolin are involved in endocytosis of WGA, indirect immunofluorescence staining techniques were applied. Surprisingly, co-localisation of neither stained coated pits nor caveolae and WGA was detected. However, it has to be taken into account, that coated vesicles exhibit a rather rapid turnover so that the clathrin coat of the vesicles containing WGA is broken down during the chase period [53]. In turn, this finding underlines the immediate uptake of cell-bound WGA.

However, as observed on horizontal cross sections of Caco-2 monolayers, the dot-like accumulation of fluorescent labelled WGA all over the cytoplasm clearly indicates vesicular accumulation of the lectin. Additionally, the decrease in cell-associated fluorescence intensity at 37 °C by time as mentioned above points to shielding or quenching of the acid-sensitive fluorescein label. To get evidence for intracellular localisation of internalised WGA in acidic compartments, the pH between acidic compartments of the cell and the cytosol was equilibrated with monensin. Monensin represents a carboxylic ionophore that catalyses the exchange of protons for potassium ions. When energy consuming transport processes were suppressed by incubation at 4 °C, the presence of monensin exerted no influence on cell-associated fluorescence intensity (Fig. 4). Upon chase incubation at 37 °C, however, the time dependent decrease of cell-associated fluorescence intensity was fully compensated by addition of monensin even approaching the 4 °C values. The restoration of the quantum yield of the acid-sensitive labelled lectin confirms accumulation of WGA within acidic compartments of the cells. Calculation of the quenched proportion of the label revealed that 65% of cell-bound lectin was enriched within the lysosomes or the trans-Golgi network after 4 h. To discriminate between both acidic compartments by confocal laser scanning microscopy, the trans-Golgi complex was stained with anti-adaptin-gamma and the lysosomal cathepsin D as well as the lysosome associated membrane protein-1 were detected with labelled monoclonal antibodies. Co-localisation analysis demonstrated presence of WGA in lysosomes, whereas no coexistence in the trans-Golgi network was observed. According to quantitative image analysis at least 50% of the internalised lectin was accumulated in the lysosomes within 1 h.

Additionally, the cellular uptake of WGA was limited to the extranuclear compartment. There was no colocalisation of propidium iodide stained nucleus and fluorescein-labelled lectin. According to colocalisation analysis and fluorimetry of artificial intestinal tissue, about 60% of internalised WGA enters the degradative lysosomal route. The lysosomal route can be advantageous for intracellular delivery of acid-labile prodrugs or nanoscaled carriers releasing the

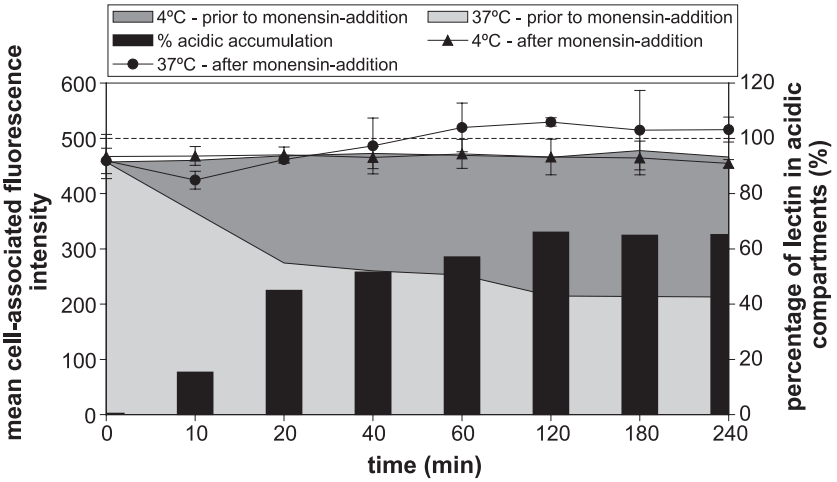

Fig. 4. Mean cell-associated fluorescence intensity of Caco-2 monolayers pulse incubated with F-WGA at 4 °C and chase incubated at 4 or 37 °C prior and after addition of monensin. The percentage of WGA accumulated in acidic compartments was calculated from the difference in fluorescence intensity prior and after addition of monensin upon incubation at 37 °C. Reproduced with permission from [53].

acid-insensitive drug upon acidic or hydrolytic degradation of the matrix. That way, the intracellular availability of poorly absorbable, acid-insensitive drugs is increased. On the other hand, the intracellular localisation of the remaining 40% is unclear. The non-lysosomal pathway can open a route for intra- or transcellular delivery of drugs susceptible to degradation such as peptides, proteins or DNA.

3.2.4. Transcytosis of lectins

From experiments with Caco-2 monolayers there is also evidence that part of the lectin, but by far not all, enters the direct shuttle pathway of transcytosis. Using confluent Caco-2 monolayers grown on transwell inserts, 3% of apically applied F-WGA was detected in the basolateral compartment within the mean life time of enterocytes of about 3 days. For comparison, the transcytosis of F-dextran of similar molecular weight and equal concentration was only 0.3% (Fig. 5). Accordingly, uptake studies by Russell-Jones et al. revealed that about 2.5% WGA were transported across Caco-2 monolayers to the filter inserts and 22% were associated with the cells after incubation for 18 h [61]. As the uptake rate of labelled WGA was reduced in presence of excess unlabelled lectin as well as the complementary carbohydrate, the processing was specific.

In an effort to utilise lectins for enhanced transcytosis of colloidal drug delivery systems, the transcellular transport of 50 nm fluorescent latexparticles was investigated. Upon surface modification with WGA the transcytosis rate of the nanoparticles increased by 20% as compared to the plain spheres. Similarly the coating with snowdrop lectin increased the transcellular transport by 16%.

These results clearly indicate that the transcellular transport of nanoscaled carriers is enhanced by surface modification with lectins, at least in vitro (Fig. 1). Due to their higher payload, colloidal drug carriers can shuttle higher amounts of drugs across the epithelial barrier. As the transport rate of lectins was quite lower than that of the lectinised nanoparticles, it is likely that the intracellular trafficking route is different. But it remains to be shown whether the transport capacity is sufficient to reach therapeutic levels of drugs.

4. Factors limiting peroral lectin-mediated drug delivery

4.1. Influence of nutrition and enzymes

As the human diet is usually rich in carbohydrates, glycotargeting with lectins might be counteracted by food-derived glycans. Obviously, this source of premature inactivation is strongly dependent on the carbohydrate specificity of the lectins. It is likely that glucose or galactose-targeted drug delivery systems are neutralised by foodstuff, but formulations grafted with WGA or lectins specific for complex glycans are

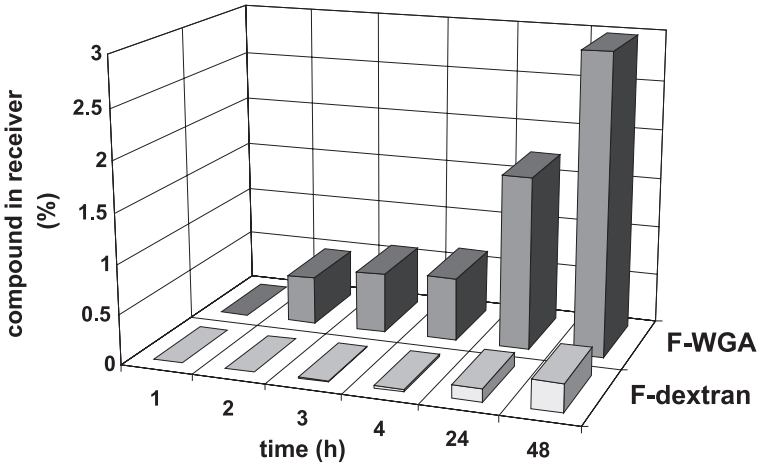

Fig. 5. Transcellular transport of fluorescein-labelled WGA (36 kDa) and fluorescein-labelled dextran (40 kDa) across Caco-2 monolayers.

not expected to be influenced. The common diet of man contains, if any, only low amounts of chitin, *N*-acetyl-glucosamine or sialic acid so that any interference is not expected. This assumption was confirmed by unaltered Caco-2 binding characteristics of WGA co-incubated with wheat flour, starch from potatoes or wheat germ, saccharose, glucose, basic, and neutral amino acids. The sole exception were acidic amino acids such as glutamic acid. In the latter case, however, the reduced binding rate is rather due to lowering the pH than blocking of binding sites (unpublished results).

Another issue to be considered upon peroral administration of lectin-grafted formulations is the exposition of lectins to gastrointestinal enzymes. In vitro, after preincubation of WGA and tomato lectin with abnormal high amounts of pepsin, trypsin, pancreatin, and elastase, no degradation products were observed and the cell-binding characteristics were fully retained. In contrast, potato lectin was highly susceptible to proteolytic degradation [42]. In vivo, 95.2% of intact WGA were recovered after peroral administration to Balb/c mice [62]. For comparison, about 60% of intact WGA were detected in the faeces after peroral administration in rats [63]. The high proteolytic stability of some, by far not all, lectins was attributed to the highly conserved structure of the protein molecules and to receptor binding in the gut.

The resistance against proteolytic attack and the lacking reactivity with oligosaccharides of common foodstuff provide the binding of lectin-containing drug delivery systems to the mucosal epithelium. Out of these reasons, the peroral administration of WGA-grafted formulations seems to be advantageous from the technological as well as from the patients point of view.

4.2. Toxicity of lectins

One of the well-known examples of lectin toxicity was given in London. In 1973, an undercover agent was murdered by the soviet secret service using the tip of an umbrella impregnated with ricin. Another well-known example of lectin toxicity is incomplete cooking of red kidney beans, especially at reduced atmospheric pressure in the highlands of Mexico. The content of 0.5–5% lectin in red kidney beans causes diarrhea, malabsorption, and growth reduction, but also stimulates overgrowth of mannose-sensitive *E. coli* [64]. There are also numerous reports about the toxic effects of certain other lectins [65]. In contrast, about 30% of the human diet, raw or cooked, contains ingredients with significant haemagglutinating activity and 53 eatable plants are considered to contain lectins [66]. Additionally, some lectins such as tomato lectin are regarded as safe since 100–200 mg tomato lectin are consumed per person and year in the USA [67].

Representing a dietary lectin, the WGA is putative non-toxic. Wheat fluor contains about 300 mg of WGA per kg and wheat germ is consumed either in unprocessed form as muesli or in processed form as bread. Whereas the muesli most likely contains the native lectin, exposure to heat such as baking bread reduces the biorecognitive properties of the lectin significantly. The prolonged incubation of an aqueous solution of WGA at 100 °C reduced the cell-binding to 10% (1 h) and 0.1% (2 h), respectively. But WGA in its solid state is less susceptible to heat induced denaturation since cell-binding was slightly reduced to 87% (1 h exposure) or 83% (2 h). Even the treatment of WGA with microwaves (1500 W, 10 s) reduced the Caco-2 binding rate to 63% regardless of solid or dissolved lectin (unpublished results). Consequently, bread is supposed to contain rather minimal amounts of intact lectin but the dietary intake of bread is rather high. Another hint towards negligible WGA-toxicity is the observation that the viability of neither Caco-2 monolayers nor rats' intestinal epithelial cells was reduced [68].

On the other hand, daily peroral administration of 42 mg WGA to rats for 10 days provoked antinutritive effects such as an increasing crypt size and crypt cell proliferation in the small intestine. Additionally hypertrophic growth of the pancreas and thymus atrophy were observed [33]. It should be considered, however, that about 400 mg of pure lectin were administered per rat with an average weight of 80 g. According to a rough calculation this corresponds to a daily intake of 38 g pure WGA in man.

To date, a final judgement on toxicity or atoxicity of WGA cannot be given due to lack of studies in vivo. Nevertheless, the amounts of lectins as necessary for glycotargeting of prodrugs or colloidal carrier systems are in the microgram range so that toxic effects should not be provoked.

4.3. Immunogenicity

Representing foreign proteins of appreciable molecular weight with structural rigidity, it is likely that lectins can elicit an immune response. The resistance against degradation by digestive enzymes and the cytoinvasive properties can favour elicitation of local and/or systemic immune responses. As exemplified by tomato lectin, oral administration can provoke formation of specific antibodies in mice and in humans [69,70]. In consequence, IgG and the secretory IgA can neutralise the glycotargeting-tags and reduce uptake of lectin-decorated formulations.

Upon use of lectins for drug delivery it should be considered that natural antibodies to dietary proteins including plant lectins exist in the blood of healthy humans. About 140 μg of each, WGA, soybean lectin, and peanut agglutinin-reactive antibodies were isolated from 100 ml human serum by affinity chromatography on immobilised lectins [71]. The peanut agglutinin-antibodies were polyreactive and cross-reacted with non-related proteins as well. The WGA-antibodies reacted with native and denaturated WGA as well as lysozyme, but not with the other proteins tested. Interestingly, protein–protein interactions and not carbohydrate–protein interactions were involved in the interaction between the antibodies and the lectins. As the binding of the natural antibodies did not interfere with the agglutinating properties of the lectins, the antigenic and carbohydrate-binding sites are different. Thus, for drug delivery purposes, the modification of the lectin structure by recombinant DNA technology can be useful for preparation of less or non-immunogenic lectins.

As demonstrated by oral and intranasal immunisation of mice, plant lectins widely differ in their immunogenicity. Tomato lectin provoked high local and systemic immune responses but WGA, red kidney bean lectin, and *U. europaeus* isoagglutinin I elicited low or no specific immune response [72]. Another issue to be concerned is the adjuvant effect of plant lectins. Using ovalbumin as a bystander, mistletoe lectin I strongly stimulated the immune response to the protein. In contrast, WGA and *U. europaeus* isoagglutinin I exhibited slight adjuvant activity [73]. Whereas immunogenicity is undesirable for drug delivery, the immunostimulatory effect is beneficial for peroral vaccination [74]. For the latter purpose, the type of immune response is supposed to be strongly dependent on the site of absorption. The uptake via the Peyer's patches favours the secretory IgA-mediated response, the transport across the enterocytes may facilitate the systemic IgG-mediated response.

Additionally, probable involvement of WGA in induction of celiac disease is discussed controversially in the literature. Whereas the serum levels of WGA-antibodies were elevated in children on a gluten-containing diet [75], other studies gave no evidence for the presence of lectin-like components in wheat gluten preparations [76]. To date, rather genetic factors, enzyme deficiency, and abnormal glycosylation of enterocytes are assumed to induce celiac disease than WGA antigenicity [77].

5. Lectin-grafted formulations for drug delivery to the small intestine

In terms of drug delivery, the carbohydrate-mediated biorecognition of lectins, resulting in mucoadhesion, cytoadhesion, and/or cytoinvasion, might be advantageous for drug delivery to the small intestine. To put this concept of glycotargeting into practice, two approaches to formulation are basically pursued (Fig. 6). The first approach is the preparation of prodrugs consisting of the lectin as the glycotargeting moiety, the drug as the active ingredient, and the spacer as a link. The second approach is the development of lectin-grafted carrier systems. A reservoir such as microparticles, nanoparticles or liposomes contains the drug and lectins are immobilised at the surface of the reservoir. The lectin should guide the drug container to the site of absorption getting closer to the dreams of Paul Ehrlich.

5.1. Lectin-grafted prodrugs

The most striking characteristics of macromolecular prodrugs are water solubility, low spatial extension and cleavability of the spacer. Although lipophilic drug molecules are conjugated, the solubility of prodrugs is mostly retained due to the hydrophilic protein backbone. This enables dissolution in the aqueous environment of the body representing a prerequisite for absorption. As compared to large particles, the low

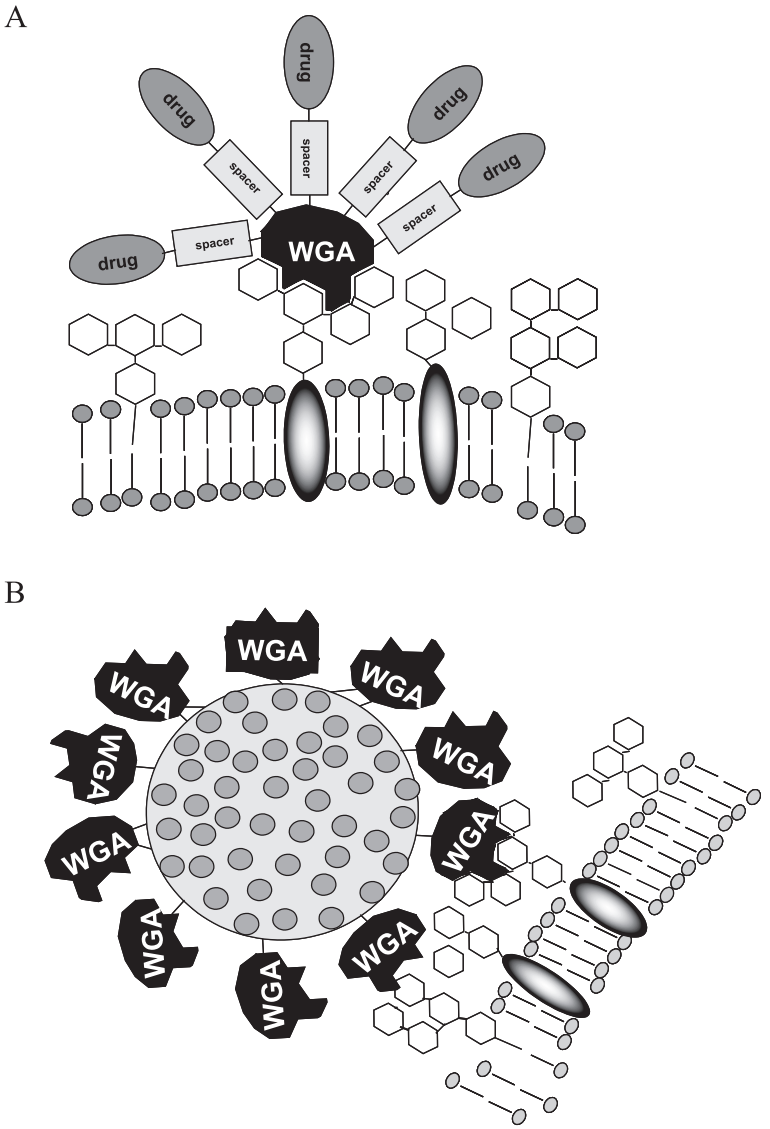

Fig. 6. Scheme of lectin-grafted prodrugs (A) and carrier systems (B) and their interaction with the glycocalyx of intestinal cells. Reproduced with permission from [96].

spatial extension facilitates transport across the cell membrane. Finally, the type of spacer can determine the release of the active drug under certain conditions. For example, doxorubicin was coupled to WGA by an *cis*-aconityl spacer [78]. The targeting effect of this colon-cancer directed prodrug derives from both the high WGA-binding capacity of colon-cancer cells and the release of the cytostatic agent not until reaching the acidic lysosomal milieu of the target cell.

To date, unique demands on pharmaceutical technology are imposed by the delicate physicochemical and biological properties of peptide and protein drugs. In terms of peroral drug delivery, formulations are required which prevent inactivation and improve absorption. To get evidence whether lectin-mediated cytoadhesion and cytoinvasion can facilitate absorption of proteins, fluorescent labelled BSA was coupled to WGA via stable amide bonds [79]. The molecular

weight of the water soluble conjugate was about 80 kDa corresponding to a coupling ratio of 1:1. About 75% of conjugate binding to the artificial intestinal tissue was mediated by specific biorecognition of the lectin moiety, whereas non-specific binding was attributed to the serum albumin content. By course of time, the interaction with Caco-2 monolayers was characterised by four phases (Fig. 7): (1) initially, the conjugate is rapidly bound to the cell surface independent from temperature. The binding rate of the conjugate is 8.7 times higher than that of glycine conjugated F-BSA. (2) Between 0.5 and 3 h, the decrease in cell-associated fluorescence intensity is stronger in absence of monensin than in presence of the pH-equilibrating agent. Thus, the area in between the curves corresponds to the amount of conjugate accumulated in the lysosomes. Concurrently, some fluorescent compound appears in the supernatant and the cell-associated fluorescence intensity is not fully restored by addition of monensin. This indicates for enzymatic degradation of cell-bound conjugate by brush border hydrolases present in differentiated Caco-2 monolayers. (3) After 3 h the vast increasing fluorescence intensity in the supernatant indicates intralysosomal degradation of the conjugate and release of degradation products into the overlying medium. (4) Finally, after 4 h the cell-associated fluorescence intensity strongly increases after monensin addition pointing to presence of high amounts of low molecular weight degradation products in the lysosomes. This vast increase in cell-associated fluorescence is due to deshielding of the intramolecularly quenched fluorescein label as confirmed by proteolysis of F-BSA.

These results illustrate that WGA can mediate cellular uptake of even high molecular weight proteins. But the cut-off for uptake of proteins is still higher since IgG, exhibiting a molecular weight of 160 kDa, is also transported into the cells by active transport mechanisms after conjugation to WGA (unpublished results). The cellular fate of IgG–WGA was similar to that of BSA–WGA but the amount of protein accumulated within the cytoplasm was lower. Thus, the membrane barrier can be surmounted by aid of lectins. However, at the same time it becomes evident that the enzymatic barrier, represented by luminal and cell-associated hydrolases, has to be overcome for successful peroral therapy. Diverse strategies like incorporation of enzyme inhibitors or shielding by matrix systems are expected to solve the problem.

5.2. Lectin-grafted carrier systems

The incorporation in appropriate carrier systems not only protects drugs against enzymic and acidic degradation in the intestine but also affords an increase in the payload. Contrary to prodrugs, it is more likely to achieve therapeutic levels of drugs by administration of microparticles, nanoparticles or liposomes. Furthermore, the matrix of drug carrier systems, which is preferably biocompatible and biodegradable, renders controlled release of drugs possible. Decoration of such Trojan Horses with lectins

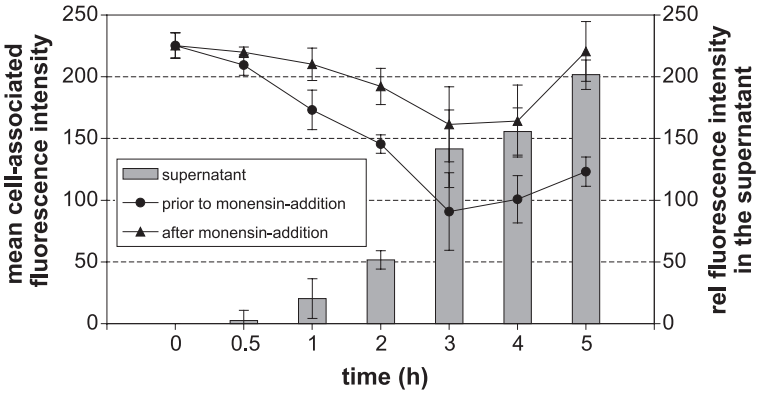

Fig. 7. Caco-2 monolayer associated fluorescence intensity of WGA-conjugated, fluorescein-labelled BSA loaded cells prior and after addition of monensin. The columns reflect the fluorescence intensity in the supernatant. Reproduced with permission from [79].

will enrich the drug on glycosylated surfaces of the gastrointestine.

Throughout the small intestine, the mucins of the mucous layer can represent the first site for glycotargeting. To what extent mucoadhesion dominates the glycotargeting effect of lectinised formulations seems to be strongly dependent on the size of the matrix system. Whereas nanoparticles are assumed to penetrate the mucous layer, microparticles bigger than 1 μm in diameter are excluded by the viscous gel layer [80,81]. On the other hand, a reversible mucin–lectin interaction may be advantageous since enrichment of the formulation at the gel layer will reduce rapid propulsion of the formulation together with the chyme.

Provided that the mucus is sufficiently penetrated, the second target of lectin-decorated drug delivery systems is the glycocalyx of the underlying intestinal epithelial cells. To what extent cytoadhesion is followed by cytoinvasion depends on the spatial extension of the formulation as well. Experiments with conjugates of cholera toxin B subunits revealed that even the glycocalyx can act as a sieve [82,83]. Conjugates of 6.4 nm in diameter had free access to the corresponding receptor located at the cell membrane. Conjugates with colloidal gold with a diameter of 28.8 nm exhibited higher binding to M-cells, featuring a thinner glycocalyx, than to enterocytes. Increasing the diameter to 1.13 μm resulted in inaccessibility of the receptors on M-cells, enterocytes, and Caco-2 cells as well.

Within the small intestine, both the M-cells and the enterocytes are the sites for glycotargeted cytoinvasion. Morphologically, there is not only a difference in the thickness but also in the glycosylation pattern of the glycocalyx [84–86]. But both M-cells and enterocytes represent potential sites for intestinal absorption of orally administered drug delivery systems. As M-cells are uniquely adapted to antigen sampling, they open a route for vaccination resulting in local and/or systemic immune response. Although the transcytotic activity of enterocytes is lower, they vastly outnumber M-cells probably leading to underestimation of enterocytes for drug absorption at least in the past.

Early work on lectin-mediated drug delivery to the small intestine concentrated on the lectin from red kidney beans [87]. In vivo studies demonstrated significant retardation of lectin-coated microspheres (2 μm) in rats small intestine as compared to controls coated with lectins of different carbohydrate specificity. At high concentrations, however, the lectin-induced morphological and metabolic changes in enterocytes. Although the toxic effects are fully reversible and unnoticeable at the low concentrations as required for bioadhesive formulations, practical utility is supposed to be precluded [64].

Since the mid 1980s a lot of research focused on extensive characterisation of tomato lectin as a tool to improved absorption. As there are some excellent reviews ([70,88], contribution of J. Woodley in this issue), only one putative milestone for drug delivery will be highlighted [89,90]. In vivo studies in rats demonstrated no evidence for uptake after single oral administration of tomato lectin coated nanoparticles with 500 nm in diameter. However, 18% of the dose were detected in the systemic circulation after daily gavage for 5 days. As lectin-induced tissue damage was not observed, the enhanced uptake by repeated administration was attributed to induction of receptor expression. Since saturation of the carbohydrate-binding site of the lectin reduced the uptake to 0.5%, the improved absorption of the nanoparticles was really due to glycotargeting. According to J. Woodley, a pioneer in tomato lectin-mediated drug delivery, retardation of the intestinal transit was not realised but the enhancement of particle absorption in the gastrointestine is very exciting [70]. Thus, the concept will be useful for drug delivery as well as for oral vaccination. Moreover, tomato lectin exhibited adjuvant activity by priming systemic and mucosal immune responses [91].

In case of WGA, fluorescein-loaded microparticles (4 μm) coated with the lectin were shown to enhance the intracellular accumulation of the model drug [92]. At saturation conditions, about 60% of the apical surface of Caco-2 monolayers were covered with lectinised microspheres as compared to 19% in case of BSA-coated microparticles. After incubation of WGA-grafted microspheres for 1 h, the amount of cell-associated model drug was 200-fold higher than that of the drug in solution. Consequently, the improved intracellular accumulation is due to both, an increase in the number of microspheres adhering to the artificial epithelium and the intimate contact of the

formulation to the epithelial barrier. Although the uptake of low amounts of similar sized microparticles was reported, confocal laser scanning microscopy gave no hint to transmembrane transport of WGA-grafted spheres. Thus, the lectin coat provided for the narrow contact of the large PLGA-spheres to the cell membrane, which resulted in shortening of the diffusional pathway, followed by improved uptake into the enterocyte-like cells.

Contrary to larger sized drug delivery systems, colloidal carriers such as nanoparticles or liposomes can also benefit from the cytoinvasive characteristics of the lectin coat. As confirmed by transport studies across polarised Caco-2 monolayers, about 25% of WGA- and 20% of ConA-modified nanospheres (50 nm) were recovered in the filter pores of transwell inserts after incubation for 18 h [61]. For comparison, only 4% of plain nanospheres were transcytosed. Interestingly, the coupling of the lectins to nanoparticles led to higher transcytosis than that of the free lectins. Additionally, the rate of transcytosis increased with the lectin density on the surface of the nanoparticles. Altogether this points to the fact that nanoparticles open a trafficking route different to that of the free lectin. It was suggested that immobilisation of lectins on colloidal formulations induces enhanced receptor clustering which results in increased transcytosis of the carrier.

The characterisation of the interaction between the colloidal carriers and the cells is most important for in vitro evaluation of lectinised nanospheres. The cytoassociation rate, meaning binding and uptake, strongly depends on the orientation of the device and agitation of the suspension. Upon vertical orientation of the Caco-2 monolayers and stirring the nanoparticle suspension by gassing, the number of cytoassociated nanospheres was reduced to 25% (WGA coating) or 16% (BSA coating) as compared to the unstirred horizontal system. Nevertheless, the amount of cell-associated WGA coated nanospheres was four times higher than that of BSA coated nanospheres. Any binding or uptake of plain nanospheres was hardly detected in the stirred vertical system (unpublished results). These observations underline that the biorecognitive interaction between the lectin and the cells is not only strong enough for fixation of the nanospheres at the cell surface but also resists mechanical agitation.

In an approach towards oral vaccination, lectinised liposomes (100 nm) were administered to fasted mice [93]. After single oral administration, 3.2% of lectin-free liposomes, 5.8% of WGA-liposomes and 10.5% UEA-I-liposomes were taken up from the gastointestinal tract into circulation. The difference in uptake was explained by the different specificity of the lectins. WGA bound to both types of intestinal epithelial cells with equal specificity but UEA-I almost exclusively interacted with murine M-cells [84,94]. Although the amount of WGA-liposomes absorbed via M-cells is unknown, the dogma of gastrointestinal absorption of particles exclusively via the M-cells becomes uncertain. As the number of M-cells in the gastrointestine amounts to less than 1% of all enterocytes and M-cells are located at the base of the villi, it is likely that the enterocytes contribute to WGA absorption. However, the mucus layer overlying the M-cells is thinner than that of enterocytes facilitating absorption via follicle associated epithelium. According to the present knowledge it is concluded that both cell types, enterocytes and M-cells, are involved in transcytosis of particulate matter. Regardless of which cell type is involved, this pathway for drug delivery can be extended by use of lectins.

6. Conclusions

Although many scientific problems and technical developments need to be solved, lectin-mediated oral drug delivery is more than an interesting idea. Relying on the specific biorecognition of gastrointestinal oligosaccharides by lectins, the elucidation of the underlying mechanisms brought three potential glycotargets to light. According to the carbohydrate composition of mucins, attachment to the mucous layer can elicit at least local and temporary retardation of lectinised formulations similar to non-specific mucoadhesives. The second target is the glycocalyx of the absorptive epithelium. In case of identical oligosaccharide structures of the mucin and the glycocalyx, partitioning of the formulation to the cell surface is facilitated due to full reversibility of the mucin–lectin interaction. In case of lectin-matching carbohydrates only at the glycocalyx, the formulation has to penetrate the mucuos layer. Both pathways

result in fixation of the drug delivery system closer to the site of absorption. That way cytoadhesion will increase the concentration gradient between the extracellular and intracellular compartment, which facilitates at least passive diffusion of the drug into the cell. The third target is represented by glycosylated receptors at the cell membrane. The binding of some lectins, such as WGA to the EGF-receptor, induces active receptor mediated endocytosis, which can improve cytoinvasion of prodrugs as well as nanoscaled carrier systems. There is evidence that part of the vesicular transport ends in the degradative lysosmal compartment releasing stable drugs to the cytoplasm of absorptive enterocytes. Another part of the drug can enter the transcytotic pathway as already confirmed by transport studies.

These underlying mechanisms are expected to improve absorption and probably bioavailability of poorly absorbable drugs as well as peptides and proteins. Because of the extracellular and intracellular enzymic barrier, the latter require matrix systems to prevent premature degradation. As the glycosylation pattern of the cells is altered upon malignant transformation, lectin-mediated delivery might be useful for anticancer therapy. Furthermore, the selective glycosylation of immune inductive M-cells opens an attractive route for oral vaccination. Finally, the cytoinvasive properties of some lectins offer an alternative to viral gene delivery.

Prior to practical utilisation of the proposed advantages, a number of issues have to be considered in future. As the toxicity of individual plant lectins varies considerably, safety concerns such as toxicity, immunogenicity and allergenicity will require rigorous testing. But it is quite possible to overcome these limitations by genetic engineering and biotechnology; e.g. recombinant mistletoe lectin 1 with similar binding properties to that from the plant was already produced [95]. A further step forward will be the expression of truncated recombinant proteins representing only the binding domain, which targets an absorption window and induces specific endo- or transcytosis.

Additionally, the glycosylation pattern varies from species to species. To date, our knowledge is based on in vitro, ex vivo and animal experiments. As already initiated by A. Pusztai for rats and mice, a glycosylation map of the human intestine deriving from human biopsy material is highly desirable to identify targets for drug delivery [32].

Ongoing from a sophisticated understanding of the lectin–cell interaction and further progress in biotechnology, glycotargeting with lectin-mediated drug delivery systems, offering cytoadhesion and cytoinvasion, will help to realise Paul Ehrlich's dream of targeted drug delivery.

Acknowledgements

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