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Lectin-mediated drug delivery:
Influence of mucin on cytoadhesion of plant lectins in vitro

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INHALTSVERZEICHNIS

EINLEITUNG

Nach peroraler Applikation stellen die Epithelzellen und die darauf befindliche Mucus-Schicht die bedeutendste Diffusionsbarriere für die Resorption von Wirkstoffen dar. Zur Überwindung dieser Barriere rückte in der Pharmazeutischen Technologie das Konzept der Bioadhäsion in den Mittelpunkt.

Die erste Generation von Bioadhäsiva umfasst Polymere wie Polyacrylsäure und deren Derivate sowie Chitosan. Diese Hilfsstoffe haften an der Mucusschicht des intestinalen Epithels, verlängern dadurch die Kontaktzeit und verkürzen die Diffusionsstrecke des Wirkstoffes [1], sodass ein erhöhter Konzentrationsgradient eine verbesserte Resorption des Arzneistoffes bewirkt. Zusätzlich führt die erhöhte Permeabilität durch Öffnung der tight junctions sowie die Hemmung proteolytischer Enzyme zu einer verbesserten Bilanz, die sich in der bereits erfolgten Markteinführung von Präparaten mit Bioadhäsiva der ersten Generation widerspiegelt.

Da die Effizienz von Bioadhäsiva der ersten Generation durch die rasche Erneuerungsrate des Mucus und Abschilferung von Mucus begrenzt ist, wurden Lektine als Bioadhäsiva der zweiten Generation vorgeschlagen [2]. Im Gegensatz zu Mucoadhäsiva binden einige pflanzliche Lektine direkt an kohlenhydrathältige Rezeptoren der Oberfläche von intestinalen Epithelzellen [3]. In-vitro wurde an Hand von artifiziellem Dünndarmgewebe nicht nur die Bindung an die Zellmembran beobachtet, sondern auch eine Aufnahme von membrangebundenem Weizenlektin (wheat germ agglutinin, WGA) und Kartoffel-Lektin (Solanum tuberosum Lektin, STL) in das Zytoplasma von absorbierenden Enterozyten [4]. Sowohl Zelladhäsion als auch Internalisation dieser Lektine beruhen auf der Wechselwirkung mit Oligosaccharidrezeptoren an der Oberfläche von intestinalen Epithelzellen. Derartige Glykoproteine sind jedoch nicht nur Bestandteil der Zellmembran, sondern auch Komponenten der Mucusschicht auf intestinalen Epithelzellen. Die Mucusschicht im menschlichen Dünndarm ist im Mittel 192 µm dick und enthält 96 – 99,5 % Wasser, sodass sie als zusätzliche Diffusionsbarriere für Wirkstoffe bzw. Formulierungen fungiert [5]. Die gelbildenden Komponenten sind Glykoproteine, sogenannte Mucine, deren Oligosaccharidmuster jenem der Lektin-Rezeptoren an der Oberfläche von intestinalen Zellen ähnlich ist.

Zur Untersuchung des Einflusses von Mucus auf die Wechselwirkung zwischen Lektinen und Zellen ist ein Testsystem erforderlich, das einerseits Zellen des Dünndarmes und andererseits

Mucus umfasst. Mucus-produzierende Zelllinien sind zwar aus der Literatur bekannt [4, 7-9], der Differenzierungsgrad und damit die Reproduzierbarkeit der Versuchsergebnisse sind jedoch gering.

Zusätzlich sollten sowohl die Wechselwirkung zwischen Lektin und Mucus als auch die Interaktion zwischen Lektin und Zelle unabhängig voneinander erfassbar sein, um diese komplexen Abläufe genauer charakterisieren zu können.

Dementsprechend sollte im Handel erhältliches Mucin aus dem Schweinemagen als Modell für humanen Mucus eingesetzt werden. Sowohl die Oligosaccharidzusammensetzung als auch der Gehalt an nativen Glykoproteiden sind jenem von humanem, intestinalem Mucus ähnlich. Entsprechend Literaturangaben enthält intestinaler Mucus 77,5% Kohlenhydrate bestehend aus N-Acetyl-galactosamin, N-Acetylglucosamin, Galactose, Fucose und Sialinsäure in einem molaren Verhältnis von 1,0 : 0,6 : 0,7 : 0,3 : 0,5 bezogen auf das Trockengewicht [5].

Als Modell für das humane intestinale Epithel sollten Caco-2 Zellen verwendet werden, die seit Jahren sowohl in der pharmazeutisch-technologischen Forschung etabliert sind als auch von der FDA (food and drugs administration) als Zelllinie für Permeabilitätsuntersuchungen empfohlen werden. Caco-2 Zellen wurden bereits 1972 von John Fogh aus dem Colon-Adenokarzinom eines weißen Patienten isoliert, sie differenzieren unter geeigneten Kulturbedingungen jedoch zu einem Epithel, das morphologisch und funktionell dem menschlichen Dünndarmepithel entspricht.

Entsprechend dem Kohlenhydratmuster auf Dünndarmepithelzellen und Mucusproteinen sollten Lektine mit unterschiedlicher Zuckerbindungsspezifität eingesetzt werden: Weizenlektin (WGA) interagiert mit Oligomeren von N-Acetyl-D-glucosamin und Sialinsäure, während Kartoffellektin (STL) nur an Sialinsäure bindet. Das Lektin aus der Kichererbse (*Dolichos biflorus* agglutinin, DBA) erkennt N-Acetyl-galactosaminreste, während das Lektin von Stechginstersamen (*Ulex europaeus* isoagglutinin I, UEA-I) an α-L-Fucose enthaltende Oligosaccharide bindet. Erdnußagglutinin (PNA) aus *Arachis hypogea* und das Linsenlektin (LCA) aus *Lens culinaris* interagieren mit Galactosaminyl- bzw. α-Mannosyl-Resten.

Im Rahmen der vorliegenden Arbeit sollte unter Verwendung dieser ex-vivo Modelle ein Testsystem zur Charakterisierung der Wechselwirkung zwischen Lektinen und Mucus etabliert werden, um damit den Einfluss der Mucusschicht auf die Zellassoziation von Bioadhäsiva der zweiten Generation abschätzen zu können.

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Lectin-mediated drug delivery: influence of mucin on cytoadhesion of plant lectins in vitro

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Abstract

As the mucous layer represents the first barrier to peroral lectin-mediated drug delivery, the influence of mucin on the cytoadhesive properties of lectins was studied in vitro by establishing a rapid and simple microplate format assay using pig gastric mucin (PGM) for coating the wells. The lectin-binding capacity of mucin followed the order WGA ≫ UEA-I ≫ LCA=STL>PNA>DBA. The PGM-binding of wheat germ agglutinin (WGA) was strongly dependent on pH being highest at pH 5.0. In comparison, PGM-binding of WGA was about 15% at gastric pH and 60–70% at intestinal pH. This points to unimpeded gastric transit of WGA-grafted formulations and favorable conditions within the intestine for binding to mucus coated enterocytes. Moreover the WGA–PGM interaction was concentration-dependent, specific and fully reversible. According to a competitive assay in the presence of Caco-2 monolayers, the PGM-binding of WGA was saturated and influenced by the lectin-concentration yielding 28% Caco-2 bound WGA (125 ng WGA/0.29 cm^2 monolayer) and 68% Caco-2 bound WGA (4 μg WGA/0.29 cm^2 monolayer), respectively. Following on from these results, lectins are expected to suffer at least partially from premature inactivation by shed off mucus like bioadhesives of the first generation, however initial but reversible mucus-binding of lectins offers partititioning to the cell membrane followed by uptake into the enterocyte.

Keywords: Bioadhesion; Caco-2; Cytoassociation; Lectin; Mucin; Wheat germ agglutinin

1. Introduction

The mucus layer and the epithelial cell lining represent the major diffusional barriers to absorption of drugs after peroral administration. To overcome these obstacles, the concept of bioadhesion gained some attention in pharmaceutical technology.

Within the last 15 years the first generation of bioadhesive excipients relying on mucoadhesion was investigated intensively. Polymers such as polyacrylic acid or chitosan and their derivatives stick to wet mucosal surfaces prolonging and intensifying the contact with the absorptive tissue [1]. Moreover modulation of epithelial permeability and inhibition of proteolytic enzymes by mucoadhesives contributed to practical application. Meanwhile mucoadhesive formulations are commercially available.

As premature inactivation by shed off mucus and the relatively fast mucus turnover limits utility of mucoadhesive excipients, lectin-mediated drug delivery was proposed as the second generation of bioadhesives [2]. In contrast to mucoadhesives, some

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lectins directly adhere to carbohydrate-containing receptors at epithelial surfaces [3]. Moreover not only membrane binding, but also uptake of membrane-bound wheat germ agglutinin (WGA) and *Solanum tuberosum* lectin (STL) into the cytoplasm of absorptive enterocytes was observed in vitro [4].

The interaction between the glycocalyx of the absorptive enterocytes and the lectin-mediated drug delivery systems involves oligosaccharide receptors located at the cell surface. As the human gastrointestinal epithelium is lined by a protective mucus layer of 192 μm in thickness at the mean in humans, this unstirred water layer acts as diffusional barrier [5]. Additionally the gel-forming components of mucus are glycoproteins, the so-called mucins. Though the mucin-content of wet gastrointestinal mucus is only 0.5–4% by weight, similarities in the carbohydrate pattern of mucins and glycocalyx-associated lectin-receptors might limit access of lectins to the cell-surface due to premature mucin-binding [6].

Though mucus producing cell lines are known from the literature [4,7–9], establishment of a test system is necessary to study the influence of mucus glycoproteins on cytoassociation of lectins at the molecular level. As commercially available pig gastric mucin (PGM) reflects the carbohydrate composition of human one and the content of native gylcoproteids is comparable to intestinal mucus, this mucin was used in the assays. As known from the literature, native glycoproteins from intestinal mucus contain 77.5% carbohydrate deriving from *N*-acetyl-galactosamine, *N*-acetyl-glucosamine, galactose, fucose and sialic acid at a molar ratio of 1:0.6:0.7:0.3:0.5 as refered to the dry weight [5]. According to the qualitative composition of mucin, a panel of lectins with different carbohydrate specificity was selected for screening the mucus binding: wheat germ agglutinin (WGA) from *Triticum vulgare* interacts with oligomers of *N*-acetyl-D-glucosamine and sialic acid, whereas *Solanum tuberosum* lectin (STL) from potato tubers binds to *N*-acetyl-D-glucosamine exclusively. The lectin from horse gram (*Dolichos biflorus* agglutinin, DBA) recognizes *N*-acetyl-galactosamine-residues, whereas the lectin from furze seeds (*Ulex europaeus* isoagglutinin I, UEA-I) binds to α-L-fucose-containing oligosaccharides. Additionally the peanut agglutinin (PNA) from *Arachis hypogaea* and the lentil lectin from *Lens culinaris* (LCA) interacting with galactosamine- and α-mannose-residues were included.

The aim of this contribution was to develop a simple procedure for characterisation of the lectin–mucin interaction and to get an idea of the impact of mucus proteins on the cytoassociation of bioadhesives of the second generation.

2. Materials and methods

2.1. Chemicals

The fluorescein labelled lectins from *Triticum vulgare* (molar ratio fluorescein/protein (F/P) = 3.2), *Solanum tuberosum* ($F/P = 2.9$), *Arachis hypogaea* ($F/P = 4.7$), *Ulex europaeus* (Ulex europaeus isoagglutinin I, $F/P = 4.0$), *Lens culinaris* ($F/P = 6.3$) and *Dolichos biflorus* ($F/P = 5.2$) were purchased from Vector laboratories (Burlingame, USA). Pig gastric mucin (type II) and bovine serum albumin (fraction V) were obtained from Sigma (St. Louis, MO, USA). 96-Well microplates (high protein binding capacity) and tissue culture-treated 96-well microplates were obtained from Greiner (Kremsmünster, Austria). All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.2. Tissue culture

The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and used between passage 50 and 60; tissue culture reagents were from Sigma and Gibco Life Technologies (Vienna, Austria).

Caco-2 cells were grown in RPMI 1640 cell culture medium containing 10% fetal calf serum, 4 mM L-glutamine and 150 μg/ml gentamycine in a humidified 5% CO_2/95% air atmosphere at 37 °C and subcultivated by trypsination.

For lectin binding assays, cells were seeded on tissue culture-treated 96-well microplates at a density of 1.7×10^4/well. The cells were fed every other day

with culture medium and used 12–14 days after seeding.

2.3. PGM-coating of microplates

Each well of a 96-well microplate (high protein binding capacity) was incubated with 100 μl 0.1% PGM-solution in PBS for 12 h at 4 °C. After washing three times with 100 μl PBS each, free binding sites were blocked by incubation with 100 μl of 1% BSA-solution in PBS for 2 h at 37 °C. The wells were washed again with PBS as described above and stored in PBS at 4 °C until use.

2.4. Confirmation of the PGM-coating

PGM-coated wells blocked with BSA, PGM-coated wells without BSA-blocking, BSA-coated wells (1% in PBS, 2 h/37 °C) as well as untreated wells as controls were incubated with 50 μl of a serial dilution of F-WGA in PBS (4–0.5 μg/well) for 1 h at 37 °C. After removal of unbound lectin by repeated washing with 100 μl PBS (three times) the relative surface bound fluorescence intensity was determined by a fluorescence microplate reader (SpectraFluor SLT; Tecan, Austria) using the Biolise™ software for calculation.

To estimate the autofluorescence of the coated wells, the relative fluorescence intensities (RFI) of the coated wells after incubation with PBS were determined. These values were subtracted from all the binding data quoted.

2.5. Determination of the lectin-binding capacity of PGM

Using a 96-well PGM-coated microplate, 50 μl of a dilution series of fluorescein-labelled lectins in PBS (4.0, 2.0, 1.0, 0.5, 0.25, 0.125 μg/well) were incubated for 2 h at 37 °C. After washing three times with 100 μl PBS each, the amount of PGM-bound lectin was determined by fluorimetry as described above.

Negative controls were included in every experiment consisting of PBS instead of the lectin-solution and considered upon calculation. Each concentration was tested in triplicate and repeated at least twice.

2.6. Specificity and reversibility of the F-WGA–PGM interaction

To assess specificity and reversibility of the carbohydrate-mediated binding of WGA to PGM, PGM-coated wells were incubated with 50 μl of F-WGA solution in PBS (2.0 μg/well) for 1 h at 37 °C followed by addition of 50 μl of a serial dilution of either *N*,*N*′,*N*″-triacetyl-chitotriose (50.0–1.5625 μg/well) or *N*-acetyl-D-glucosamine (500–15.625 μg/well) in PBS. After incubation for 1 h the wells were washed three times with 100 μl PBS each to remove both, soluble carbohydrate-bound and -unbound lectin. The amount of PGM-bound WGA was quantified by fluorimetry as described above.

Each concentration was analysed in triplicate. Negative controls were carried out as described above. Positive controls representing the maximum amount of PGM-bound F-WGA were prepared as above but using PBS instead of the solutions containing the complementary carbohydrate.

2.7. Influence of pH on the PGM–WGA interaction

To estimate the influence of pH on PGM-binding of F-WGA, washed PGM-coated microplate wells were incubated with 50 μl of F-WGA (4.0–1.0 μg/well, serial dilution) in buffer-solutions of different pH between 1.1 and 9.0. The following buffers were used: 0.1 M glycine/HCl (pH 1.1and 2.0), 0.1 M citric acid/Na_2HPO_4 (pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0), 0.1 M $NaHCO_3$/NaOH (pH 9.0). After incubation at 37 °C for 2 h the wells were washed with PBS (100 μl, three times) and the PGM-bound lectin was determined by fluorimetry.

As a negative control the assay was performed as above but using PBS instead of the lectin solutions. Each experiment was carried out in triplicate.

2.8. Influence of mucin ageing on the PGM–WGA interaction

To study the effect of prolonged storage of the mucin solution on Caco-2 adhesion of F-WGA in the presence of mucin, Caco-2 monolayers were washed and incubated with 50 μl F-WGA solution in PBS

(2.0–0.5 μg/well, serial dilution) and 50 μl PGM solution in PBS (1.0 mg/ml) for 2 h at 4 °C. Prior to addition the PGM solution was stored for 1, 2, 4 or 8 days at 4 °C. The Caco-2 monolayers were washed three times with 100 μl PBS each and the amount of cell-bound lectin was determined.

The same assay as above but using PGM-solution supplemented with phenylmethylsulfonyl-fluoride (PMSF, 170 μg/ml) during storage was performed to examine the influence of proteases on the WGA-binding capacity of PGM. Additionally negative controls were carried out as described above.

2.9. Binding of F-WGA to Caco-2 monolayers in presence of PGM

Caco-2 monolayers were grown in 96-well microplates for 12 to 14 days and washed twice with 100 μl PBS prior to the binding assay. To estimate saturation conditions of the WGA–PGM interaction Caco-2 monolayers were incubated with 50 μl PGM solution in PBS (1 mg/ml) and 50 μl F-WGA solution containing increasing amounts of lectin (0.125–4.0 μg/well) for 2 h at 4 °C to inhibit uptake of the lectin into the cells. By washing with 100 μl PBS each, both PGM-bound and excessive F-WGA were removed and the cell-bound fluorescence intensity was determined.

Tests were carried out in duplicate and repeated at least twice. Positive controls representing the Caco-2 binding of F-WGA in the absence of PGM as well as negative controls considering autofluorescence of Caco-2 monolayers grown in the wells were included in each experiment.

3. Results

3.1. Development of the PGM-assay

To elucidate the interaction between WGA and PGM, a microplate-format test system was established taking advantage of the nonspecific adhesion of proteins on polystyrene-surfaces. As conventional methods for determining the protein content such as the Micro-BCA assay were inappropriate (beyond the limit of sensitivity) to confirm PGM-coating of the wells, binding of fluorescent labelled WGA to *N*-acetyl-D-glucosamine- or sialic acid-containing oligosaccharide chains of PGM was used to verify successful immobilisation of PGM. Screening of PGM-binding to three different types of microplates (low, medium, high protein binding capacity) at three different PGM-concentrations (0.01, 0.1 and 1.0%) yielded most densely PGM-coated wells when microplates with high protein binding capacity and 0.1% (w/v) PGM solutions were used (data not shown). Upon coating the wells with 1% BSA-solution, the amount of surface bound F-WGA decreased to an extent of about 77% at the mean ($n=15$, Fig. 1) as compared to untreated polystyrene wells due to blocking of non-specific WGA-binding to the non-coated wells. In contrast, coating the wells with freshly prepared 0.1% PGM-solution F-WGA-binding was reduced by only 27% at the mean probably because of specific interaction between the carbohydrate-containing PGM and the lectin. Since additional blocking of possibly residual binding sites of PGM-pretreated wells with BSA resulted in unaltered F-WGA binding capacity of the PGM-coated wells, all the F-WGA binding sites in the wells were saturated by incubation with PGM overnight. To confirm the specificity of the interaction between F-WGA and the PGM-coating a competitive assay was performed using soluble complementary carbohydrates for inhibition of lectin binding to immobilized PGM (see Table 1).

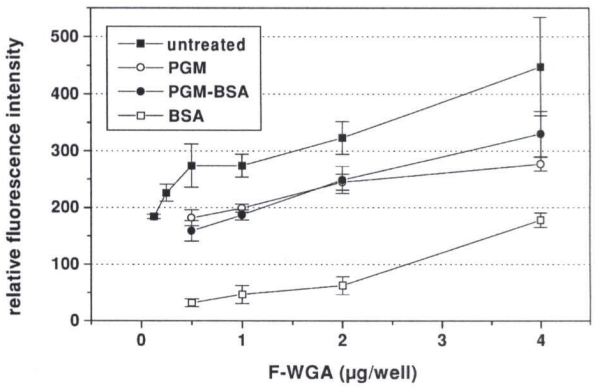

Fig. 1. Binding of F-WGA to polystyrene microplate wells coated with pig gastric mucin (PGM) followed by saturation with bovine serum albumin (PGM–BSA) or BSA in comparison to untreated wells (mean±S.D., $n=6$).

Table 1
Competitive inhibition of WGA binding sites on PGM-coated microplates by addition of increasing amounts of the complementary carbohydrate (mean±S.D., $n=3$)

N,N′,N″-triacetyl-chitotriose		*N*-acetyl-D-glucosamine	
μg carbohydrate/ PGM coated well	PGM-bound F-WGA (%)	μg carbohydrate/ PGM coated well	PGM-bound F-WGA (%)
1.56	63.0±7.6	15.62	94.8±4.5
3.12	42.3±5.3	31.25	81.0±9.4
6.25	20.6±8.3	62.50	57.0±5.8
12.50	19.1±1.5	125.00	62.8±8.0
25.00	0.0±9.4	250.00	55.7±9.5
50.00	0.0±9.6	500.00	57.8±9.6

3.2. Lectin-binding capacity of PGM

According to the carbohydrate composition of human mucus and to previous studies dealing with lectin-adhesion to enterocyte-like cell lines [3], a panel of lectins with different carbohydrate specificities were tested for PGM-binding. When PGM-coated wells were incubated with increasing amounts of fluorescein labelled lectins, the mean PGM-bound fluorescence intensity increased with increasing amounts of each lectin added (Fig. 2). Considering the F/P ratio of lectins as well as the autofluorescence of the PGM-coated wells for calculation, the highest PGM-binding rate was observed in case of F-WGA exhibiting an increase in PGM-bound fluorescence intensity concurrent with concentration ranging from 14.7±3.1 to 49.0±2.5 (mean±S.D., $n=3$). Whereas PGM-binding of F-UEA-I elicited a relative fluorescence intensity between 6.0±0.5 and 39.7±3.0, the adhesion of F-LCA, F-STL, F-PNA and F-DBA was quite lower resulting in mean PGM-bound fluorescence intensities in the range of 21.2±2.8 (F-LCA) to 12.7±0.0 (F-DBA) at the highest. According to these experiments the WGA-binding capacity of PGM is 1.2-fold higher than that of UEA-I and about 2.7-fold higher as compared to the rest of the lectins under investigation. Due to its high binding properties to both, PGM as well as enterocyte-like cell lines, WGA was used for the following experiments exclusively.

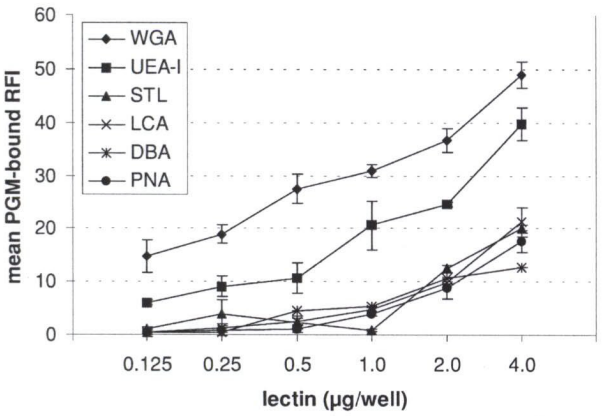

Fig. 2. Saturation analysis of lectin-binding sites on PGM-coated microplate wells (mean±S.D., $n=3$). Mean PGM-bound RFI is adjusted for the different F/P-ratios of each lectin.

3.3. Specificity and reversibility of the WGA–PGM interaction

The F-WGA-binding rate to constant amounts of PGM increased with increasing lectin-concentration and indicated for specificity of the WGA-PGM interaction. In order to confirm specificity of binding, a competitive assay was performed using soluble *N, N′, N″*-triacetyl-chitotriose and *N*-acetyl-D-glucosamine for displacement of the PGM-bound lectin. When 2 μg of F-WGA were allowed to interact with PGM for 1 h at 37 °C followed by addition of increasing amounts of dissolved complementary carbohydrate and further incubation for 1 h, the amount of PGM-bound WGA decreased with increasing the carbohydrate concentrations (Table 1). Upon addition of *N, N′, N″*-triacetyl-chitotriose the

WGA–PGM interaction was inhibited completely at 25 μg carbohydrate/well (corresponding to 0.5 mg/ml) at the minimum. In contrast, not even 50% of the total amount of PGM-bound WGA could be detached with *N*-acetyl-D-glucosamine even up to concentrations of 500 μg/well (corresponding to 10 mg/ml). Calculation of the amount of corresponding carbohydrate as necessary for 50% inhibition of lectin-binding to PGM (IC_{50}) from the plot according to Boltzman resulted in 2.5 μg/well in case of *N*, *N′*, *N″*-triacetyl-chitotriose representing a 72-fold molar excess as compared to WGA. The IC_{50} of *N*-acetyl-D-glucosamine could not be calculated exactly due to limited solubility of this carbohydrate.

3.4. pH-dependency of F-WGA-binding to PGM

To estimate the influence of pH on the interaction between WGA and PGM, PGM-coated microplate wells were incubated with F-WGA at different pH levels ranging from 1.1 to 9.0. Independent from lectin-concentration, highest F-WGA binding rates were observed at pH 5.0 (Fig. 3). At pH 4.0 and pH 6.0 the amount of PGM-bound F-WGA decreased by about 20–30%. Lowest adhesion rates were detected in acidic milieu amounting to 10% (pH 1.1), 15% (pH 2.0) or 22–30% (pH 3.0) in comparison to lectin-binding at pH 5.0. Incubating WGA and PGM at pH levels higher than pH 6.0 resulted in rather constant PGM-binding of the lectin in the range of 50–70% of pH 5.0 values.

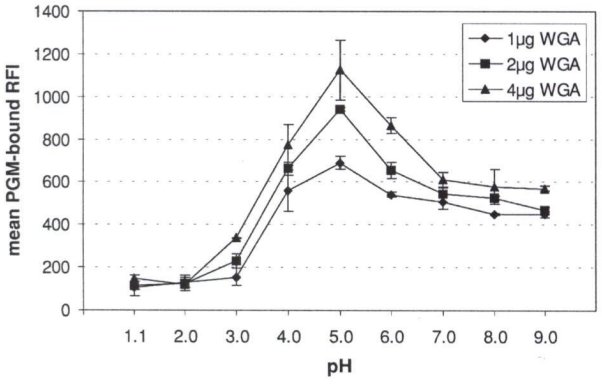

Fig. 3. Influence of pH on the PGM–WGA interaction at different concentrations of the lectin (mean±S.D., $n=6$).

3.5. Influence of mucin ageing and protease-inhibition on the PGM–WGA interaction

Besides pH, ageing of the PGM-solution had an impact on the WGA-binding capacity of PGM. When Caco-2 monolayers were incubated with 2 μg F-WGA in presence of PGM (50 μg/well) stored for 24 h the cell-bound relative fluorescence intensity was 173.0±1.8 (Fig. 4). Using PGM after storage for 8 days at 4 °C for the same assay, the amount of Caco-2 bound lectin decreased to 124.2±0.2. Incubating the cells with lower amounts of F-WGA (1.0 or 0.5 μg/well) led to an even higher decrease in cell-bound fluorescence intensity of 35–38%.

The decreasing Caco-2 binding of lectin might be due to proteolytic degradation of PGM causing opening of hidden oligosaccharide-chains of mucin, which probably resulted in increased PGM-binding of the lectin. Accordingly the experiments as above were performed with PGM stored in the presence of protease inhibitor PMSF (8.5 μg/test), but considerable alterations in cell-bound fluorescence intensity due to presence or absence of PMSF were not observed.

3.6. Binding of F-WGA to Caco-2 monolayers in presence of PGM

To simulate the situation at the site of intestinal absorption, WGA-binding experiments were performed using Caco-2 monolayers corresponding to

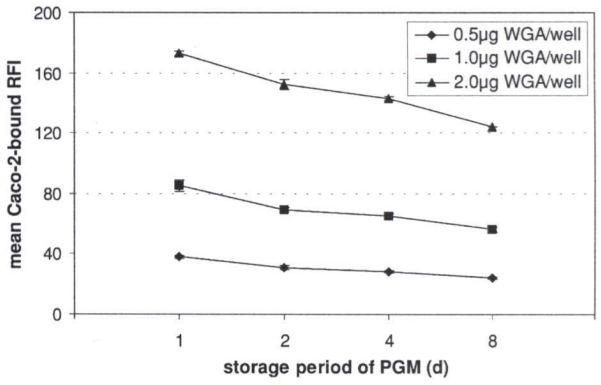

Fig. 4. Caco-2 binding of F-WGA at different concentrations in presence of PGM, which was stored for increased time at 4 °C (mean±S.D., $n=3$).

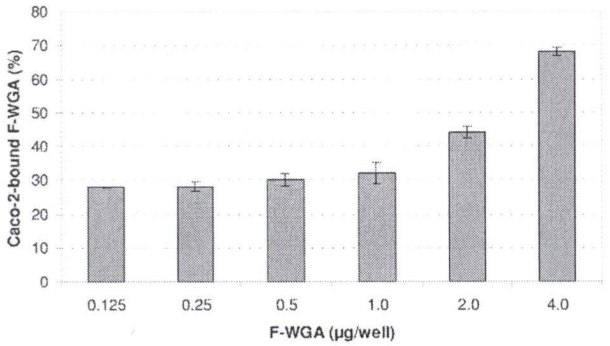

Fig. 5. Caco-2 binding of F-WGA in the presence of PGM (mean±S.D., $n=4$).

intestinal artificial tissue and PGM-solutions as a limited model for the mucus layer simultaneously. As WGA-binding to Caco-2 monolayers was highest after cultivation of the cell-layers for 12 to 14 days (data not shown), the Caco-2 cells were grown in 96-well microplates for at least 12 days prior to utilisation for binding assays. When Caco-2 monolayers were incubated for 2 h at 4 °C with increasing amounts of F-WGA in the presence of PGM (0.5 mg/ml), the amount of cell-bound WGA increased upon addition of F-WGA concentrations higher than 0.25 μg/well (Fig. 5). As compared to adhesion of WGA in the absence of PGM, in the presence of PGM only 27.9±0.1% were bound to the monolayer after incubation with 125 ng F-WGA/well. But the amount of Caco-2-bound lectin increased up to 68.1±1.3% upon addition of F-WGA at concentrations of 4.0 μg/well.

4. Discussion

Within the gastrointestinal tract, the mucus layer protects the underlying tissue from acidic gastric juice, proteases, pathogenic microorganisms and mechanical trauma. As known from some in vitro and a few in vivo studies, some lectins directly interact with the apical membrane of absorptive enterocytes [4,10]. But there is less information about the interaction between lectins and the mucus layer representing the first barrier against absorption of lectin-grafted prodrugs or lectin-modified carrier systems.

As an alternative to gel permeation chromatography of mucin-lectin complexes [11], a rapid and simple protocol was established using PGM-coated microplate wells for characterisation of the mucin-lectin interaction. Though the mucus represents a mixture of large glycoproteins, enzymes, water, electrolytes, sloughed epithelial cells, bacteria and bacterial products, the chief determinants of the functional and physical properties termed mucin glycoproteins were used in the assays [12]. Pig intestinal mucus contains about 1% glycoprotein, but due to high viscosity and poor reproducibility a 0.1% solution of PGM was used for coating the wells. The results indicate that F-WGA is bound to PGM-coated microplate wells mainly via specific interaction between the lectin and carbohydrate chains of the PGM (Fig. 1, Table 1).

Using this protocol for screening the interaction between lectins and PGM, the lectin-binding capacity of mucin followed the order WGA ≫ UEA-I ≫ LCA = STL > PNA > DBA. This order not necessarily reflects the molar composition of the mucin, but rather the sterical configurations of the mucus glycoproteins. According to the molar ratio of carbohydrates building up the mucin, the sialic acid content of mucin is only half of *N*-acetyl-galactosamine, but sialic acid often operates as a chain terminator at the linear or branched oligosaccharide sidechains of mucin. Thus the WGA-binding capacity was found to be highest of all lectins under investigation. Similarly the fucose-content of the mucin glycoprotein is rather low, but due to free access to terminal fucosyl residues at the lateral oligosaccharide chains UEA-I-binding was rather high. In contrast, the *N*-acetyl-galactosamine is most abundant, but it is linked *O*-glycosidically to projecting hydroxyls of serin and threonine at the peptide backbone. The steric hindrance of access resulted in lowest PGM-binding capacity in the case of DBA. Among others, the PGM-binding rate of PNA corresponds to accessible galactosyl- and galactosaminyl-residues. The *N*-acetyl-glucosamine residues involved in STL-binding are localized close to the protein-backbone of mucins [11]. The amount of PGM-bound STL was comparable to that of mannose-specific LCA. Usually mannose is not reported to be part of mucin-associated oligosaccharides, but binding of mannose-specific fimbriae of *E. coli* was

found to bind to exposed oligomannosyl residues [13].

Not only the PGM-binding capacity was highest in case of WGA binding specifically to sialic acid- and *N*-acetyl-glucosamine residues, but the interaction was found to be specific too as indicated by the PGM-binding assay in presence of the inhibitory carbohydrates *N*-acetyl-D-glucosamine ($IC_{50} > 500$ μg/well) or *N*,*N*′,*N*″-triacetylchitotriose ($IC_{50} = 2.5$ μg/well). As the WGA-combining site is proposed to be complementary to a trisaccharide, the inhibitory potency of the trisaccharide was considerably higher than that of the monomeric carbohydrate [14]. Additionally, the extent of inhibition was independent from sequence of addition of carbohydrate and WGA, either first the carbohydrate followed by the lectin or vice versa. In contrast to the WGA-Caco-2 interaction, these results point to full reversibility of the WGA–PGM interaction.

According to the results of WGA-binding to PGM at different pH-levels, premature adhesion of WGA-grafted drug delivery systems to gastric mucin might be negligible. By use of the Heidelberg capsule, the gastric pH in fasted humans was reported to range between 1.3 and 2.1 [15]. At this pH-range, the PGM-binding of WGA was lowest being independent from the amount of lectin added. This points to most unfavorable conditions for WGA-mucus interaction in the acidic environment. Additionally independent from pH, the extracellular mucus of normal human gastric epithelial cells was found to be negative for WGA-labelling, whereas mucus cytoplasmic vacuoles were positive [16]. As WGA resists proteolytic degradation [3] and mucus-binding in acidic gastric milieu is rather low, WGA-modified drug delivery systems are expected to survive gastric transit undamaged.

But it is likely that WGA adheres to intestinal mucus, since the intestinal pH in fasted humans was reported to range between 5.5 and 6.5 [17]. At pH 6.0 the amount of PGM-bound WGA was five to seven times higher than that at pH 2.0 and strongly increased concurrent with increasing the amount of lectin available.

To estimate the influence of PGM on Caco-2 binding of F-WGA, an in vitro model consisting of Caco2-monolayers as an artificial intestinal absorptive tissue and supernatant PGM-solution was used. To exclude cellular uptake of the lectin, the assay was performed at 4 °C. At this temperature level the fluidity of the cell membrane and the metabolism of the cells is reduced resulting in repression of energy dependent transport processes. For this reason the amount of cell-associated lectin refers mainly to membrane-bound lectin. Upon incubation with F-WGA up to 1 μg/well, about 70% of the lectin were inhibited from cell-binding by PGM. In contrast, when the amount of lectin added was increased to 4 μg/well, only 30% of the lectin were bound to PGM. In turn this corresponds to 70% cell-bound lectin. As the adhesion of F-WGA to Caco-2 monolayers in presence of PGM increases with increasing amounts of lectin added, the WGA-combining sites on PGM are saturable. Disregarding at first the fact, that membrane-bound lectin is taken up into the cell, which shifts the balance between mucin- and membrane-bound WGA towards cytoadhesion, the ratio between mucus and amount of lectin-grafted formulation might be a crucial factor for lectin-mediated drug delivery due to initial saturation of the mucus.

Additionally, mucin ageing influences the lectin-binding capacity of PGM notedly. Since an increase in storage period of the mucin solution leads to a decrease of Caco-2 bound lectin, more carbohydrate side chains representing lectin-combining sites seemed to become accessible during storage. But addition of the omnipotent protease inhibitor phenylmethylsulfonyl-fluoride to the storage solution of PGM resulted in no considerable alteration of the binding data. Consequently disclosure of *N*-acetyl-D-glucosamine-residues located near the backbone of mucin by proteases contaminating the PGM preparation can be excluded. But it is not likely that the increase in mucus-lectin interaction with mucin ageing adversely affects lectin-mediated drug delivery in vivo as the mucus is constantly turned over with an turnover time between 47 and 270 min [18]. But upon performing experiments with mucin solutions in vitro, this observation should be kept in mind.

5. Conclusions

Following on from the results of the interaction between WGA and PGM, binding of WGA within the stomach is not crucial after peroral application. Since highest binding rates were observed at pH 5

most of the lectin is expected to bind within the upper duodenum after peroral administration. Though the mucus layer represents a diffusional barrier, the mucins might act as 'catchers' for lectin-grafted drug delivery systems due to the specificity of the PGM–WGA interaction. But the mucosal barrier can be surmounted due to reversibility of the mucin–WGA interaction, which facilitates partitioning of the lectin-modified drug delivery system from mucins to free lectin receptors at the cell membrane. As membrane binding is immediately followed by cytoinvasion provided by the lectin, intracellular uptake of the formulation makes free lectin binding sites available again. These characteristics can result in accelerated and facilitated intracellular uptake of drugs from lectin-functionalized formulations into the absorptive enterocyte, which has to be confirmed by further experiments.

All in all lectins as bioadhesives of the second generation offer the main advantage of mediating cellular uptake after diffusion through the intestinal mucus layer, which is more permeable than the gastric one [19]. On the other hand it should be kept in mind that they suffer at least partly from premature inactivation by shed off mucus like those of the first generation. In contrast initial mucin binding of lectins even can be advantageous, since the mucus layer might act as a first, but fully reversible binding site followed by distribution of lectin-mediated drug delivery systems to the cell layer.

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ERGEBNISSE UND DISKUSSION

Im Rahmen der vorliegenden Diplomarbeit konnte zur Publikation

„ Lectin-mediated drug delivery: influence of mucin on cytoadhesion of plant lectins in vitro“
(Autoren: M. Wirth, K. Gerhardt, C.Wurm, F. Gabor)
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(siehe vorliegende Diplomarbeit Seite 6-14)

maßgeblich, insbesondere durch Experimente zur Untersuchung der Mucus-Lektin Interaktion beigetragen werden.

Obwohl in einigen *in-vitro* und wenigen *in-vivo* Studien die Wechselwirkung zwischen speziellen Lektinen als Bioadhäsiva der zweiten Generation und der apikalen Membran von intestinalen Epithelzellen beschrieben wird, ist wenig über den Einfluss der Mucusschicht auf diese Interaktion bekannt.
Aufgrund gewisser Ähnlichkeiten im Kohlenhydratmuster von Zelloberfläche und Mucinen ist jedoch ein Effekt zu erwarten, der insbesondere für die Einsetzbarkeit von Lektin-hältigen prodrugs und Lektin-hältigen Formulierungen entscheidet.
Alternativ zur Gelpermeationschromatographie von Mucin-Lektin Komplexen [11] wurde ein schnelles und einfaches ELISA-Verfahren entwickelt, das auf der Verwendung von Mucin-beschichteten Mikrotiterplatten zur Charakterisierung der Mucin-Lektin Interaktion beruht. Obwohl die Mucusschicht eine Mischung von hochmolekularen Glycoproteinen, Enzymen, Wasser, Elektrolyten, abgelösten Epithelzellen, Bakterien und Bakterienprodukten darstellt, wurden die als Hauptdeterminanten der physiologischen Eigenschaften, die Mucinglycoproteine, in den Versuchen eingesetzt (12). Intestinaler Schweinemucus enthält etwa 1 % Glycoprotein, aber wegen der hohen Viskosität und der geringen Reproduzierbarkeit wurde eine 0,1 %ige Lösung von Mucin aus Schweinemagen (pig gastric mucin, PGM) zur Beschichtung der wells verwendet.
Die Experimente ergaben, dass die Bindung von Fluorescein-markiertem WGA an PGM-beschichtete Mikrotiterplattenwells überwiegend durch spezifische Interaktion zwischen dem Lektin und den Kohlehydratketten des PGM zustande kommt (fig.1, Tabelle 1).
Die Anwendung dieses Protokolls zum Screening der Lektin-PGM Interaktion ergab, dass die Lektinbindungskapazität in folgender Reihenfolge abnimmt: WGA >>UEA-

I>>LCA=STL>PNA>DBA. Diese Reihenfolge spiegelt nicht unbedingt die molare Zusammensetzung des Mucins wieder, jedoch die sterischen Konfigurationen der Mucusglycoproteine. Gemäß dem molaren Verhältnis der Kohlenhydrate in den Mucin-Molekülen beträgt der Sialinsäuregehalt von Mucin nur die Hälfte jenes von N-Acetyl-D-galactosamin. Jedoch bildet Sialinsäure oft das Kettenende der linearen oder verzweigten Oligosaccharidseitenketten des Mucins, sodass die WGA-Bindungskapazität von allen untersuchten Lektinen am Höchsten war.

Ähnlich dazu ist auch der Fucosegehalt der Mucinglycoproteine sehr gering, aber Aufgrund der freien Zugänglichkeit terminaler Fucosereste an den seitlichen Oligosaccharidketten war die UEA-I-Bindung sehr hoch.

Im Gegensatz dazu ist der N-Acetyl-galactosamin-Gehalt sehr hoch, es ist jedoch O-glycosidisch an exponierte Hydroxylgruppen von Serin und Threonin an der Peptidkette gebunden. Diese sterische Hinderung des Zutritts bewirkt die geringe PGM-Bindungskapazität von DBA.

Die PGM-Bindungsrate von PNA entspricht den frei zugänglichen Galactose- und Galactosamin-Teilstrukturen. N-Acetylglucosaminyl-Reste als Bindungsstellen für STL befinden sich direkt am Proteingerüst von Mucin [11], sodass die PGM-Bindungsrate von STL gering und jener von Mannose-spezifischem LCA vergleichbar war. Mannose ist zwar kein Bestandteil von Mucin-assoziierten Oligosacchariden, aber an Hand von mannosespezifischen Fimbrien von E.coli wurde eine Bindung an offene Oligomannosylreste nachgewiesen [13].

WGA zeigte nicht nur die höchste PGM-Bindungskapazität, sondern auch die Spezifität der Bindung an Sialsäure- und N-Acetyl-glucosaminylreste war am höchsten wie an Hand von kompetitiven Interaktionsstudien in Gegenwart der inhibierenden Kohlenhydrate N-Acetyl-D-glucosamin (IC_{50} >500 g/Well) oder N,N´,N´´-Triacetylschitotriose (IC_{50} = 2,5 g/well) nachgewiesen werden konnte. Da die WGA-Bindungsstelle komplementär zu einem Trisaccharid ist, war die Inhibitionsstärke des Trisaccharids weit höher als jene des monomeren Kohlenhydrates [14]. Außerdem war das Ausmaß der Inhibition unabhängig von der Reihenfolge des Zusatzes der Kohlenhydrate und WGA. Im Gegensatz zur WGA-Caco-2-Zell Interaktion, weisen diese Ergebnisse auf eine volle Reversibilität der WGA-PGM Interaktion hin.

Entsprechend den Ergebnissen der WGA-PGM Bindung bei unterschiedlichen pH-Stufen ist die vorzeitige Adhäsion von WGA-hältigen Formulierungen an Mucin des Magens vernachlässigbar. Da mit Hilfe der Heidelberg Kapsel in nüchternen Probanden ein pH-Wert

von 1,3-2,1 gemessen wurde und die PGM-Bindung von WGA in diesem pH-Bereich unabhängig von der Menge des zugesetzten Lektins am Geringsten war, dürften die Bedingungen für eine WGA-Mucus Interaktionen im sauren Milieu des Magens äußerst unvorteilhaft sein [15].

Darüber hinaus konnte unabhängig vom pH-Wert der extrazelluläre Mucus von menschlichen Magenepithelzellen nicht mit WGA markiert werden, obwohl Mucus zytoplastische Vakuolen mit Mucin mit WGA markiert werden konnten [16].

Da WGA proteolytisch stabil ist [3] und die Mucusbindung in saurem Milieu sehr gering ist, dürften WGA-modifizierte Formulierungen die Magenpassage unbeschadet überstehen.

Aber höchstwahrscheinlich bindet WGA an intestinalen Mucus, da der intestinale pH im nüchternen Probanden zwischen 5,5 und 6,5 [17] liegt. Bei pH 6,0 war die Menge an PGM-gebundenem WGA 5-7 mal höher als bei pH 2,0 und nahm gleichzeitig mit steigender Menge an verfügbarem Lektin zu.

Um den Einfluss von PGM auf die Caco-2-Bindung von F-WGA abzuschätzen, wurde ein *in-vitro* Modell bestehend aus Caco-2-Monolayern als ein künstliches intestinales Gewebe und PGM-Lösung eingesetzt und gleichzeitig die Versuchstemperatur auf 4°C gesenkt, um eine zelluläre Aufnahme des Lektins zu verhindern.

Bei dieser Temperatur ist die Fluidität der Zellmembran und der Metabolismus der Zellen reduziert und bewirkt eine Untedrückung energieabhängiger Transportprozesse. Dadurch wird sichergestellt, dass die Menge an zellassoziiertem Lektin nur membrangebundenes Lektin darstellt.

Bei Inkubation mit bis zu 1 µg F-WGA/well, wurde die Zellbindung des Lektins durch PGM zu etwa 70 % inhibiert. Eine Erhöhung der Lektinmenge auf 4 µg/well bewirkte jedoch eine 70%ige Zellbindung, was einer Bindung von 30 % des Lektins an PGM entspricht. Da die Adhäsion von F-WGA an Caco-2-Monolayer in Gegenwart von PGM mit steigender Lektinmenge zunimmt, sind die WGA-Bindungsstellen an PGM gesättigt.

Ungeachtet der Tatsache, dass membrangebundenes Lektin in die Zellen aufgenommen wird und somit durch Freiwerden von Lektinbindungsstellen an der Zelloberfläche das Gleichgewicht in Richtung Cytoadhäsion verschiebt, könnte das Verhältnis zwischen Mucus und vorhandener Menge an Lektin-hältiger Formulierung einen entscheidenden Parameter für Lektin-vermittelte Arzneiformen darstellen, da der Mucus initial abgesättigt sein muß.

Darüber hinaus beeinflusst das Alter der Mucin-Lösung die Lektinbindungskapazität von PGM deutlich. Da eine verlängerte Lagerungszeit der Mucinlösung einer Abnahme der Caco-2 gebundenen Lektin-Menge bewirkt, dürfte die Zugänglichkeit der Lektin-bindenden

Kohlenhydratseitenketten während der Lagerung zunehmen. Jedoch bewirkte der Zusatz des Proteaseinhibotors Phenylmethylsulfonylfluorid zur gelagerten PGM-Lösung keine merkbare Änderung der Bindungsraten, sodass eine erleichterte Zugänglichkeit der N-Acetyl-D-glucosamin-Reste nahe der Peptidkette von Mucin durch einen möglichen Protease-Gehalt der PGM-Präparate ausgeschlossen werden kann.

Es ist aber unwahrscheinlich, dass sich der Anstieg der Mucus-Lektin Interaktion mit der Mucinalterung *in-vivo* nachteilig auf die Lektin-hältigen Arzneiformen auswirkt, da sich der Mucus mit einer Erneuerungszeit zwischen 47 und 270 Minuten konstant erneuert [18].

AUSBLICK

Entsprechend den Ergebnissen der Studien zur Wechselwirkung von PGM und WGA stellt der Magen kein Hindernis für die Bioadhäsion Lektin-hältiger Arzneiformen dar. Da die höchsten Bindungsraten bei pH 5 beobachtet wurden, dürfte eine derartige Arzneiform erst im Duodenum adhärieren.

Obwohl die Mucus-Schicht eine Diffusionsbarriere darstellt, fungieren die Mucusproteine quasi als „Fänger“ für Lektin-hältige Arzneiformen aufgrund ihrer spezifischen Interaktion mit Lektinen. Aber aufgrund der Reversibilität der Wechselwirkung, die eine Verteilung der Mucin-gebundenen Lektin-hältigen Formulierung zu freien Bindungsstellen an der Zellmembran erleichtert, kann die mucosale Barriere überwunden werden. Da Membran-gebundenes Lektin in die Zellen aufgenommen wird, stehen wieder freie Lektinbindungsstellen an der Zellmembran zur Verfügung, die ihrerseits wieder eine Verteilung von Mucus-gebundenem Lektin zur Zellmembran begünstigen. Diese Charakteristika könnten eine beschleunigte und erleichterte intrazelluläre Aufnahme von Arzneistoffen aus Lektin-hältigen Formulierungen in resorbierende Enterocyten bewirken, was jedoch durch weitere Experimente abgesichert werden muss.

Lektine als Bioadhäsiva der zweiten Generation bieten somit den wesentlichen Vorteil, die Aufnahme des Wirkstoffes in die Zelle nach Diffusion durch die intestinale Mucusschicht zu ermöglichen, die zusätzlich leichter permeabel ist als jene im Magen. Es sollte jedoch auch berücksichtigt werden, dass die Effektivität von Bioadhäsiva der zweiten Generation wie jene von Mucoadhäsiva durch abgeschilferten Mucus eingeschränkt wird. Die initiale Mucusbindung von Lektinen kann jedoch sogar von Vorteil sein, da der Mucus als erste, jedoch vollständig reversible Bindungsstelle für Lektin-hältige Formulierungen fungiert, auf die eine Verteilung der Formulierung zur Zellmembran und Aufnahme in die Enterozyten folgt.

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