Gastrointestinal Absorption of Recombinant Hirudin-2 in Rats

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Abbreviations:
HP-β-CD, hydroxypropyl-β-cyclodextrin; SDCh, deoxycholic acid sodium salt; PBS, phosphate buffer solution.

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ABSTRACT

To investigate the absorption of recombinant hirudin-2 (rHV2) after oral administration to rats and its possible absorption mechanism, a series of experiments were carried out. The degradation of $^{125}$I-rHV2 in the luminal contents and variant mucosal subcellular fractions, as well as the effect of degradation inhibition of some adjuvant was investigated. The bioavailability of rHV2, with or without degradation inhibitor after oral administration to rats was estimated, while the in situ loop test and everted sac experiment were also conducted to understand more about the gastrointestinal absorption of rHV2 in rats. It was demonstrated that the rHV2 was not stable in the luminal contents and sub-fraction of the intestinal mucosa. Some enzyme inhibitor like bacitracin or casein could inhibit the degradation to certain degrees. The intact rHV2 molecules were found in the rat plasma after oral administration, and the bioavilability varies obviously, dependent on the analytical method. Some of the enzyme inhibitor could enhance the rHV2 oral absorption. There is no site difference on rHV2 absorption in different segments of small intestine. The possible transport mechanism of rHV2 across the GI tract is concerned with the endocytosis process.
Hirudin is a peptide composed of 65 amino acids residues with a molecular weight of 7000Da. Hirudin reacts with thrombin in a 1:1 molar ration to form an enzyme-inhibitor complex in which the proteolytic activity of the enzyme is totally blocked (Markwardt et al., 1970). The dissociation constant of the hirudin-thrombin complex was determined to be as $6.3 \times 10^{-11}$mol/L (Chang JY, 1983). Hirudin holds many advantages over the commonly used anticoagulants such as heparin. It does not need a cofactor for the interaction with the enzyme neither it is inactivated by platelet factors. Also it has no toxicity or antigenicity. These characteristics make hirudin a promising candidate for proteinase inhibitor therapy in clinical states associated with thrombosis and disseminated intravascular coagulation (Nowak and Markwardt, 1991). It has become possible to produce biologically active peptide and proteins that are therapeutically applicable such as hirudin by means of recombinant DNA technique. Generally, recombinant hirudin has three variants: rHV1, rHV2 and rHV3. All of them have a high degree of homology (13 variable positions only), but in contrast to the natural molecule they lack a sulfate group at Tyr$^{63}$. They all have the similar effect of anticoagulation as nature hirudin (Dolt et al., 1985; Vindigni et al., 1994; Scharf et al., 1989). Most studies about hirudin are concerned with the pharmacological evaluations and choice the intravenous injection as the administration route. In clinic, frequently injecting of hirudin are needed for the purpose of therapy, therefore the patients experience great discomfort. To improve the quality of life of such patients, an alternative method of administration is desired. Oral administration is the most convenient route, and development of an oral formulation for hirudin would be of great value. rHV2 has been proved has the effect of anticoagulation when administered to the duodenal of rats (Wang et al., 1999). But there
is no information on the GI absorption of rHV2 administered by directly oral administration. The oral delivery of biologically active peptides and proteins is a very attractive but tough job. There are two main barriers to the GI absorption of peptide drugs: one is the poor membrane permeability that results from their macromolecular weight and hydrophilic characteristics, and another is the degradation of peptides by proteases in the GI lumen and mucosa (Lee VH, 1990). Scientists have made lots of attempts in this field for decades and investigated on the methods to enhance the absorption of peptides and proteins, as well as the mechanisms of their absorption. In the studies reported, most of model drugs are small peptides containing less than five amino acids (Shen and Xu, 2000; Berteloot et al., 1981), or endogenous peptides and proteins, such as insulin (Cheeseman and Johnston, 1982; Stoll et al., 2000; Due et al., 1985; Uchiyama et al., 1999; Hosny et al., 1997), insulin like growth factor I (Donovan et al., 1997; Nakagawa et al., 1997; Xu and Wang, 1996; Toshikiro et al., 1996) and epithelial growth factor (Shen and Xu, 1998; Adachi et al., 1993; Rao et al., 1990). Few of the studies were concerned with exogenous therapeutic peptides and proteins with large molecular weight besides calcitonin (Tozaki et al., 1998) and cyclosporin A (Drewe et al., 1992; Patrick et al., 2000). Though lots of approaches have been made to enhance the absorption of peptides and proteins, the absorption mechanisms of biologically active peptides and proteins in GI tract have not yet been firmly established. In the present paper, we chosen rHV2 as the model drug and rats as the model animals then studied the possibility of oral administration of rHV2 and the possible mechanisms of its GI absorption. The improvements of absorption in rats after oral administration of rHV2 by using several protease inhibitors were also investigated.
Materials and Methods

Materials. Recombinant hirudin (rHV2, rHV-Lys47, College of Life Science, Peking University, Beijing, China) was obtained by polymerase chain reaction-directed mutagenesis and expressed in *Escherichia coli.*, the specific activity is 10347 ATU/mg determined by Chromogenic assay. Bacitracin, casein, bovine serum albumin (BSA), sodium deoxycholate (SDCh), poly-L-lysine hydrobromide (molecular weight about 1000), Tris and colchicine were purchased from Sigma-Aldrich Chemical Co. (Shanghai, China). Chromozym TH was from Roche (Mannheim, German), and Carbopol 941 was a product of Noveon Chemical Company (Cleveland, Ohio, USA). hydroxypropyl-β-cyclodextrin (HP-β-CD) was supplied by Xi’an Deli Biological and Chemical Engineering Co. Ltd. (Xi’an, China). 2,4-dinitrophenol (DNP) was purchased from Beijing Chemical reagent factory (Beijing, China). All other chemicals were analytical grade and used as received.

Animals. Male Sprague-Dawley rats weighing 270-300g were provided by Vital Laboratory Animal Center, Beijing, China. All cares and handling of animals was performed with the approval of Institutional Authority for Laboratory Animal Care.

Preparation of luminal contents and mucosal subcellular fractions. The luminal contents of the GI tract were prepared according to the method of Asada (Asada et al., 1994). Rats were euthenized to death with an overdose intraperitoneal injection of urethane, and the GI tract was excised. The luminal contents of the stomach were collected by flushing with 15ml of artificial gastric juice (pH 1.2), and the contents of the jejunum (20 cm below the ligament of Treitz), proximal and distal part of ileum (40 cm above the ileocecal junction, each segment was 20 cm long) were collected by flushing...
with 15 ml of phosphate buffer solution (PBS, pH 7.4). Mucosal subcellular fractions of the small intestine were prepared according to the method of Bai (Bai and Chang, 1995), with slight modification. The intestinal mucosa of each segment was scraped off, suspended in 0.3 M sucrose buffer (pH 7.0) and then homogenized. The homogenate was centrifuged at 100,000 × g at 4°C for 1h to separate each fraction. The resulting supernatant (15ml) was used as the cytosol fraction, and the pellet was divided into two fractions. One was resuspended with 15 ml sucrose buffer (0.3 M, pH 7.0) as a brush border membrane (BBM) fraction, while the other with 15 ml acetate/NaOH buffer (1 M, pH 4.5) as a lysosomal fraction.

**Degradation Studies.** The degradation of ¹²⁵I-rHV2 in the luminal contents and mucosal subcellular fraction was examined according to the method of Bai (Bai and Chang, 1995). The incubation mixture consisted of 50mM Tris/HCl buffer (pH 7.5) for luminal content of small intestine, or artificial gastric juice (pH 1.2) for luminal content of stomach, or 1 M acetate/NaOH buffer (pH 4.5) for subcellular fraction, as well as 125mM NaCl, 1%BSA, 8µg/ml of ¹²⁵I-rHV2, some adjuvant (0.8mg/ml of bacitracin, 10mg/ml of casein, 20mg/ml of HP-β-CD or 2.4 mg/ml SDCh) and a luminal content or a subcellular fraction. The experiment of ¹²⁵I-rHV2 degradation was performed at 37°C, the samples were taken at 0, 1, 5, 10, 15 and 30 min and determined by trichloroacetic acid (TCA)-precipitable method.

**Oral administration experiments.** The rats were fasted for 16 hours prior to the experiments, with free access to water. Oral administration of ¹²⁵I-rHV2 alone or with bacitracin or casein was carried out by using a curved bulb-ended gavage tube. ¹²⁵I-rHV2 dissolved in saline solution was administered to rats at the dose of 1.0 mg/kg. When
co-administration with bacitracin or casein their doses were 4.0 and 10.0 mg/kg respectively. The animals were anesthetized with an intraperitoneal injection of urethane (1.5 g/kg). Blood samples (0.5 ml) were periodically taken from the orbit venous plexus at 0, 15, 30, 60, 120, 240 and 360 min using a heparinized capillary tubes and estimated by TCA-precipitable method. In another experiment, unlabeled rHV2 was administered to rats at doses of 5.0 mg/kg, while bacitracin and casein were 4.0 and 10.0 mg/kg respectively. The blood sample was anticoagulated with 3.8% trisodium citrate solution in a ratio of 8.25:1.75(V/V) and the rHV2 in the plasma was determined by chromogenic assay. For the calculation of bioavailability in the two sets of experiments, $^{125}$I-rHV2(0.5 mg/kg) and rHV2 (1.0 mg/kg) solution were given intravenously to rats by tail veins, respectively.

**In situ loop experiments.** The rats were anesthetized with an intraperitoneal injection of urethane (1.5 g/kg). Anesthesia was maintained with additional urethane as needed throughout the studies. The absorptions of $^{125}$I-rHV2 from the jejunum, proximal and distal part of ileum were examined by an *in situ* loop method (Bai and Chang, 1995). Having been washed out the luminal contents, the anesthetized rats were administered the drug solution at the same doses as those used in the oral administration study. Blood samples (0.5 ml) were periodically taken from the orbital venous plexus at 0, 15, 30, 60, 120, 240 and 360 min, and determined by TCA-precipitable method.

**In vitro everted sac experiments.** The rats were euthanized to death with an overdose intraperitoneal injection of urethane. The segment of jejunum was excised after washing the luminal side with 20 ml of PBS (pH 7.4) (Toshikiro et al., 1996). The isolated jejunum was everted and cut into 5-cm segments. Half a milliliter of the PBS was
introduced into the serosal side, and both ends of the segment were ligated. The resulting
everted sac was placed in the 10 ml PBS (pH 7.4) incubation media, which contained 100
µg/ml of rHV2 and 10 mg/ml casein with or without adjuvant (1mM poly-L-lysine, 1
mM Carbopol 941, 1mM DNP or 0.1 mM colchicine), and was bubbled with 95% O₂: 5%
CO₂ throughout the experiment. The incubation was performed for 30 min at 37°C, and
then the everted sac was twice immersed in 20 ml of the fresh PBS (pH 7.4, 4°C) for 5
min to remove the nonspecifically adsorbed rHV2 on the mucosal surface. To remove the
rHV2 bound on the membrane, the everted sac was immersed in the ice-cold basic
washing solution (0.05 M NaOH/0.5 M NaCl, 10ml) for 10 min. After recovery of the
serosal solution, the tissue was homogenized and mixed with 4% acetic acid. The mixture
was centrifuged at 2000 × g for 10 min. The rHV2 in the resulting supernatant and in the
serosal solution were determined by chromogenic assay.

Assay procedures. The ¹²⁵I-rHV2 in the blood or GI-luminal contents was determined
by TCA precipitation method (Toshikiro et al., 1996). For GI-luminal contents, 200 µl of
15% TCA was added to 200 µl of the incubation mixture, as for plasma, 1 ml of 15%
TCA was added to the mixed solution after 1ml of Krebs-Henseleit bicarbonate buffer
(119mM NaCl, 4.7mM KCl, 2.5mM CaCl₂, 1.2mM MgSO₄, 1.2mM KH₂PO₄, 25mM
NaHCO₃ ) containing 5% BSA was added to 50µl of each plasma sample. Afterwards,
the mixtures were centrifuged at 2000 × g for 15 min, then the radioactivities in the
precipitate were counted by a γ -counter (SNB-69513, Shanghai, China).

Chromogenic assay was performed according to Groetsch (Groetsch et al., 1991)
with a little modification. The blood samples were denaturated by heating for 15 min at
65°C, then 0.05 ml HCl (1 M) was put into 0.5 ml blood sample. The samples were
neutralized after cooling by addition of 0.05 ml NaOH (1 M) and then centrifuged at 3000 ×g for 10 min. 100 µl of supernatant was added to 200 µl Tris buffer (50 mM, containing 154 mM NaCl and 0.12U thrombin), and incubated at 37 °C for 5 min. Afterwards, 16 µl chromozym TH (1.9mM) was added into the incubation mixture and kept on incubating for another 10 min at 37 °C. The reaction is terminated by addition of 200µl acetic acid (33%) and then the absorbance of the sample was read at 405 nm with a spectrophotometer (TU-1901, Puxi versatile instrument Co. China).

**Data analysis.**

The plasma concentration of rHV2 was calculated by dividing the total volume of plasma isolated. The plasma concentration-time data were analyzed non-compartmentally on the basis of the statistical moment theory. The $AUC_{0\rightarrow360}$ was estimated by the trapezoidal method and the bioavailability was calculated by comparing the $AUC_{0\rightarrow360}$ obtained after intra-intestinal or oral administration to that obtained after intravenous injection. Statistical evaluations were performed using the Student $t$-test. Differences with a $p$-value less than 0.05 were considered significant.
Results

Degradation of rHV2 in the GI contents and mucosa. The degradation of rHV2 in the GI contents is given in figure 1. As we can see, $^{125}$I-rHV2 rapidly degraded to the TCA-soluble form in the gastric contents and small-intestinal contents. The residual percentages of $^{125}$I-rHV2 in the GI contents are summarized in table 1. The maximal degrading activity is in the stomach, only 27.04% $^{125}$I-rHV2 remained after 30 min of degradation. The degrading activity in distal ileum is almost the same as in stomach, while those in jejunum and proximal ileum are much lower. Some adjuvant could inhibit the degradation, especially bacitracin and casein, with the residual percentage of hirudin of 62% and 58%, respectively.

Table 1 also shows the degrading activity in the intestinal mucosa. Degrading activity in sub-fraction of the intestinal mucosa is nearly the same as in GI contents, except that the activity in cytosal fraction is a little lower than other parts of sub-fraction. There was no site difference through the entire small intestine.

Oral administration experiments. The plasma concentration-time curves of TCA-perceptible radioactivity after oral administration of $^{125}$I-rHV2 (1.0 mg/kg) with or without bacitracin (4.0 mg/kg) and casein (10.0 mg/kg) are demonstrated in figure 2. The bioavailability of rHV2 without adjuvant was 21.23±3.73%. When co-administered with bacitracin or casein the bioavailability was increased to 37.41±3.55% ($p < 0.05$) and 29.23±3.08% ($p > 0.05$), respectively.

To further confirm the above absorption results obtained from the $^{125}$I-rHV2 oral administrations, chromogenic assay of rHV2 in the plasma was also performed after oral administration of unlabelled rHV2. The plasma concentration-time curves such obtained
are shown in figure 3. The bioavailability values of rHV2 administered alone, with bacitracin or casein were 6.99±0.32%, 12.70±1.00% (p < 0.01) and 8.36±1.02% (p > 0.05), respectively.

**In situ loop experiment.** The site difference of rHV2 absorption in the intestine was also examined by an *in situ* loop method. There was no significant variance of absorption in different segments of small intestine. The bioavailabilities of rHV2 in variant parts of intestine are expressed in figure 4, and the values ranged from 6.33% to 8.98%. The rank order of the absorption was proximal ileum ≥ distal ileum > jejunum with no statistical differences, whether with or without adjuvant (p > 0.05).

**In vitro everted sac experiment.** To clarify the transport mechanism of rHV2 across the GI tract, we examined the effect of variant inhibitors on the uptake and transport of rHV2 across the jejunal mucosa. The results are summarized in table 2. The transport of rHV2 to the serosal side was significantly inhibited by low temperature, DNP (an uncoupler of oxidative phosphorylation), Carbopol 941 (a polyanion) and colchicines (an inhibitor of microtubular assembly), but not by poly-L-lysine (a polycation). The amount of rHV2 found in the intestinal tissue was also decreased, and the total amount of rHV2 taken in and transported across the GI tract tended to be inhibited by these treatments except for the addition of poly-L-lysine.
Discussion

Chang demonstrated that hirudin is a very stable compound, it may be irreversibly denatured unless under elevated temperatures in alkaline solution (Chang JY, 1991). Wang also proved that rHV2 remained its most of anticoagulation activity after incubated with trypsin, pepsin or chymotrypsin (Wang et al., 1995), but it can be degraded by pepsin. In above-mentioned studies only one kind of protease acts with hirudin in each experiment, however the effects of various digestive enzymes in GI tract should be more complex, especially in small intestine. Therefore, the luminal contents and mucosal subcellular fractions were used in our investigation in order to simulate the complicated surroundings in GI tract.

It was indicated from the degradation experiments that rHV2 was degraded rapidly by the GI tract luminal contents and mucosal subcellular fraction. The rHV2 concentration decreased below 50% of the initials within 30 min. The significant inhibitory effects of bacitracin and casein on rHV2 degradation have also been noticed. Xian reported that casein can protect IGF-I from degradation in the stomach or duodenal flushing, but the mechanisms of this protection are not clear (Xian et al., 1995). The inhibitory effect of HP-β-CD and SDCh are relatively weak compared with bacitracin and casein, though some studies suggested that DM-βCD and SDCh have the ability of inhibiting the enzyme activities (Lars and Helle, 1995; Kakemi et al., 1970). Anyhow it seems possible to use some protease inhibitors to protect rHV2 from the degradation by digestive enzymes in GI tract.

The bioavailability of oral administration of $^{125}$I-rHV2 in rats is 21.23±3.73% estimated by TCA-precipitable method, which is not consistent with the value of
6.99±0.32% determined by chromogenic assay. The variance was obviously due to the different methods used and each of the two methods has advantages and drawbacks. TCA-precipitable method is more sensitive and could prove the transport of the intact rHV2 molecules into the body circulation, while the chromogenic assay could demonstrate at least the absorption of biologically active segments of rHV2 molecules into the blood of the animals. When co-administration of rHV2 with bacitracin or casein, the bioavailability was increased markedly, no matter which of the two analytical methods was applied.

As we can see from the results of in situ loop, the absorption of rHV2 is more slowly than oral administration. That may be related to the way of administration and the state of animals. In the perfusion process, the drug solution was pumped into intestinal tract slowly, while in the study of oral administration the drug solution was administered very rapidly. Another reason might be that the animals used in the in situ loop experiment were under anesthesia and their systemic circulation is much more slow than that of the normal states (Holzer et al., 2003). Therefore, the absorption of drug in the in situ loop experiment was also slow. On the other hand, no significant differences in the rHV2 absorption among different parts of small intestine were found, suggesting that rHV2 has no preference on the absorption sites. Co-administration with bacitracin or casein could attain a small increment in bioavailability of rHV2, possibly due to the fact that intestinal tract was washed well and cleared of most digestive enzymes before perfusion studies.

Furthermore, as demonstrated in the experiments using the everted sac of jejunum, the uptake of rHV2 was inhibited by polyanion (Carbopol 941) and low temperature but not by polycation (poly-L-lysine). The significant decrease of rHV2 transport under 4°C
suggested that the transport was energy depended, so the transport routine may be related to transcellular way. The polylysine was reported to bind anionic sites of gall bladder epithelial cell membranes, thereby producing morphological changes such as collapse of the microvillar structure, membrane folding from the apical border into the terminal web, or ‘fused’ membranes with pentalaminar substructure (Quinton and Phillpot, 1973). These changes limited the absorption by transcellular way. Madara reported that polylysine could interact with anionic components of the glycoproteins on the surface of the epithelial cells (Madara JL, 1989). Moreover, the interiors of the tight junction (pores) are highly hydrated and negative charged. An alteration in the relative concentration of specific ion species in the pore would result in substantial changes in tight junction resistance, which might lead to loosening or opening of the pore and enhancing the absorption by paracellular routine.

Presuming rHV2 was absorbed by endocytosis, the transcellular way would be blocked whereas the paracellular routine opened after polylysine was added. These two adverse effects on the epithelial cells would result in no increase of the rHV2 transport. Polysine and rHV2 are being oppositely charged and might interact with each other, so the amount of transport of rHV2 in the mucosal tissue has a little decrease. The results of our experiments are consistent with above presumption, so it seems logical to deduce that rHV2 was absorbed mainly by endocytosis.

The transport of rHV2 was inhibited by DNP and cholchicine. DNP is an uncoupler of oxidative phosphorylation, while colchicine is an inhibitor of microtubular assembly. Both of them can damage the functions of epithelial cells and inhibit the transport of rHV2 through transcellular way.
It was reported that carbopol showed a clear effect on opening of intercellular junctions, thereby enhancing the paracellular permeability for hydrophilic macromolecules (Luesen et al., 1996). Carbopol also displays strong mucoadhesive properties (Bai et al., 1995; Mortazavi SA, 1995) and may therefore be able to localize its enzyme inhibiting and absorption enhancing activities to a confined area in the intestinal tract. However, it was showed in this study that carbopol inhibited the rHV2 transport. The possible reason is that a stagnant layer formed by a highly viscous carbopol solution and repellence between both negatively charged carbopol and rHV2 may prevent rHV2 molecules from getting close to epithelial cell membrane, thus leading to the decrease of transport of rHV2 cross the membrane.

Toshikaro demonstrated that rhIGF-I could be absorbed into systemic circulation (Toshikiro et al., 1996). The molecular weight of rhIGF-I is 7000 Da, which is almost equal to that of rHV2. The transport mechanism of rhIGF-I was inferred to be absorptive-mediated endocytosis. It was supposed from above results that rHV2 has the similar absorption mechanism as rhIGF-I, i.e. endocytosis might be the main route by which rHV2 molecules were absorbed into the circulation.

In conclusion, the maximum degradation was found in the stomach and distal ileum, slightly high than that in proximal ileum, jejunum and mucosal sub-fraction. Some enzyme inhibitor like bacitracin or casein could inhibit the degradation to certain degree. The bioavilability after oral administration of rHV2 to rats varies obviously, dependent on the analytical method, and some of the enzyme inhibitor could enhance the rHV2 oral absorption. There is no site difference on rHV2 absorption in different segments of small intestine. The possible transport mechanism of rHV2 across the GI tract is concerned
with the endocytosis process.
References


normal and papain-treated brush border membrane vesicles from mouse intestine. Biochim Biophys Acta 649: 179-188.


in the mouse in the absence of salivary insulin-like growth factor binding protein.


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Legends for Figures

**Figure. 1.** Degradation of $^{125}$I-rHV2 alone or with adjuvant in luminal contents of rat jejunum (A), proximal ileum (B) or distal ileum (C). $^{125}$I-rHV2($\blacklozenge$), +bacitracin($■$), +casein($▲$), +HP-β-CD($\times$), +SDCh($+$). Data represent mean; $n = 3$.

**Figure. 2.** Plasma concentration profiles following oral administration of $^{125}$I-rHV2 alone or with adjuvant in rats, determined by TCA-precipitable method. $^{125}$I-rHV2($\blacklozenge$), +bacitracin($■$), +casein($▲$). Data represent mean ± S.E.; $n = 5$.

**Figure. 3.** Plasma concentration profiles following oral administration of rHV2 alone or with adjuvant in rats, determined by chromogenic assay. $^{125}$I-rHV2($\blacklozenge$), +bacitracin($■$), +casein($▲$). Data represent mean ± S.E.; $n = 5$.

**Figure. 4.** Bioavailability of $^{125}$I-rHV2 after coadministration with bacitracin or casein into different sites of rat small intestine in the *in situ* loop experiment. a: $p > 0.05$ vs jejunum without adjuvant; b: $p > 0.05$ vs the same part of intestine without adjuvant. ± S.E.; $n = 5$. 

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Table 1. Degradation of $^{125}$I-rHV2 in the contents and mucosal subfractions of rat GI tract and its inhibition by some adjuvant

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Adjuvant</th>
<th>Stomach</th>
<th>Jejunum</th>
<th>Proximal ileum</th>
<th>Distal ileum</th>
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<tr>
<td>Luminal contents</td>
<td>None</td>
<td>27.04±1.28</td>
<td>36.94±5.56</td>
<td>41.80±2.21</td>
<td>30.29±2.80</td>
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<td></td>
<td>Bacitracin</td>
<td>47.17±0.59**</td>
<td>55.59±3.73*</td>
<td>62.34±5.10**</td>
<td>52.16±8.22*</td>
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<td>Casein</td>
<td>39.29±1.89*</td>
<td>50.75±4.91</td>
<td>58.54±9.92</td>
<td>42.01±2.35*</td>
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<td>HP-β-CD</td>
<td>63.62±4.49*</td>
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<td>SDCh</td>
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<td>47.75±4.87**</td>
<td>41.84±8.44</td>
<td>40.50±4.57*</td>
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<td>Bacitracin</td>
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<td>65.58±3.96**</td>
<td>59.34±2.11*</td>
<td>46.82±3.20*</td>
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<td>48.07±7.00</td>
<td>60.05±4.17**</td>
<td>44.32±4.07*</td>
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<tr>
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<td>41.70±4.08**</td>
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<td>-</td>
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<td>31.70±1.50</td>
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<tr>
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<td>Bacitracin</td>
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<td>61.39±4.75</td>
<td>51.74±4.24*</td>
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<td>51.29±0.02*</td>
<td>53.88±4.38*</td>
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<td></td>
<td>Casein</td>
<td>-</td>
<td>47.29±7.67</td>
<td>62.62±2.83*</td>
<td>60.42±1.14</td>
</tr>
<tr>
<td></td>
<td>HP-β-CD</td>
<td>-</td>
<td>48.19±3.71</td>
<td>53.45±8.73</td>
<td>56.87±3.32</td>
</tr>
<tr>
<td></td>
<td>SDCh</td>
<td>-</td>
<td>45.48±1.88</td>
<td>53.72±1.81</td>
<td>64.04±13.55</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01 vs none. Data represent mean ± S.D.; n = 3.
Table 2. Effect of several inhibitors on the transmucosal passage of rHV2 in jejenum *in vitro*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (mM)</th>
<th>Amount Transported in 30 Min (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Serosal solution</td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>141.4±16.1</td>
</tr>
<tr>
<td>None (4°C)</td>
<td>-</td>
<td>71.8±4.1 ***</td>
</tr>
<tr>
<td>DNP</td>
<td>1.0</td>
<td>72.4±4.7 **</td>
</tr>
<tr>
<td>Colchicine</td>
<td>0.1</td>
<td>17.4±3.0 ***</td>
</tr>
<tr>
<td>Carbopol 941</td>
<td>1.0</td>
<td>45.0±4.1 ***</td>
</tr>
<tr>
<td>Poly-L-Lysine</td>
<td>1.0</td>
<td>156.8±9.7</td>
</tr>
</tbody>
</table>

* p < 0.05, ** p < 0.01, *** p < 0.001 vs none. Data represent mean ± S.D.; n = 5.
Figure 1

Residual $^{125}$I-rHV2 (%) vs. Time (min)
Figure 1
Figure 1
Figure 2

Plasma concentration of $^{125}$I-rHV2 (µg/ml) over time (min).

**Legend:**
- Square: Group A
- Triangle: Group B
- Diamond: Group C

**Time (min):** 0, 100, 200, 300, 400

**Plasma concentration:** 0.0, 0.2, 0.4, 0.6, 0.8
Figure 3

Plasma concentration of rHV2 (µg/ml)

Time (min)
Figure 4

Different part of intestine

Jejunum
Proximal ileum
Distal ileum

125I-rHV2  Barcitracin  Casein

F(%)