

HM15912, a Novel Long-Acting Glucagon-Like Peptide-2 Analog, Improves Intestinal Growth and Absorption Capacity in a Male Rat Model of Short Bowel Syndrome

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ABSTRACT

Extensive bowel resection caused by various diseases that affect the intestines, such as Crohn's disease, volvulus, and cancer, leads to short bowel syndrome (SBS). Teduglutide is the only approved glucagon-like peptide-2 (GLP-2) drug for SBS; however, it requires daily administration. A novel GLP-2 analog with a prolonged duration of action to reduce dosing frequency and promote a greater efficacy may provide patients with a better quality of life. In the present study, the sustained exposure of HM15912 was characterized in normal male rats. The efficacy of HM15912 on intestinal growth and absorption capacity was also evaluated in normal male mice, rats, and SBS rats. HM15912 exhibited a remarkably extended half-life (42.3 hours) compared with teduglutide (0.6 hours) in rats. Despite somewhat lower in vitro potency on GLP-2 receptor than human GLP-2 or teduglutide, this longer-lasting mode of action promotes HM15912 to be more effective in terms of small intestinal growth than existing GLP-2 analogs even with a less frequent dosing interval of as little as once a week in rodents, including SBS rats. Furthermore, the small

intestinal weight was approximately doubled, and the D-xylose absorption was significantly increased after pre-treatment of existing GLP-2 analogs on the market or under clinical development followed by HM15912 in rodents. These results indicate that HM15912 possesses a significant small bowel trophic effect driven by continuously increased exposure, supporting that HM15912 may be a novel treatment option with greater efficacy and the longest dosing interval among existing GLP-2 analogs for SBS with intestinal failure.

SIGNIFICANCE STATEMENT

HM15912, a novel long-acting glucagon-like peptide-2 (GLP-2) analog, has a significant small bowel hypertrophic effect in rodents with a reduced frequency of administration compared to the existing GLP-2 analogs on the market or currently under clinical development. This study supports the possibility that HM15912 could be administered much less frequently than other long-acting GLP-2 analogs for patients with short bowel syndrome.

Introduction

The clinical hallmarks of short bowel syndrome (SBS) include a reduced intestinal length and insufficient absorptive surface area, leading to malabsorption of essential macronutrients and micronutrients from a normal diet (Buchman, 2016). SBS is clinically defined as having less than 200 cm of remaining small bowel (Pironi L et al., 2016) in adults, and intestinal failure is defined as the reduction of gut function

below the minimum necessary for the absorption of nutrients, water, and electrolytes such that intravenous supplementation is required to maintain health and/or growth.

SBS can be congenital or acquired (Buchman, 2016). In most cases, SBS occurs as a result of surgical intervention for other diseases; however, in a few cases, the small intestine is abnormally shortened at birth. Acquired SBS results from either surgical resections or disease-associated loss of intestinal absorption (Spencer et al., 2005). In case of adults acquiring SBS, postoperative intestinal resection necessary to treat traumatic injuries, mesenteric ischemia, volvulus, tumors, or Crohn's disease has emerged as the most common cause (O'Keefe et al., 2006; Messing et al., 1999), whereas for children with SBS, necrotizing enterocolitis, followed by gastroschisis and midgut volvulus (Wilmore and Robinson, 2000) is the predominant reason.

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ABBREVIATIONS: AUC_{last}, area under the curve; C_{max}, maximum observed concentration; Fc, fragment crystallizable region; FDA, Food and Drug Administration; GLP-2, glucagon-like peptide-2; hGLP-2, human glucagon-like peptide-2; LA-GLP2A1, GLP-2 analog with identical sequence to glepaglutide; LA-GLP2A2, GLP-2 analog with identical sequence to apraglutide; MEM alpha, minimum essential medium alpha; PEG, polyethylene glycol; SBS, short bowel syndrome; SD, Sprague-Dawley; T_{max}, time of maximum observed concentration; t_{1/2}, half-life.

Although various treatment options are available for treating SBS, such as dietary, pharmacologic, surgical, and transplant interventions (U.S. NIDDK, 2015), the management of SBS focuses on ensuring adequate nutrition absorption as well as balance of fluid and electrolyte to alleviate the risk of malabsorption.

Although the Food and Drug Administration (FDA) approved a recombinant human growth hormone for the treatment of SBS in 2003, administration is limited to only short-term usage (U.S. FDA, 2003). With such medical limitation, glucagon-like peptide-2 (GLP-2) was suggested as a novel treatment option over a decade ago. GLP-2 is an intestinal hormone produced in endocrine L-cells of the distal jejunum, ileum, and colon that affects multiple facets of intestinal physiology, including growth, barrier function, digestion, absorption, motility, and blood flow (Drucker et al., 1996; Rowland and Brubaker, 2008; Rowland and Brubaker, 2011). It acts through the GLP-2 receptor (GLP-2R) and is predominantly expressed in intestinal subepithelial myofibroblasts. Stimulation of GLP-2R results in the production and release of insulin-like growth factor-1 in intestinal subepithelial myofibroblasts via phosphatidylinositol-3-kinase/Akt-dependent signaling in the small intestine (Leen et al., 2011). In turn, the secreted insulin-like growth factor-1 stimulates the proliferation of crypt cells and contributes to the growth of the intestine. The therapeutic target and mechanism of action via the intestinotrophic activity of GLP-2 agonism have been demonstrated and supported by the FDA New Drug Application approval of the GLP-2 peptide analog teduglutide in 2012 (U.S. FDA, 2014).

Teduglutide is a single drug approved in the United States for long-term usage in the management of SBS. According to medical consensus, this drug may be considered after a period of stabilization following surgery, during which intravenous fluids and nutritional support should have been optimized. However, the widespread use of teduglutide remains limited due to high cost and daily subcutaneous administration requirement caused by its short half-life (1.3–2.2 hours) (U.S. FDA, 2012).

HM15912 is a conjugate of the GLP-2 analog, which was designed to have more potent intrinsic activity than native GLP-2, with a human immunoglobulin G4 fragment crystallizable region (hIgG4 Fc) joined by a flexible polyethylene glycol linker. We hypothesized that this unique structure would enable HM15912 to have a substantially extended elimination half-life, thus ensuring that the body is continuously exposed to this potent GLP-2 receptor agonist. In this study, we characterized the *in vitro* potency on human GLP-2 receptor and *in vivo* long-acting properties of HM15912, the ability to induce intestinal growth as well as the increase in the absorption capacity of the intestines in an SBS animal model.

Materials and Methods

Peptide Synthesis and Compound Manufacturing. HM15912 was manufactured at Hanmi Bio Plant Complex (Pyeongtaek-si, Gyeonggi-do, South Korea) in the following method. The GLP-2 analog GT15912 was conjugated with the 3.4 kDa size of the aldehyde bi-functional polyethylene glycol (PEG) linker. Thereafter, the mono PEGylated GT15912 was purified and conjugated with an aglycosylated hIgG4 Fc. The reductive amination chemistry between amine and aldehyde groups was used for all of the conjugation reaction steps. HM15912 conjugate, which was generated through a two-step

conjugation reaction and composed of GT15912, Linker, and Fc, was purified and prepared with a high purity of > 95%. Teduglutide (Gattex, Revestive) was purchased from Merlonipharm (Chiasso, Switzerland). The sequences of the long-acting GLP-2 analog 1 (LA-GLP2A1) and long-acting GLP-2 analog 2 (LA-GLP2A2) used in these studies were identical to those of glepaglutide (Zealand Pharma A/S) (Larsen et al., 2006) and apraglutide (VectivBio AG) (Hargrove et al., 2020), respectively, and were prepared as acetate salts by the Chinese Peptide Company Ltd. (Hangzhou, China). The identities of these peptides were verified by mass spectral analysis. The purity of the peptides was >95%, as confirmed by high-performance liquid chromatography analysis. Table 1 shows the amino acid sequences.

Animals and Housing. Mice: Adult male C57BL/6 mice weighing approximately 20–25 g were obtained from Orient Bio, Inc. (Seongnam-si, Gyeonggi-do, South Korea). The mice were housed in ventilated polysulfone cages at ambient temperature (22±2°C) and maintained on a 12-hour light/dark cycle. Pellet chow (Picolab rodent diet 5053, Lab-Diet, USA) and water were provided *ad libitum*. The mice were acclimatized to the facility for at least six days prior to the initiation of the experiments and assigned to two separate efficacy studies.

Rats: Adult male Sprague-Dawley (SD) rats weighing approximately 249–345 g were obtained from Orient Bio, Inc. (Seongnam-si, Gyeonggi-do, South Korea). The rats were housed in ventilated polysulfone cages at ambient temperature (22±2°C) and maintained on a 12-hour light/dark cycle. Pellet chow (Picolab rodent diet 5053, Lab-Diet, USA) and water were provided *ad libitum*. The rats were acclimatized to the facility for at least seven days prior to the initiation of the experiments and assigned to one pharmacokinetic and two efficacy studies, including a surgical resection model for SBS rats. All animal studies were conducted at Hanmi Research Center (Hwaseong-si, Gyeonggi-do, South Korea), and the protocols were approved by the Animal Care and Use Committee of the Hanmi Research Center.

Cell Culture. Human GLP-2 receptor overexpressed Chinese hamster ovary cells (DiscoverX, 95-0112C2) were maintained in minimum essential medium alpha (MEM) alpha containing 5% (v/v) heat inactivated and dialyzed fetal bovine serum, 1% (v/v) penicillin-streptomycin, 1 mg/ml of G418, and 10 nM of methotrexate at 37°C under 5% CO₂ in humidified atmosphere. Cells were passaged 1:5 to 1:10 by trypsinization every 3–4 days.

cAMP Assay. Human GLP-2 receptor overexpressed Chinese hamster ovary cells grown in flasks were dispersed with 0.05% (v/v) trypsin-EDTA solution and harvested in MEM alpha containing 5% (v/v) heat inactivated and dialyzed fetal bovine serum, 1% (v/v) penicillin-streptomycin, 1 mg/ml of G418, and 10 nM of methotrexate. And, the cells were plated on to 384-well white clear bottom plates at 6×10³ cells per well and incubated overnight at 37°C under 5% CO₂ in humidified atmosphere.

Intra-cellular cAMP accumulation level was measured by LANCE cAMP detection kit (PerkinElmer, USA). Each compound was typically tested in serial dilutions as concentration ranges of 0.003 to 166.67 nM for human glucagon-like peptide-2 (hGLP-2), teduglutide, and GT15912, and 0.006 to 333.33 nM for HM15912. The assay was initiated by addition of the pre-diluted compounds (5 µl per well), and 5 µl per well of stimulation buffer containing the Alexa fluor 647-anti cAMP antibody was added into the cell-plated 384-well plates. After 30 minutes of incubation at room temperature, 10 µl per well of pre-mixed detection mix was added to the 384-well plates. After 180 minutes of incubation at room temperature, fluorescence intensity was measured by multi-mode microplate reader (EnVision, PerkinElmer, USA). Each assay compound was typically tested in duplicate and in three independent assays. Relative activity compared with human GLP-2 was calculated by the following formula.

Relative activity (%): EC_{50} of hGLP-2/ EC_{50} of test compounds *100

Surgical Procedures. SD rats were fasted overnight before surgery and operated on to achieve 80% resection of the small bowel. In the sham operation group, transection and re-anastomosis of the jejunum were conducted 10 cm distal from the ligament of Treitz toward the ileocecal junction. In the resection groups, transection was conducted at the small

TABLE 1
Amino acid sequence of hGLP-2 and GLP-2 analogs

Peptide	Amino acid sequence																																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34		
hGLP-2	H	A	D	G	S	F	S	D	E	M	N	T	I	L	D	N	L	A	A	R	D	F	I	N	N	W	L	I	Q	T	K	I	T	D	OH	
Teduglutide	H	G	D	G	S	F	S	D	E	M	N	T	I	L	D	N	L	A	A	R	D	F	I	N	N	W	L	I	Q	T	K	I	T	D	OH	
LA-GLP2A1	H	G	E	G	T	F	S	S	E	L	A	T	I	L	D	A	L	A	A	R	D	F	I	N	N	W	L	I	A	T	K	I	T	D	KKKKKK-NH ₂	
LA-GLP2A2	H	G	D	G	S	F	S	D	E	Nle	f*	T	I	L	D	L	L	A	A	R	D	F	I	N	N	W	L	I	Q	T	K	I	T	D	NH ₂	
GT15912	C ₆ H	G	D	G	S	F	S	D	E	M	N	T	I	L	D	N	L	A	A	R	D	F	I	N	N	W	L	I	Q	T	R	I	T	D	K	OH

C₆H, [1H-imidazol-4-yl)-acetyl 1]; f*, D-phenylalanine; LA-GLP2A1, same amino acid sequence to gilepaglutide; LA-GLP2A2, same amino acid sequence to apraglutide.

bowel starting at 10 cm distal from the ligament of Treitz and ending at 10 cm proximal to the ileocecal valve. These procedures were followed by an end-to-end jejunoileal anastomosis between the resection sites. The non-operated group was given pellet chow (Picolab rodent diet 5053, Lab-Diet, USA) ad libitum. The sham operation and resection groups were given a rodent liquid diet (F1259SP, BioServ, USA) two days before and three days after surgery. The rats were fed chow and tap water ad libitum thereafter.

Pharmacokinetics of HM15912 in Rats. SD rats were administered a single subcutaneous injection of teduglutide at 2,500 µg/kg (equal to 670 nmol/kg) or HM15912 at 705 µg/kg (equal to 13 nmol/kg). The exposure to both teduglutide and HM15912 was dose-normalized to fairly compare. Blood (0.3 ml) was then collected from the jugular vein at 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, and 8 hours for teduglutide and 1, 4, 8, 24, 48, 72, 96, 230, 244, 268, 192, 216, 240, 264, 288, 312, and 336 hours for HM15912. Serum was obtained by centrifugation at 10,000 rpm for 10 minutes. The serum concentrations of teduglutide or HM15912 were determined using a GLP-2 ELISA kit (ALPCO, Salem, NH, USA) or an in-house developed and qualified ELISA at the Hanmi Research Center (Hwaseong-si, Gyeonggi-do, South Korea), respectively. The pharmacokinetic parameters, maximum observed concentration (C_{max}), time of maximum observed concentration (T_{max}), half-life (t_{1/2}), and area under the curve (AUC_{last}), were measured with the non-compartmental analysis tool of Phoenix WinNonlin 8.0 software (Certara, Princeton, NJ, USA).

Assessment of Intestinal Growth in Rodents. C57BL/6 mice and SD rats were sacrificed after anesthesia by ethyl ether at the end of each study. The small intestine (from the pyloric sphincter to the ileocecal junction) and large intestine (from the distal end of the cecum to the anal verge) were dissected from the animals. The intestine was opened longitudinally, cleared of its contents with Dulbecco's phosphate-buffered saline, and weighed. The weight of the small intestine is presented as a percentage increase over the mean of the vehicle group.

For SBS model rats, the animals were sacrificed after anesthesia by ethyl ether at the end of the study. Then, the length of the small intestine (from the pyloric sphincter to the ileocecal junction) was measured. The first 5-cm segments of the intestine from the site of re-anastomosis in both the jejunal and ileal directions were discarded (Nygaard, 1967), and the next 5-cm segments in both the jejunal and ileal directions were cut and weighed. The weights of the jejunum and ileum were normalized by length.

D-xylose Absorption Test. Before sacrifice, the mice were fasted for 4 hours, after which 0.2 ml of D-xylose solution (500 mg/ml) was administered by oral gavage. Blood samples (300 µl) were obtained from the orbital plexus 0.5 hours after the administration of D-xylose solution and centrifuged at 12,000 rpm for 10 minutes to collect serum. The rats were fasted overnight, after which 2 ml of D-xylose solution (500 mg/ml) was administered by oral gavage. Blood samples (300 µl) were obtained from the jugular vein 1 hour after administration of D-xylose solution and centrifuged at up to 15,000 rpm for 15 minutes to collect serum. For the SBS model rats, 2 ml of D-xylose solution (50 mg/ml) was administered by oral gavage after 4 hours of fasting. Blood samples (300 µl) were obtained from the jugular vein 0.5–4 hours after administration of D-xylose solution and centrifuged at up to 15,000 rpm for a maximum of 15 minutes to collect serum. The serum concentration of D-xylose was determined using a commercially available kit (Chondrex, Inc., Woodinville, WA, USA) and then calculated using SoftMax Pro software (version 7.0; Molecular Devices, San Jose, CA, USA).

Histologic Analysis. The dissected jejunum and ileum from each SBS model rat were fixed in 10% neutral formalin. The fixed samples were dehydrated and embedded in paraffin and then cut perpendicularly to the axis of their length. Serial sections were stained with hematoxylin and eosin (H&E). The stained slides were scanned and observed under a microscope, and the villus height, crypt depth, and mucosal area were evaluated using Olympus cellSens 1.3 software (Olympus Corporation, Tokyo, Japan). The heights of 10 villi were

measured (from the tip of the villi to the villus-crypt junction) and then averaged to derive the mean value for each animal. The depth of 10 crypts between the basement membrane was measured and then averaged to obtain the mean value for each animal. The mucosal area was calculated by subtracting the luminal area from the total intestinal area of the transverse section (excluding the muscular layer).

Statistical Analyses. Statistical analyses were performed with GraphPad Prism version 6 (GraphPad Software, Inc., San Diego, CA, USA). Significant differences among multiple groups were identified by one-way ANOVA with Dunnett's post hoc test. A p -value < 0.05 was considered statistically significant.

Results

HM15912 Possesses Unique Structure to have a Prolonged Duration of Action. To develop a highly potent GLP-2 analog with long-lasting effects, we developed a GLP-2 analog, GT15912, after minimal sequence modification from the human GLP-2, and conjugated a 3.4 kDa size of PEG linker between GT15912 and the hIgG4 Fc (Fig. 1, A and B). The conjugation of GT15912 and hIgG4 Fc was carried out through the formation of amine bonds between the bi-functional PEG and Lys34 in GT15912 or the N-terminal amino acid in hIgG4 Fc. The molecular weight of HM15912 is 57 kDa for the whole molecule and 53.7 kDa for the protein content.

Fig. 1C and Table 2 summarized the pharmacokinetics of HM15912 compared with teduglutide in male rats after a single subcutaneous injection. Endogenous GLP-2 has a very short elimination half-life of approximately 7 min in human (Hartmann et al., 2000). Although teduglutide acquired extended half-life by possessing dipeptidyl peptidase IV resistance, 1.3–2.2 hours in human or 0.64–1.3 hours in SD rats (EMA, 2012) is still insufficient to cover a day. In contrast, HM15912 exhibited 70-fold extended elimination half-life (42.4 hours) compared with teduglutide in SD rats (0.6 hours). Furthermore, based on the evidence of dose-proportionality for both teduglutide (EMA, 2012) and HM15912, exposure was dose-normalized to fairly compare the two. The dose-normalized exposure of HM15912 was 283.3 kg·h/L, which was approximately 283.3-fold higher than that of teduglutide (1.0 kg·h/L) after a single subcutaneous injection.

HM15912 is Potently Stimulating GLP-2 Receptor Even After Conjugation of IgG4 Fc Through Highly Potent GLP-2 Peptide Analog and Structural Feature. The GLP-2 analog, GT15912, of HM15912 is responsible for inducing crypt cell proliferation for treatment of SBS. GLP-2 acts through the GLP-2 receptor (GLP-2R), a member of the G protein-coupled receptor family, predominantly expressed in gastro-intestinal tract. Upon GLP-2R stimulation, GLP-2 activates adenylate cyclase to generate intra-cellular cAMP. To investigate the biologic activity of GLP-2 analogs, intracellular cAMP accumulation was examined using human GLP-2 receptor overexpressed Chinese hamster ovary cells in which the endogenous rodent GLP-2 receptor was replaced with the human GLP-2 receptor. The experimental results exhibited that native human GLP-2 or GLP-2 analogs accumulated the cAMP as well as dose-dependent increase in intracellular cAMP was also observed in GT15912, HM15912, and teduglutide, with maximal efficacy similar to the native human GLP-2. The EC_{50} of teduglutide, GT15912, and HM15912 were calculated be 0.120 ± 0.036 , 0.052 ± 0.008 , and 0.327 ± 0.096 nM, respectively, while the native human GLP-2 was 0.173 ± 0.056 nM. Based on these observed EC_{50} values, teduglutide, GT15912, and

HM15912 were $146.9 \pm 46.3\%$, 331.7 ± 84.5 , and $52.5 \pm 2.7\%$ as potent as native human GLP-2 (Fig. 1D).

HM15912 Significantly Induces Small Bowel Expansion in Dose-Dependent Manner. The dose-dependent intestinotrophic efficacy of HM15912 was investigated in C57BL/6 mice and SD rats. The small intestinal wet weight was presented as a percentage increase over vehicle-control treatment after once-a-week (Q1W) subcutaneous administration for two weeks. The dose ranges of 0.17–56.14 mg/kg (3.16–1,045.8 nmol/kg) and 0.03–10.24 mg/kg (0.56–190.8 nmol/kg) of HM15912 were used for mice and rats, respectively. At the end of the experiment (Day 14), HM15912 increased the small intestine weights in a dose-dependent manner in mice (Fig. 1E) and rats (Fig. 1F), and the maximum efficacy was reached approximately 2-fold of vehicle in both mice and rats. The ED_{50} values were 2.582 mg/kg (48 nmol/kg) for mice and 0.496 mg/kg (9.2 nmol/kg) for rats. Such promising results support the notion that the long-acting GLP-2 analog with increased and prolonged systemic exposure promoted enhanced pharmacological action.

HM15912 Exhibited Long-acting and Potent Pharmacodynamic Properties Associated with Intestinal Growth in a Rat Model of SBS. The effects of HM15912 on intestinal growth and the absorption capacity of the intestinal mucosa were evaluated in a rat model of SBS after repeated administration of HM15912 or teduglutide for two weeks. HM15912 was used with 4.5 nmol/kg and 30 nmol/kg every other day, and 30 nmol/kg of teduglutide was used twice a day to elicit the maximum hypertrophic effect on the small intestine (EMA, 2012). At each dose, HM15912 significantly increased the jejunal weight per centimeter in SBS model rats compared with the resection vehicle ($p < 0.001$) and teduglutide-treated ($p < 0.05$) groups while showing no meaningful increase in teduglutide compared with the resection vehicle (Fig. 2A). Similar to the results of jejunal weight per centimeter, 30 nmol/kg of HM15912 significantly increased the villus height, crypt depth, and mucosal area in the jejunum compared with teduglutide ($p < 0.01$, $p < 0.05$, and $p < 0.05$, respectively; Fig. 2, B–D). Teduglutide showed negligible increase in the villus height, crypt depth, or mucosal area in the jejunum beyond the adaptive responses to the resection vehicle. Hematoxylin and eosin (H&E) staining of jejunal sections also supported the series of results including villus height, crypt depth, and mucosal area in the jejunum (Fig. 2J). In the ileum, neither HM15912 nor teduglutide induced a significant increase in weight per centimeter compared with the resection vehicle. Although 30 nmol/kg of HM15912 induced a significant increase in villus height and mucosal area compared with the resection vehicle ($p < 0.01$ and $p < 0.05$, respectively; Fig. 2, F and H), these increases were somewhat lower than those in the jejunum.

Next, D-xylose absorption capacity was reduced by approximately 50% in the resection vehicle compared with the non-operation or sham operation vehicle, as shown in Fig. 2I. This functional depression was completely restored after treatment with HM15912, which was correlated with the morphologic improvements. Both the 4.5 nmol/kg and 30 nmol/kg doses of HM15912 abruptly and significantly augmented the level of D-xylose in serum compared with the resection vehicle ($p < 0.001$) in the early phase (0.5 hours and 1 hour), which more accurately reflects the absorption capacity of this drug. In particular, at 0.5 hours, a high dose of HM15912 was associated with a significant increase in the D-xylose level compared

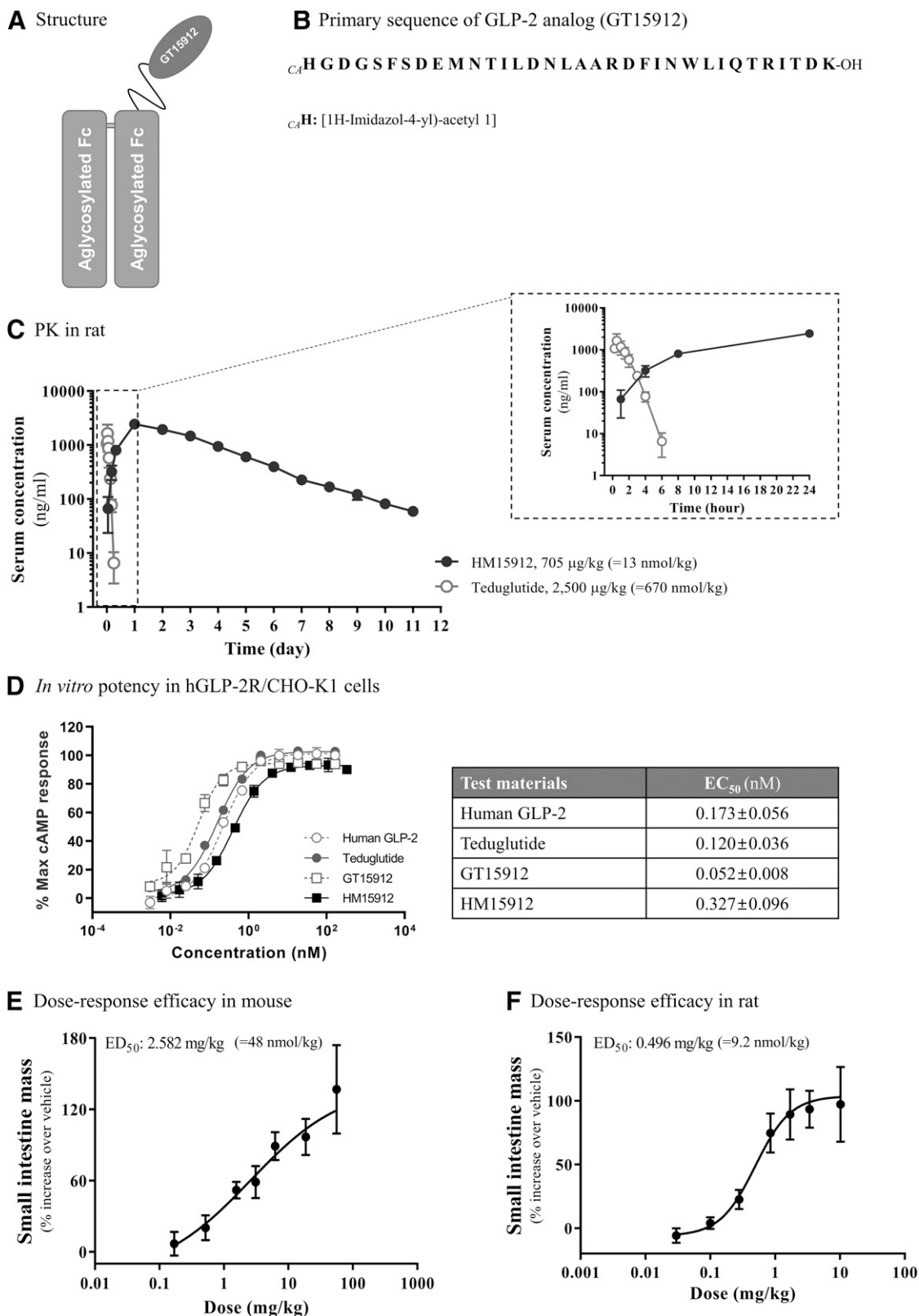


Fig. 1. Characterization of HM15912. (A) Structure of HM15912, (B) primary sequence of GLP-2 analog, GT15912, (C) time course of the serum concentrations of HM15912 and teduglutide following a single subcutaneous injection ($n = 3$ /group). Clear circle, teduglutide 2,500 μ g/kg (equal to 670 nmol/kg); black circle, HM15912 705 μ g/kg (equal to 13 nmol/kg). Data are expressed as the mean \pm S.D. (D) *In vitro* potency of GLP-2 analogs on human GLP-2 receptor in cell-based functional assays of dose-response cAMP. Data are expressed as the mean \pm S.D. of three independent assays. Clear circle, human GLP-2; gray circle, teduglutide; clear square, GT15912; black square, HM15912. (E) Dose-dependent percentage increase of small intestine weight over the mean of vehicle group in mice ($n = 6$ /group) on day 14 after once a week subcutaneous administration of HM15912. Data are expressed as the mean \pm S.D. (F) Dose-dependent percentage increase of small intestine weight over the mean of vehicle group in rats ($n = 6$ /group) on day 14 after once a week subcutaneous administration of HM15912. Data are expressed as the mean \pm S.D.

TABLE 2
Pharmacokinetic parameters of HM15912 in rats (subcutaneous administration)

Test material	Dose ($\mu\text{g/kg}$)	C_{max} (ng/ml)	T_{max} (h)	$t_{1/2}$ (h)	AUC_{last} (ng \cdot h/ml)	Dose-normalized AUC_{last} (kg \cdot h/L)
Teduglutide	2,500	1675.5 \pm 744.0	0.7 \pm 0.3	0.6 \pm 0.1	2596.6 \pm 580.7	1.0
HM15912	705	2442.1 \pm 392.6	24.0 \pm 0.0	42.3 \pm 3.4	199738.9 \pm 28544.5	283.3

Note: n = 3 per group. AUC, area under the curve.

Various pharmacokinetic parameters (AUC_{last} , C_{max} , T_{max} , $t_{1/2}$) were calculated using the results from the single subcutaneous administration pharmacokinetic study (Fig. 1). The mean values of parameters were calculated using a non-compartmental analysis method with WinNonlin software.

with teduglutide ($p < 0.01$), which even surpassed the normal condition.

Considering the overall dosage for a week, even at the dosages of 15.8 and 105 nmol/kg, which were 27- and 4-fold lower than 420.0 nmol/kg of teduglutide respectively, HM15912 significantly improved both the morphologic and functional outcomes compared with teduglutide.

HM15912 Had a Potent Small Bowel Hypertrophic Effect at Less Frequent Administration Intervals Than Teduglutide and Other Long-Acting GLP-2 Analogs in Mice. In mice, HM15912 was subcutaneously administered in the various administration intervals, including every other day, every fourth day, and once a week. Observation after two weeks of showed that HM15912 induced numerical dose-dependent increment in intestinal growth within the same administration regimen, and significant hypertrophy was observed in the small intestine compared with the treatment of teduglutide at all doses with various regimens.

At the every-other-day dosing interval, HM15912 at a medium dose (50 nmol/kg) and high dose (100 nmol/kg) significantly increased the small intestine weight compared with the vehicle by 105% and 112%, accordingly, whereas a high dose of LA-GLP2A1 and LA-GLP2A2 (100 nmol/kg) only showed increases of 47% and 51%, respectively, demonstrating that the small bowel trophic efficacy of HM15912 was significantly higher ($p < 0.001$) than that of both long-acting GLP-2 analogs under clinical development.

To further investigate the long-term properties of HM15912 compared with other long-acting GLP-2 analogs, the additional experiment was performed with extended administration interval. At a dosing interval of four days, meaningful small bowel hypertrophic effects were observed in 29 nmol/kg and 58 nmol/kg of HM15912 when compared with both 100 nmol/kg of LA-GLP2A1 and LA-GLP2A2 ($p < 0.001$). Furthermore, even at the once-a-week dosing interval, approximately 74% of small intestinal weight was augmented by treatment with 116 nmol/kg of HM15912 based on vehicle, which was more effective than the every-other-day dosing interval of 100 nmol/kg of weekly GLP-2 analogs or the twice-a-day dosing interval of teduglutide (Fig. 3).

HM15912 Exhibited a Potent and Long-Lasting Small Bowel Hypertrophic Effect After Pretreatment with Teduglutide in Mice. To demonstrate the predominant small bowel hypertrophic effect of HM15912 relative to teduglutide, four-week administration twice a day or every other day were given, respectively. For the alternative pre-treatment group, 15 nmol/kg of teduglutide was pre-treated twice a day for two weeks followed by once-a-week administration of 58 nmol/kg of HM15912 for an additional two weeks. As shown in Fig. 4A, administration of HM15912 at 58 nmol/kg once a week provided

a similar small bowel hypertrophic effect to administration of HM15912 at 15 nmol/kg every other day, as we previously observed (Fig. 3). Interestingly, the mice pre-treated with teduglutide followed by HM15912 showed a remarkable increase in the small intestinal weight on day 28 compared with the teduglutide only treated group. ($p < 0.01$). In addition, the D-xylose absorption test was performed to evaluate functional improvement following the mucosal area expansion at the end of the study. While the continuous administration of teduglutide did not significantly increase D-xylose absorption, the mice given teduglutide prior to a weekly administration of HM15912 exhibited a greater degree of increase in D-xylose absorption compared with the vehicle-treated group (Fig. 4B).

HM15912 Exhibited a Potent and Long-Lasting Small Bowel Hypertrophic Effect After Pretreatment of Other Long-Acting GLP-2 Analogs in Rats. To more clearly support the significant small bowel hypertrophic effect of HM15912 compared with the other long-acting GLP-2 analogs under clinical development, the administration of LA-GLP2A2 or LA-GLP2A1 every other day for two weeks was given followed by once a week administration of HM15912 for another two weeks. LA-GLP2A2 and LA-GLP2A1 were used at doses of 64 nmol/kg and 56 nmol/kg, respectively, that correspond to a human equivalent dose of 10 mg based on body surface area, whereas 58 nmol/kg of HM15912 was used. As shown in Fig. 5A, HM15912 treatment significantly increased the small intestinal weight compared with both long-acting GLP-2 analogs on days 14, 21, and 28 ($p < 0.05$ – $p < 0.001$), while showing no significant difference in the groups with pre-treated LA-GLP2A2 or LA-GLP2A1 and HM15912 on days 21 and 28.

Furthermore, on the D-xylose absorption test on day 28, at 1 hour after D-xylose administration (T_{max} of D-xylose absorption), the level of D-xylose in serum in the HM15912-treated group was significantly higher than that in the LA-GLP2A2- ($p < 0.05$) and LA-GLP2A1-treated ($p < 0.001$) groups. When the rats were given long-acting GLP-2 analogs prior to HM15912 administration, the serum D-xylose levels were increased to a similar extent with continuous administration of HM15912 only (Fig. 5B).

Discussion

HM15912, a novel long-acting GLP-2 analog, induces GLP-2R activation in the same way as naturally occurring hGLP-2. The apparent higher intestinotrophic effect of HM15912 is likely a consequence of the more potent intrinsic activity of the GLP-2 analog component, GT15912, than either native hGLP-2 or teduglutide. The highly potent in vitro activity of GT15912 is not significantly affected even after the conjugation of IgG4 Fc.

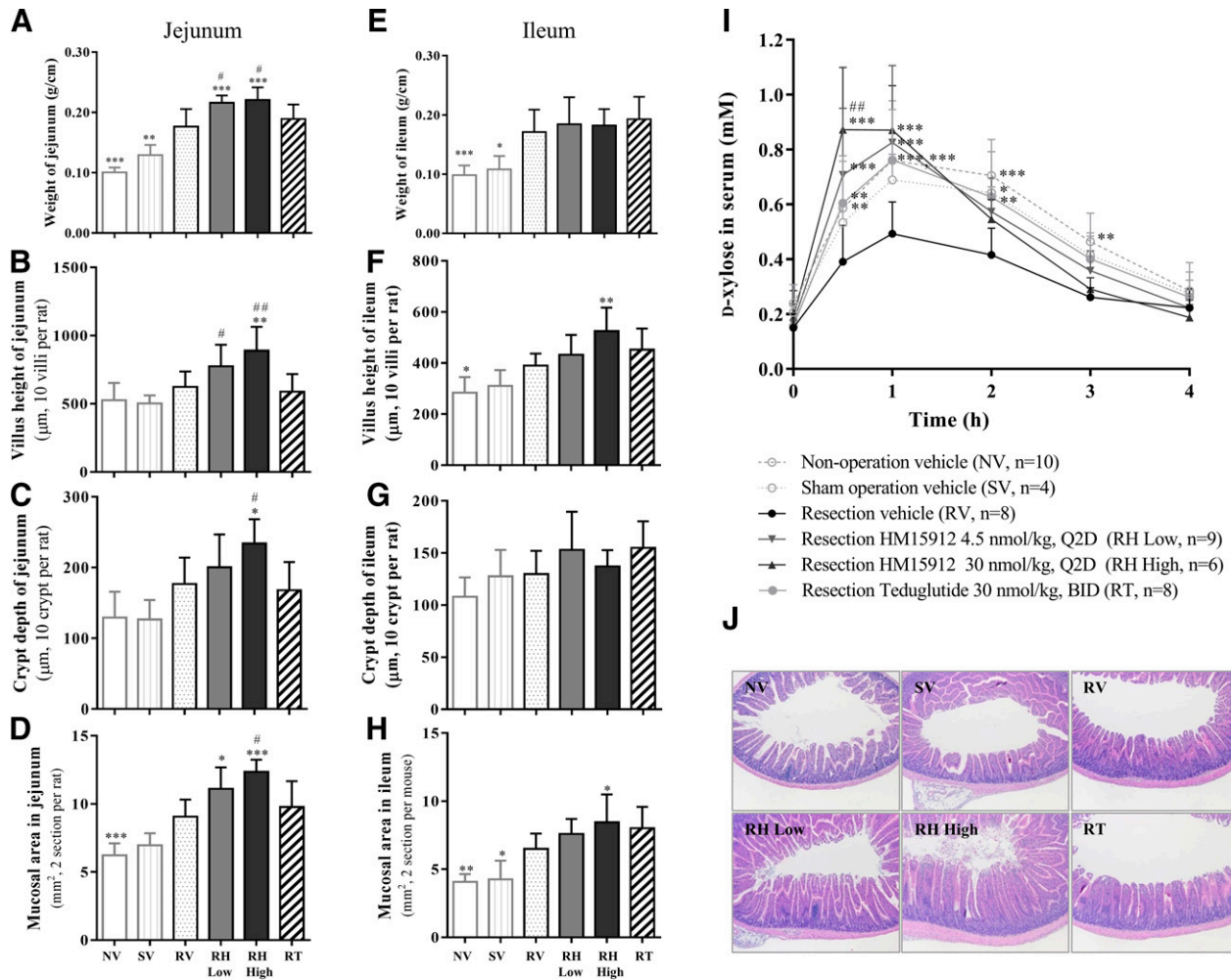


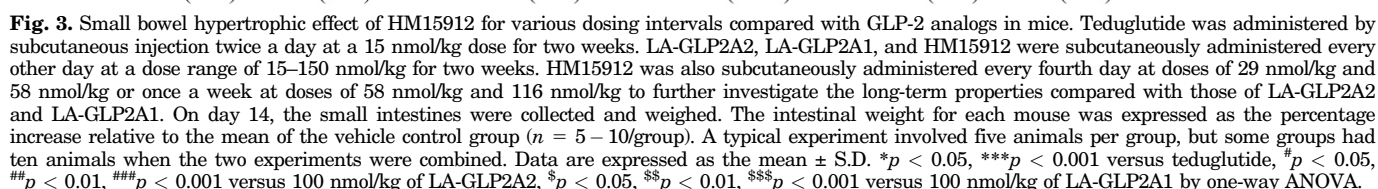
Fig. 2. Potent and long-acting pharmacodynamic properties of HM15912 for intestinal growth in a rat model of SBS ($n = 4-10/\text{group}$). Teduglutide was administered by subcutaneous injection twice a day at a 30 nmol/kg dose, and HM15912 was subcutaneously administered every other day at doses of 4.5 nmol/kg and 30 nmol/kg for two weeks. On day 13, the small intestines were collected. The first 5-cm segments of the intestine from the site of re-anastomosis in both the jejunal and ileal directions were discarded, and the next 5-cm segments in both the jejunal and ileal directions were cut and weighed. The weights of the jejunum and ileum were normalized by length. The dissected jejunum and ileum were stained with hematoxylin and eosin after fixation to measure the villus height, crypt depth, and mucosal area. The heights of 10 villi and 10 crypt depths were measured and then averaged to obtain the mean value for each animal. The mucosal area was calculated by subtracting the luminal area from the total intestinal area of the transverse section. Before sacrifice, the rats were fasted for 4 hours, after which 2 ml of D-xylose solution (50 mg/ml) was administered by oral gavage. Blood samples were obtained from the jugular vein 0.5–4 hours after administration of D-xylose solution, after which the serum concentration of D-xylose was measured. Data are expressed as the mean \pm S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the resection vehicle group. # $p < 0.05$, ## $p < 0.01$ versus the resection teduglutide 30 nmol/kg group by one-way ANOVA. (A) Weight of the jejunum, (B) villus height of the jejunum, (C) crypt depth of the jejunum, (D) mucosal area of the jejunum, (E) weight of the ileum, (F) villus height of the ileum, (G) crypt depth of the ileum, (H) mucosal area of the ileum, (I) D-xylose level in serum, (J) hematoxylin and eosin staining of jejunal sections. NV, non-operation vehicle; SV, sham operation vehicle; RV, resection vehicle; RH Low, resection + HM15912 4.5 nmol/kg; RH High, resection + HM15912 30 nmol/kg; RT, resection + teduglutide 30 nmol/kg.

We assume that the flexible polyethylene glycol linker minimizes steric hindrance of the IgG4 Fc region to the binding of the GT15912 region on GLP-2 receptors. Moreover, IgG4 Fc possibly contributes to extending the duration of action of HM15912 by promoting neonatal fragment crystallizable receptor-mediated vascular endothelial recycling (Roopenian and Akilesh, 2007) and facilitating avoidance of renal clearance by increasing the molecular weight to >50 kDa as observed in other long-acting protein drugs that applied same platform technology as HM15912 (Barrett et al., 2020). Despite the somewhat lower *in-vitro* potency of HM15912 than teduglutide, the molecular characteristics of greater and prolonged exposure of HM15912 elicited dose-dependent increase in the weight of

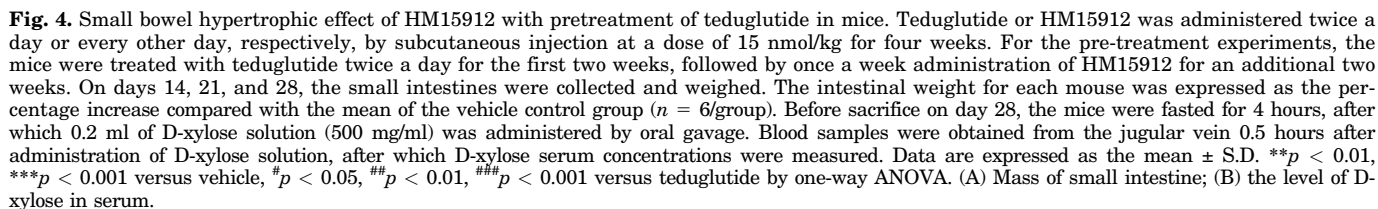
small intestine up to twice that of the vehicle group in both mice and rats even in the once-a-week administration interval.

Furthermore, the direct comparisons of HM15912 with teduglutide reported in this study conspicuously demonstrated that HM15912 proved superior small bowel trophic effect in less dosing frequency. These facts support the notion that outstanding pharmacodynamics effects of HM15912 were feasibly driven by greater and continuous exposure.

The restorative effect of HM15912 was determined in an 80% jejunoileal resection rat model of intestinal malabsorption using an extended administration interval. A dose of 30 nmol/kg of teduglutide was chosen as a human equivalent dose of 0.05 mg/kg based on body surface area, and an equimolar



reflects some portions of intestinal resections in patients with SBS, for example, jejunioileal anastomosis, jejunocolic anastomosis, and jejunostomy. In general, resection of the proximal bowel



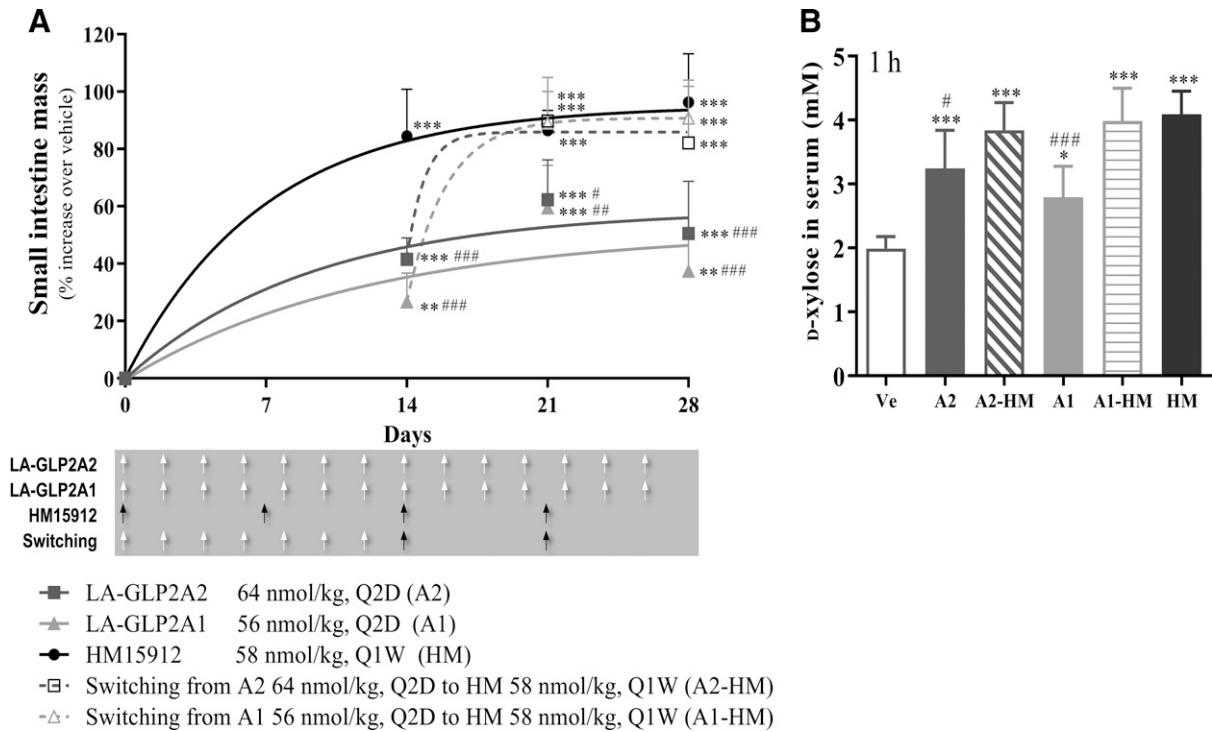


Fig. 5. Small bowel hypertrophic effect of HM15912 after pretreatment of other long-acting GLP-2 analogs in rats. LA-GLP2A2 or LA-GLP2A1 was administered by subcutaneous injection every other day at doses of 64 nmol/kg or 56 nmol/kg, respectively, for four weeks. HM15912 was subcutaneously administered once a week at a dose of 58 nmol/kg for four weeks. For the pretreatment experiments, the rats were pretreated with LA-GLP2A2 or LA-GLP2A1 every other day for the first two weeks, prior to once a week treatment of HM15912 for two additional weeks. On days 14, 21, and 28, the small intestines were collected and weighed. The intestinal weight for each mouse was expressed as the % increase relative to the mean of the vehicle control group ($n = 6/\text{group}$). Before sacrifice on day 28, the rats were fasted overnight, after which 2 ml of D-xylose solution (500 mg/ml) was administered by oral gavage. Blood samples were obtained from the jugular vein 1 hour after administration of D-xylose solution, after which the D-xylose serum concentration was measured. Data are expressed as the mean \pm S.D. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle (Ve), # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus HM15912 (HM) by one-way ANOVA. (A) Mass of small intestine; (B) the level of D-xylose in serum.

is more manageable than distal resection because the intestinal adaptation exhibited by the ileum is greater than that of the duodenum or jejunum (Dowling and Booth, 1967; Thompson et al., 1999). Therefore, patients with jejunoileal anastomosis rarely exhibit major nutrient imbalance since the remnant ileum and intact colon compensate for the absence of the resected bowel. Similar to patients with SBS with jejunoileal anastomosis, spontaneous small intestinal hypertrophy by adaptation of residual small bowel after surgical resection also occurred in this rat model. Nevertheless, rats developed SBS with an abnormally reduced intestinal absorption capacity, as demonstrated by a reduction in the normal D-xylose absorption capacity by half. After two weeks of teduglutide treatment, the deteriorated intestinal absorption capacity was restored to the normal condition. Interestingly, the more potent pharmacological GLP-2R stimulation by HM15912 exceeded the restored absorption capacity by teduglutide and the normal condition despite the lower dosage with an increase in injection interval.

Next, we further investigated the duration of action of HM15912 by varying the administration intervals (every other day, every fourth day, or once a week) in normal mice and rats. In the case of every other day administration with the same dosing regimen as the other long-acting GLP-2 analogs, HM15912 induced more than 2-fold increase in small intestinal weight compared with the vehicle, while only approximately 50% increase was observed in terms of the other long-acting GLP-2 analogs. In addition, HM15912 still had

a more pronounced intestinal hypertrophic effect in rodents than either the long-acting GLP-2 analogs or teduglutide even when administered only once a week. This longer-lasting pharmacological potential was conspicuously demonstrated by assessing the effects on pre-treatment of teduglutide, LA-GLP2A2, or LA-GLP2A1 followed by HM15912. We have obtained promising results that once-a-week administration of HM15912 to rodents led to a significant small bowel hypertrophic effect compared with every-other-day administration of other long-acting GLP-2 analogs currently under clinical development for up to weekly administration in SBS patients. This result is clearly supporting the possibility of a longer duration of action for more than a week, probably even up for a month in humans. However, the results should be carefully interpreted since the synthesized peptide analogs of long-acting GLP-2 analogs in this study only mimic the amino acid sequence of glepaglutide or apraglutide.

Kang et al. (2021) showed that the plasma citrulline level was maintained for over a month after a single subcutaneous administration of HM15912 in healthy volunteers, which demonstrated that the pharmaceutical efficacy of HM15912 could last up for a month, and potential administration for once a month in humans. Furthermore, the physicochemical stability of HM15912 facilitates the development as a ready-to-use injectable solution, which may provide improved safety and convenience for patients. Of course, given the potential risk of intestinal neoplasia caused by natural mode of action of GLP-2,

we cannot rule out the possibility that the greater proliferative effect of and more continuous exposure to HM15912 could increase the risk of intestinal neoplasia (Ring LL et al., 2018). As the clinical development stage progresses, the risk of carcinogenicity will be closely investigated.

Consistent efficacy of HM15912 in a broad range of pathophysiological conditions of SBS remains unknown; therefore, future studies should assess the structural and functional improvement in the small bowels by treatment of HM15912 in other animal models with various anatomic features. This finding must also be substantiated by positive outcomes from ongoing clinical trials for patients with SBS. Collectively, these findings could lead to the development of novel GLP-2 analogs powerful enough to be administered on a monthly basis.

Authorship Contributions

Participated in research design: J. Choi, IY. Choi.

Conducted experiments: J. Choi, Lee, Park, Kwon.

Contributed new reagents or analytic tools: D. Kim, Bae.

Performed data analysis: J. Choi, Lee.

Wrote or contributed to the writing of the manuscript: J. Choi, IY. Choi, HH. Kim.

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