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## **Incretin combination therapy for the treatment of non-alcoholic steatohepatitis**

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### **Abstract**

**Aims:** Agonists to the glucagon-like peptide 1 receptor (GLP1R) agonists and dual agonists targeting GLP1R and the glucagon receptor (GCGR) or the Glucose-dependent insulintropic peptide receptor (GIPR) are currently being developed for the treatment of non-alcoholic steatohepatitis (NASH). We have tested specific mono-agonists to these three receptors individually and in combination in a mouse model of diet-induced NASH and fibrosis, to decipher the contribution of their activities and potential additive effects on improving systemic and hepatic metabolism.

**Materials and methods:** Advanced NASH was induced by pre-feeding C57BL/6J mice a diet rich in fat, sucrose and cholesterol for 36 weeks. This was followed by eight weeks of treatment with the receptor-specific agonists 1-GCG (20 µg/kg bid sc), 2-GLP1 (3 µg/kg bid sc) or 3-GIP (30 µg/kg bid sc), or the dual (1+2) or triple (1+2+3) combinations thereof. A dual GLP1R/GCGR agonistic peptide, 4-dual-GLP1/GCGR (30 µg/kg bid sc), and liraglutide (100 µg/kg bid sc) were included as references.

**Results:** Whereas 1-GCG and 3-GIP alone, at the selected low dose, did not influence body weight, liver lipids and histology, their combination with 2-GLP1 provided additional weight loss, reduction in liver triglycerides and improvement in histological NAFLD activity score (NAS). In addition, there was a trend to further reduction in markers of hepatic inflammation and fibrosis. Notably, compared to

high-dose liraglutide, 4-dual-GLP1R/GCG as well as the dual and triple combinations of selective mono-agonists demonstrated stronger improvement in NAS at the same extent of body weight loss.

Conclusions: GCGR and GIPR agonism provide additional, body weight-independent improvement in a murine model of advanced NASH with fibrosis on top of GLP1R agonism.

## Introduction

Non-alcoholic fatty liver disease (NAFLD) covers a spectrum of hepatic abnormalities ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and liver fibrosis. Fatty liver is very common, with an estimated global prevalence of approximately 25%, and strongly associated with other systemic conditions such as obesity, diabetes and dyslipidemia.<sup>1</sup> Progression to NASH and especially to NASH with advanced fibrosis is a strong risk factor for the development of cirrhosis and hepatocellular carcinoma, has been linked to increased overall and liver-related mortality, and is becoming the leading cause of liver transplantation.<sup>2,3,4,5,6</sup> From a pathophysiological perspective, fat and triglyceride accumulation in the liver, hepatic and adipose tissue insulin resistance, inflammation as well lipotoxicity and oxidative stress are involved in the development of NAFLD. At present there are no approved pharmacological therapies available to treat NAFLD/NASH. Lifestyle intervention focusing on weight loss is regarded as first line therapy.<sup>7</sup> Thus, novel treatment options for NAFLD/NASH are highly warranted.

Amongst the various approaches which are being investigated to treat NAFLD/NASH are agonists of the glucagon-like peptide 1 receptor (GLP1R) agonists or GLP-1-based multi-agonists as they have been shown to produce significant and sustained weight loss as well as elicit favorable metabolic effects.<sup>8,9</sup> For example, treatment of patients with biopsy-confirmed NASH with liraglutide, a once daily GLP1R agonist, over 48 weeks at a dose of 1.8 mg/day (LEAN study) led to a resolution of NASH in 39% of the treated patients as well as to reduced worsening of fibrosis.<sup>10</sup>

Unimolecular dual or triple agonists activating besides the GLP-R also the glucagon receptor (GCGR) or/and the glucose-dependent insulintropic peptide receptor (GIPR) are emerging as a promising class of next generation drug molecules offering significantly improved metabolic effects and weight loss.<sup>11</sup> Besides their pronounced effects on glycemic control and body weight, dual GLP1/GCGR agonists were shown to improve lipid metabolism and hepatic steatosis in mice with diet-induced obesity (DIO).<sup>12</sup> When studied in non-human primates, treatment with an activity- balanced, lipidated dual GLP1R/GCGR agonist, MEDI0382, led to significant hepatic fat reduction, which was also seen in human clinical studies.<sup>13,14</sup> MEDI0382 is currently under advanced clinical development for treatment of NAFLD/NASH. (clinicaltrials.gov; NCT04019561)

Likewise, unimolecular dual GLP-1R /GIPR agonists as well as triple GLP-1R/GIPR/GCGR agonists have been shown to improve glycemic control and weight loss in DIO mice accompanied by improved liver function and hepatic steatosis.<sup>15,16</sup>

However, very little is known on the effects of these multi-incretin approaches in models of obesity and insulin resistance in combination with manifest NASH and advanced fibrosis. Also, the contribution of their individual components – GLP1R, GCGR and GIPR agonism – on hepatic and metabolic disease in the setting of NASH has not been thoroughly investigated. In the absence of such systematic studies, we have designed acylated, selective GLP-1R, GCGR and GIPR agonists as tool compounds and studied them alone and in combination in a mouse model of diet-induced, biopsy-confirmed advanced NASH and fibrosis to better understand their individual contribution and potential additive effects on improving systemic and hepatic metabolism.

## Methods

### Animals and experimental design

The peptides were investigated in a mouse model of diet-induced obesity, NASH and fibrosis (DIO-NASH model) as described.<sup>17</sup> All animal experiments were conducted according to the international principles for care and use of laboratory animals and were covered by personal licenses for Jacob Jelsing (2013-15-2934-00784 and 2015-15-0201-00518) issued by the Danish committee for animal research.

Male C57BL/6J mice (5 weeks old) obtained from Janvier (LanVier Labs, France) were placed on either standard rodent chow (Altromin 1324, Brogaarden, Denmark) or AMLN diet (D09100301, Research Diet, United States). AMLN diet is a NASH-inducing diet rich in fat (40%, including 18% trans-fat), carbohydrates (40%, including 20% fructose) and cholesterol (2 %) as previously described.<sup>18</sup> After 33 weeks on these diets, a baseline liver biopsy was conducted for histological assessment of individual fibrosis and steatosis staging, as described.<sup>17</sup> A total of 96 mice (n=12 per treatment group) were randomized and stratified according to body weight and liver Col1A1 quantification. Ten mice on chow diet were included as controls. Treatment commenced 36 weeks after starting on the diets and lasted for eight weeks with all animals remaining on the same diet as in the pre-treatment phase. At the end of the intervention, animals were euthanized and liver tissue and serum samples were collected.

Tested compounds and doses are summarized in [table 1](#). All compounds were administered twice daily by subcutaneous injection using phosphate-buffered saline as vehicle.

Methods used for body weight and body composition analysis, blood sampling, plasma biochemistry and liver tissue biochemistry are detailed in Supplemental Information.

### Histology assessment

Baseline liver biopsy and terminal samples were collected from the left lateral lobe (about 50-100 mg at baseline and 200mg at the end) and fixed overnight in 4% paraformaldehyde. Liver tissue was paraffin embedded and sectioned (3µm thickness). Sections were stained with Hematoxylin and Eosin and Sirius Red to assess hepatic steatosis and fibrosis respectively, followed by analysis with Visiomorph software (Visiopharm, Denmark). Col1a1 and galectin-3 were assessed using IHC staining. A blinded to the study pathologist performed the histological assessment and scoring. NAFLD activity score (NAS) (steatosis/inflammation/ballooning degeneration) and fibrosis stage were quantified applying the criteria proposed by Kleiner et al.<sup>19</sup>

## Hepatic gene expression changes

Liver tissue was harvested from the left lateral lobe, stabilized overnight in RNAlater® solution (Merck KGaA, Darmstadt, Germany) and stored at -80 °C. Total RNA isolation was performed with the miRNeasy kit following the instructions of the manufacturer (QIAGEN GmbH, Hilden, Germany). RNA was quantified with an Agilent RNA 6000 Nano kit using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Waldbronn, Germany). Gene expression was quantified using droplet digital PCR or qRT-PCR analysis as described in Supplemental Information.

## Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical significance was evaluated using Dunnett's test one-factor linear model for body composition, blood and liver biochemistry. T-test was used for the comparison of differences of gene expression between the groups.  $P < 0.05$  was set as the statistical significance level.

## Results

### Test compounds

For the systematic study of incretin hormone analogs and their combination in the described NASH animal model, specific mono-agonists of the GLP1R, GCGR and the GIPR were generated. In addition, an earlier described dual GLP1R/GCGR agonist<sup>20</sup> was used as well as Liraglutide as GLP1 standard for comparison. Compounds 1-GCG, 2-GLP1 and 3-GIP were all designed based on the exendin-4 sequence using acylation with either palmitic acid or stearic acid to prolong their half-life, similar to liraglutide ([table 1a](#)). In mice, 1-GCG is an equipotent GCGR agonist compared to glucagon itself with a much better selectivity profile towards the GLP1R (> 300-fold). 2-GLP1 & 3-GIP were at least 3000-fold selective for their corresponding receptor ([table 1b](#)).<sup>21,22</sup> In mice, the dual agonist 4-dual-GLP1/GCG was 10-fold more active at the GLP1R compared to the GCGR. All selected compounds had reasonable pharmacokinetic properties in mice with half-lives of 2.5-4.1 h after subcutaneous administration ([table 1c](#)). In order to guarantee full daily coverage in our DIO-NASH model the compounds were dosed twice daily (b.i.d.) by subcutaneous administration. The doses selected for compounds 1-GCG, 2-GLP1, 3-GIP were 20 µg/kg, 3 µg/kg and 30 µg/kg b.i.d, respectively. These comparably low doses were selected to allow for the identification of additive or synergistic activity when given in combination at the same individual doses. Liraglutide as a reference GLP-1R agonist

was administered at 100 µg/kg twice daily to provide near-maximal effects that can be achieved with a selective GLP1R agonist. The dose of compound 4-dual-GLP1/GCG was chosen as 30 µg/kg b.i.d. to achieve an extent of weight loss which is similar to liraglutide at 100 µg/kg b.i.d.

### **Body composition and food intake, blood glucose**

*Figure 1a* shows the relative change in body weight over the treatment period of eight weeks. Average body weight at the onset of treatment was 36.1 gram ( $\pm$  0.3 gram SEM) without significant differences between treatment groups, compared to 31.1  $\pm$  0.4 gram for the chow control mice. Whereas vehicle-treated mice gained about 3 % over the treatment period, body weight remained constant for mice treated with 1-GCG or 3-GIP at the tested doses. Treatment with 2-GLP1 led to body weight loss of 5 % whereas 4-dual-GLP1/GCG, liraglutide or the dual or triple combinations of 1-GCG, 2-GLP1  $\pm$  3-GIP led to 8-9 % weight loss, all significantly different from vehicle controls and getting close to the weight of lean control mice. Weight loss was driven primarily by an initial decrease in food intake in the first week of treatment which then recovered and remained stable for the rest of the treatment period (*figure 1b*). Body weight loss predominantly resulted from loss of fat (*figure 1c*) whereas there was no significant change in lean mass (*figure 1d*).

Mice with NASH had enlarged livers that had about twice the weight of those of the chow control mice (*figure 1e*). Whereas treatment with 1-GCG or 3-GIP alone had no effect on liver weight, 2-GLP1, the combination of 2-GLP1 with 1-GCG or with 1-GCG and 3-GIP as well as 4-dual-GLP1/GCG and liraglutide led to a significant reduction in liver weight.

As expected, treatment with the glucagon receptor agonist 1-GCG led to an increase in blood glucose whereas it remained constant or decreased in all other treatment groups (*figure 1f*). Of note, DIO-NASH mice are not diabetic which explains the limited glucose lowering seen for the GLP1R- or GIPR-agonist containing treatment groups.

### **Liver enzymes, hepatic steatosis and histopathology**

Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were significantly reduced upon intervention with 2-GLP1, the 1-GCG + 2-GLP1 dual combination, the 1-GCG + 2-GLP1 + 3-GIP triple combination, 4-dual-GLP1/GCG or liraglutide (*figures 2a and 2b*). In the same groups, total liver triglycerides were lower than in vehicle control mice at the end of the treatment period (*figure 2c*). Notably, there was a significant add-on effect of GCGR and GIPR

agonism to GLP1R activation in lowering liver triglycerides which was also observed for lowering total liver cholesterol (*figure 2d*).

Whereas 1-GCG or 3-GIP alone did not lead to a reduction in liver fat content, hepatic steatosis was lower in all treatment groups containing GLP1R-agonistic activity (*figure 2e*) with a significantly stronger reduction observed for the 1-GCG + 2-GLP1 + 3-GIP triple combination compared to the GLP1R agonist 2-GLP1 alone. Hepatic galectin-3 as a marker of liver fibrosis was decreased in the dual and triple combination group and upon treatment with 4-dual-GLP1/GCG or liraglutide (*figure 2f*).

The change in the histological NAFLD activity score (NAS) upon treatment is depicted in figure 3a. In the pre-treatment biopsy, DIO-NASH mice had, on average, a NAS of 6 primarily driven by a steatosis score of 3 in all DIO-NASH animals and an inflammation score of 3 in 85 % of all DIO-NASH mice whereas there was little hepatocyte ballooning (35 % with score 1, 65 % with score 0) at the onset of therapy. Treatment with 1-GCG, 2-GLP1 ± 3-GIP dual and triple combinations or the dual GLP1R/GCGR-agonist 4-dual-GLP1/GCG led to a stronger decrease in NAS compared to treatment with single GLP1R-, GCGR- or GIPR-agonistic peptides (*figure 3a*) with improvements in all three NAS components steatosis, lobular inflammation and hepatocyte ballooning (not shown). Of note, the dual GLP1/GCG agonist and the dual and triple combination treatments also provided a stronger decrease in NAS compared to liraglutide (*figure 3a*) although the amount of body weight loss between these groups was nearly indistinguishable (*figure 1a*).

The majority of the DIO-NASH mice had a fibrosis score of two in the pre-biopsy. Whereas in the 1-GCG and 3-GIP groups only two and one mice, respectively, showed an improvement by one point, about 50 % of animals treated with 2-GLP1, the dual or triple peptide combination or 4-dual-GLP1/GCG exhibited a reduction in hepatic fibrosis score by one point (*figure 3b*).

This improvement in histology is also reflected in changes in the expression of marker genes for fibrosis (Col1a1, Col3a1, Loxl2) or inflammation (Ccl2, Tlr4). In addition, dermatopontin (Dpt), previously described to be associated with NASH both in rodents and in people<sup>23</sup> was found to be regulated in DIO-NASH mice and partially normalized upon treatment (*figure 4*).

## Discussion

Using a mouse model of biopsy-confirmed, diet-induced, advanced NASH with fibrosis, we have demonstrated that combining GLP-1 receptor, glucagon receptor and GIP receptor agonism provides additive effects in improving hepatic steatosis, liver injury and NAFLD activity.



GLP1R agonists are an established therapy for the treatment of diabetes and obesity<sup>24,25,26,27,28,29,30</sup>, with positive effects on cardiovascular outcome.<sup>31,32,33,34,35</sup> Recently, dual GLP1R/GCGR<sup>14,36</sup> and GLP1R/GIPR<sup>37</sup> have demonstrated clinical proof of concept in lowering body weight and blood glucose in obese patients with type-2 diabetes. Treatment of patients with biopsy-confirmed NASH with the GLP1R agonist liraglutide led to NASH resolution and inhibition of fibrosis progression<sup>10</sup>, and GLP1R agonists were shown to improve hepatic and metabolic health in pre-clinical models of NAFLD or NASH.<sup>38,39,40,41,42,43</sup> In contrast, there is little information on the activity of dual or triple agonists in models of NASH, and the contribution of individual incretin or glucagon effects to the combined activity of these molecules has not been systematically investigated. A dual-active peptide targeting GLP1R and GCGR, G49, was described to improve hepatic steatosis and ameliorate liver injury in mice on a methionine and choline-deficient diet and partial hepatectomy.<sup>44</sup> Likewise, a unimolecular GLP1R/GCGR/GIPR triagonist led to an improvement in steatohepatitis in mice with diet-induced obesity.<sup>16</sup> However, these studies did not include specific GLP1R or GCGR agonists as comparators to delineate the relative contribution of the two components to the observed effects.

Weight loss is a strong predictor of a reduction in hepatic steatosis and resolution of NASH, independently of whether it is induced by diet and exercise,<sup>7</sup> bariatric surgery<sup>45</sup> or pharmacological intervention.<sup>10</sup> Correspondingly, weight loss observed in our study was tied to improvements in liver metabolism and histology. However, combination of sub-maximal doses of GLP1R and GCGR mono-agonists or of GLP1R, GCGR and GIPR mono-agonists as well as administration of a dual GLP1R/GCGR agonist provided a more pronounced improvement in NAFLD activity score compared to a high dose of liraglutide eliciting the maximal GLP1R-mediated response, at the same extent of weight loss. Thus, it is likely that there are additional, weight-independent effects via activation of, e.g., liver GCGR leading to inhibition of hepatic de-novo lipogenesis and stimulation of liver fat utilization.<sup>46</sup>

While providing a first systematic investigation of individual and combined effects of GLP1R-, GIPR- and GCGR agonism in diet-induced NASH, our study has certain limitations:

Firstly, peptides could only be tested at one dose because including several doses per mechanism alone and in combination would have made the study excessively large and costly. Individual doses were selected according to previous studies in other murine models to produce small effects on weight and metabolic parameters. However, the selected dose of 2-GLP1 by itself led to significant weight loss, glucose lowering and reduction in hepatic steatosis, leaving less room for additive or synergistic effects of 1-GCG and/or 3-GIP on top of 2-GLP1 to be explored. In further studies, lower doses of 2-GLP1 should be included.

Secondly, development of NASH in our model is driven by a diet artificially high in fat, especially trans-fat, fructose and cholesterol. Whether and how results obtained with this murine model

translate into clinical efficacy in humans is not clear. In reverse translational studies, several molecules with clinical efficacy in NASH, e.g., obeticholic acid, liraglutide and elafibranor also led to improvements in NASH in our model.<sup>42</sup> However, it remains to be shown that the model also predicts forward translation into humans. Notably, following an FDA ban on trans-fat as a food component,<sup>47</sup> the NASH inducing diet has recently been changed to contain palm oil instead of trans-fat.<sup>48</sup>

Finally, it was outside of the scope of our study to further investigate the molecular mechanisms of how GLP1R-, GCGR- or GIPR-specific agonists and their combinations elicit their beneficial effects on systemic metabolism and steatohepatitis, e.g. through comparative expression, proteomics or metabolomics analysis. Such studies are currently under way.

## **Acknowledgements**

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## **Conflict of interest**

AK, CK, RE, MB, TH, AE, KL, CR, ZB, JCG, VM and MW are employees of Sanofi, some hold Sanofi shares.

## **Author contributions**

AK, JCG, FP, BS, VM and MW designed the concept. AK, ANM, CK, RE, CR and ZB performed experiments and analyzed data. MB, TH, KL and MW designed and synthesized test compounds. AK and MW wrote the paper. All authors edited draft versions and approved the final manuscript.

## Table and Figure Legends

**Table 1.** Structure, activities and pharmacokinetics of test compounds. (a) Amino acid sequence and modifications of peptides used in this study. (b) In-vitro potencies ( $EC_{50}$  in pM) in HEK293 cells overexpressing the murine GLP1R, GCGR or GIPR for peptides used in this study. Potencies determined for human GLP-1, human glucagon and human GIP are shown for comparison.

**Figure 1.** (a) Body weight change (% of day 0) throughout the eight-week treatment period. (b) Comparative 24-hour food intake relative to NASH vehicle group. (c) Lean tissue mass, (d) fat tissue mass, and (e) relative liver weight at study termination. (f) Four-hour fasting blood glucose levels after six weeks of treatment. Values are mean of  $n=10-12 + SEM$ . \* $p<0.05$ ; \*\* $p<0.01$ , \*\*\* $p<0.001$  compared to NASH Vehicle

**Figure 2.** (a) Plasma AST, (b) plasma ALT activities at study termination. (c) Liver triglycerides, (d) liver total cholesterol in mg/g wet liver tissue at study termination. (e) Hepatic fat content, (f) hepatic galectin-3 content (% fractional area) as determined by histological quantitative assessment (morphometry). Values are mean of  $n=10-12 + SEM$ . \* $p<0.05$ ; \*\* $p<0.01$ , \*\*\* $p<0.001$  compared to NASH Vehicle. # $p<0.05$ , ## $p<0.01$ , ### $p<0.001$  compared to 2-GLP1 treatment group.

**Figure 3.** (a) Representative H&E stained images (20x) of liver morphology at study termination and individual changes in NAFLD activity score (pre- vs. post-treatment) for the different treatment groups. (b) Representative picrosirius red stained images (20x) and individual changes in fibrosis score for the different treatment groups.

Figure 3. Hepatic expression of fibrosis marker genes (a) Col1a1, (b) Col3a1, (c) Loxl2 and (d) Dpt, inflammation marker genes (e) Ccl2 and (f) Tlr7 at study termination as determined by digital droplet PCR. Values are mean of  $n=10-12 + SEM$ . \* $p<0.05$ ; \*\* $p<0.01$ , \*\*\* $p<0.001$  compared to NASH Vehicle.

**a) Peptide Sequences**

Compound	Sequence	Bid Dose [µg/kg]
1-GCG	Tza-s-QGTFTSDYSKQ-K[γGlu-C16]-ESRRAQEFIEWLLAGGPESGAPPPS-NH <sub>2</sub>	20
2-GLP1	H-s-EGTFTSDVSKQ-K[γGlu-C16]-EKRAA-Aib-EFIEWLKNTGPSSGAPPPS-NH <sub>2</sub>	3
3-GIP	Y-a-EGTFISDYSIA-K[γGlu-C16]-DKIHQQDFVNWLLAQKPSSGAPPPS-NH <sub>2</sub>	30
4-Dual-GLP1/GCG	H-s-QGTFTSDLKQ-K[γGlu-C18]-DSRRAGDFIEWLKNGGPSSGAPPPS-NH <sub>2</sub>	30
Liraglutide	HAEGTFTSDVSSYLEGQAA-K[γGlu-C16]-EFIAWLVRGRG-OH	100

Tza: Thiazolyl-alanine; Aib: 2-Aminoisobutyric acid

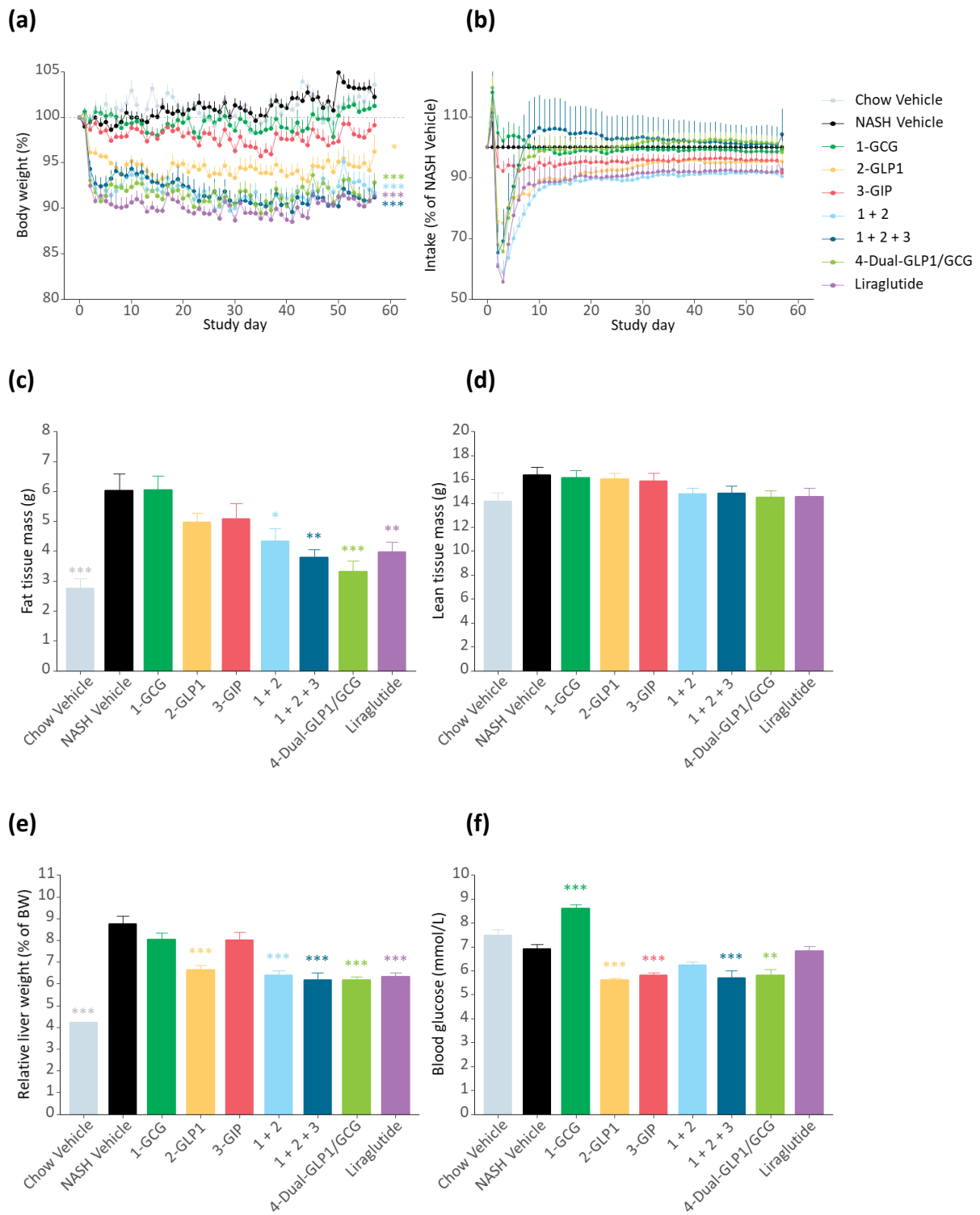
**b) In vitro receptor agonist potencies (cAMP release) in HEK-293 cell lines stably expressing mouse GLP-1, glucagon or GIP receptors**

Compound	mouse EC <sub>50</sub> [pM]		
	GLP1R	GCGR	GIPR
1-GCG	396	1.3	>10,000
2-GLP1	1	>10,000	>10,000
3-GIP	>10,000	>10,000	3
4-Dual-GLP1/GCG	2.3	25	>10,000
Liraglutide	4.4	>10,000	>10,000
hGLP-1	0.9		
hGlucagon	43.5	1.3	>10,000
hGIP			1.2

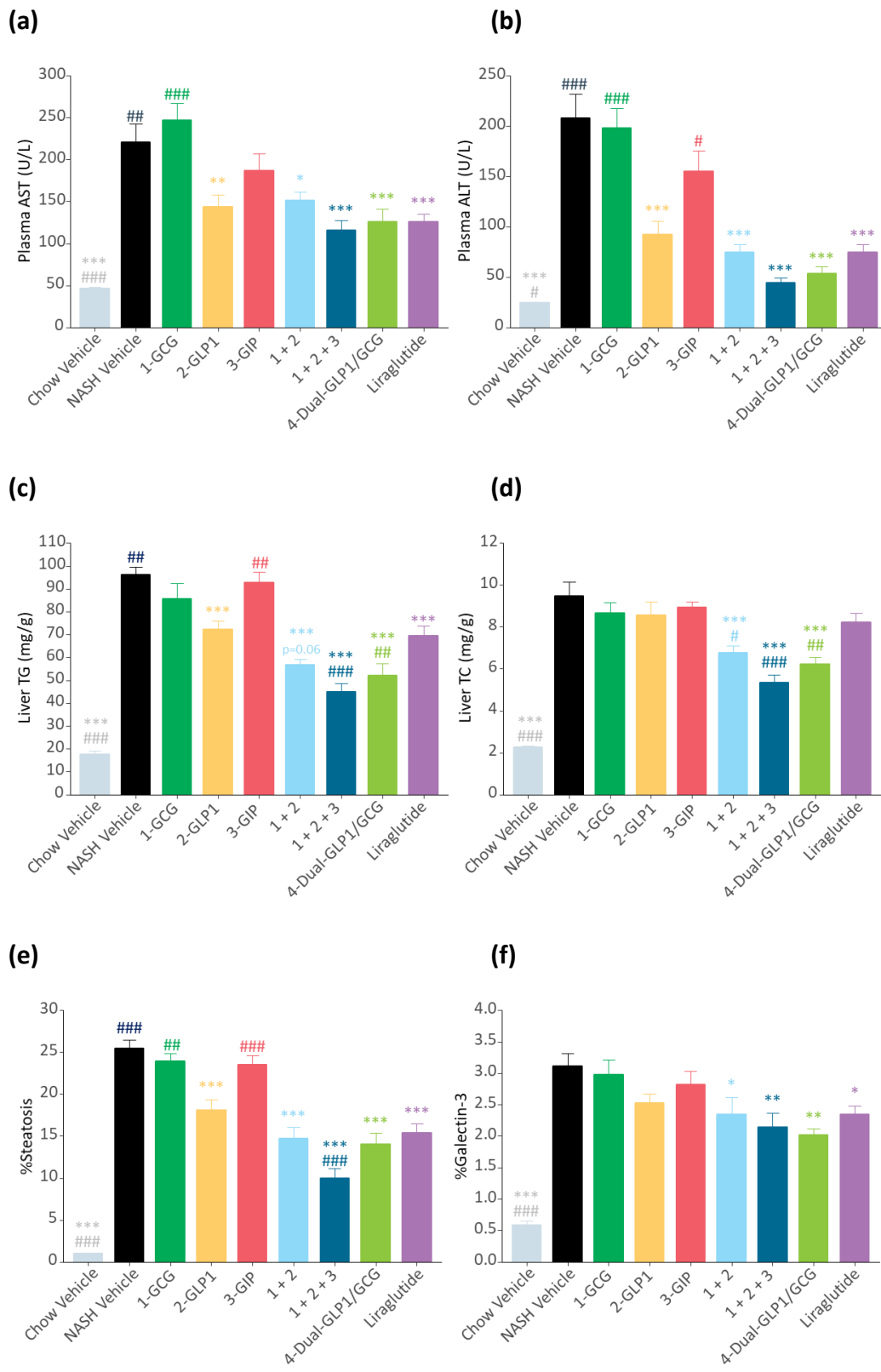
**c) Pharmacokinetic parameters after single subcutaneous administration to female C57Bl6 mice**

Compound	Dose [mg/kg]	Cmax [ng/ml]	AUC <sub>0-24</sub> [ng h/ml]	T <sub>1/2</sub> [h]
1-GCG	1	5640	36600	2.5
2-GLP1	0.5	1820	11800	3.5
3-GIP	0.5	5060	38200	4.1
4-Dual-GLP1/GCG	1	1930	11000	3.2
Liraglutide	1	7700	79100	3.4

**Table 1**

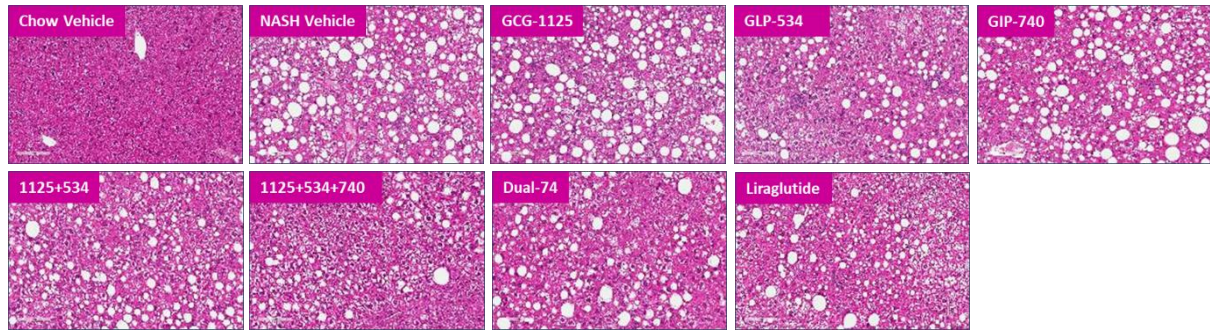


**Figure 1**

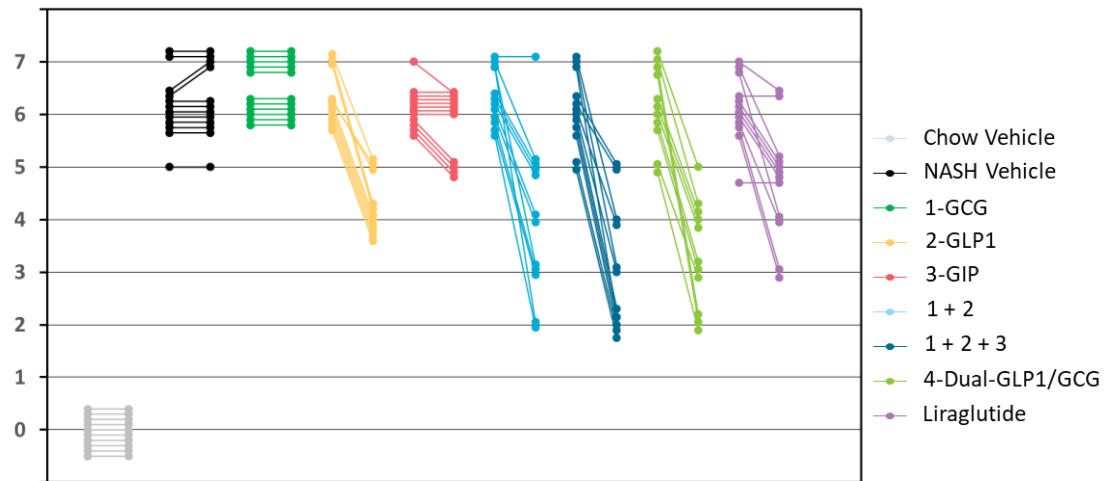


**Figure 2**

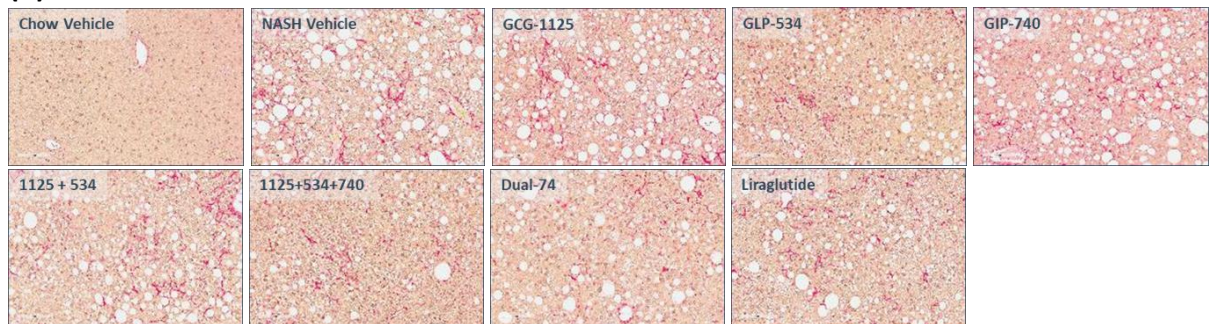
(a)



NAFLD Activity Score



(b)



Fibrosis score

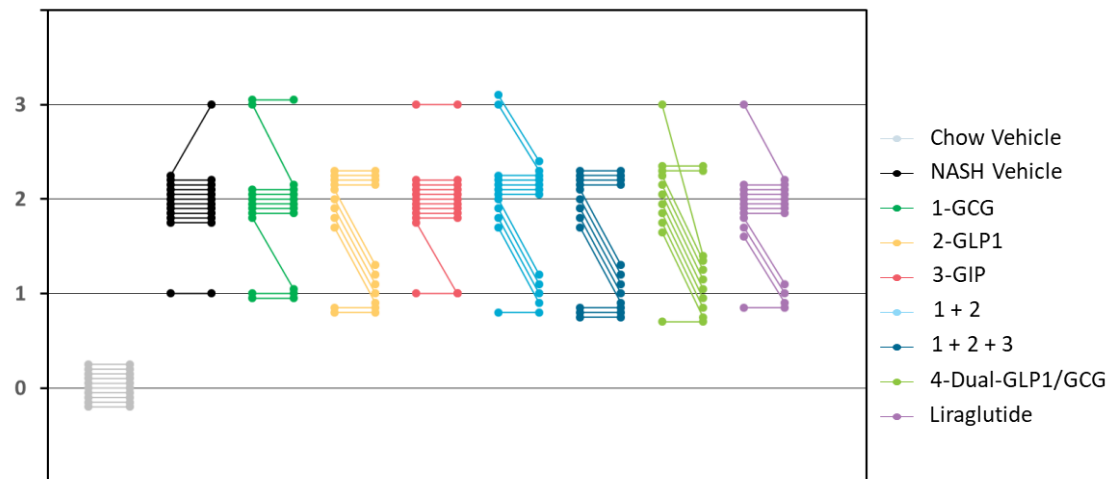
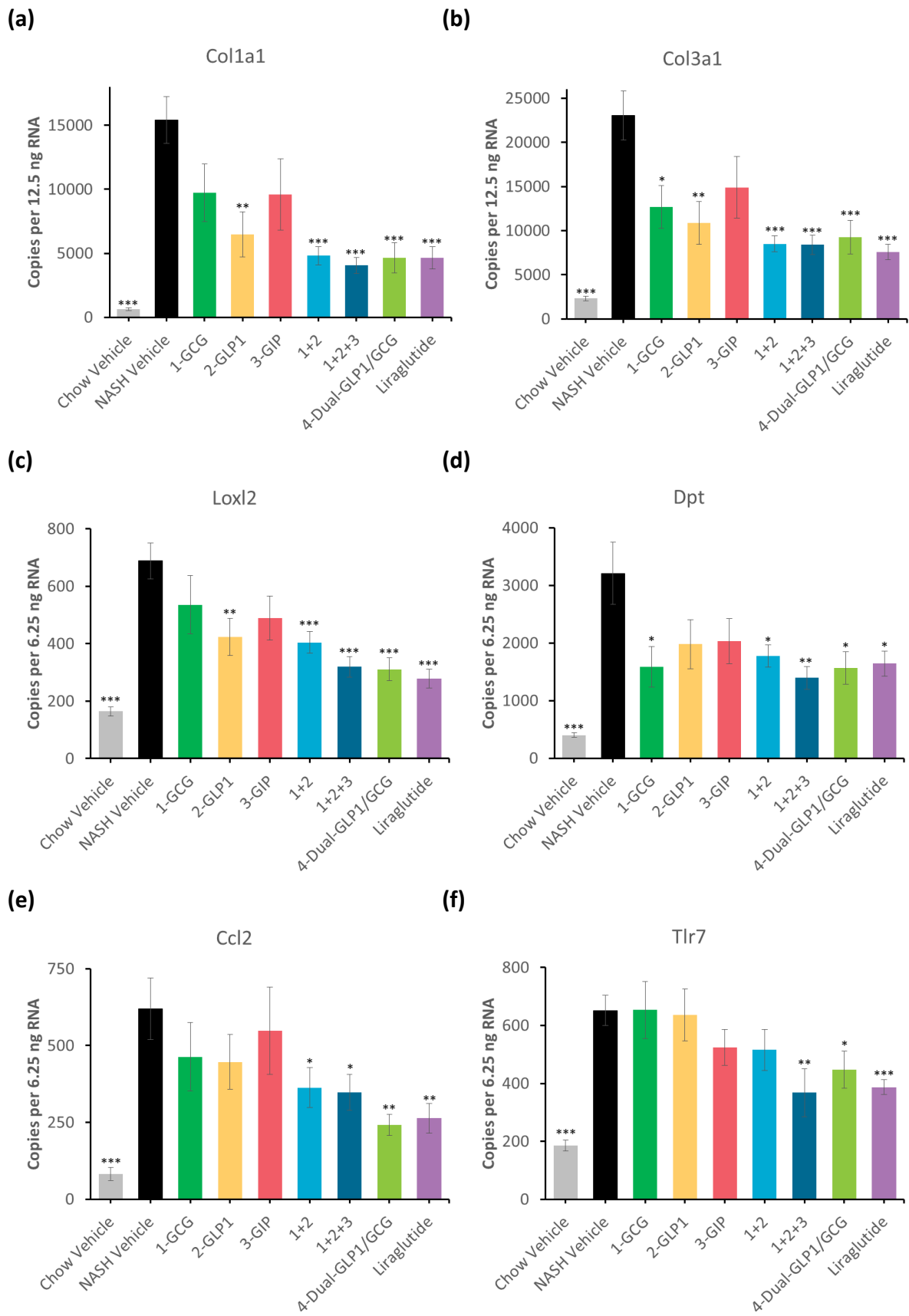


Figure 3



**Figure 4**



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