Underlying Mechanisms for Long-Acting Properties of the Novel Weekly Insulin, LAPSInsulin 115 (HM12470)

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ABSTRACT

The ultra long-acting basal insulin,HM12470, has been developed by conjugating an insulin analog (Insulin 115) with the constant region of a human immunoglobulin fragment (Fc) via non-peptidyl linker. Previously, we demonstrated that HM12470 (LAPSInsulin 115) had longer PK/PD properties than HM12460A (insulin conjugated with Fc, LAPSInsulin) in various animal models. However, the mechanism of prolonged properties remained to be fully elucidated. This study investigated the underlying molecular mechanisms of the long-acting properties of insulin 115 and HM12470 in comparison with insulin and HM12460A.

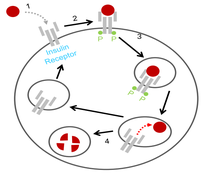
Firstly, we demonstrated that insulin 115 and HM12470 had lower affinity to the human insulin receptor-B than insulin and HM12460A. Similar results were observed when comparing receptor phosphorylation and internalization [insulin 115 (86%, 63%, 74%), HM12470 (1.7%, 2.0%, 20%), HM12460A (2.6%, 5.6%, 30%); relative activity vs. insulin for receptor binding, phosphorylation, and internalization, respectively]. The reduced *in vitro* activities were well correlated with cellular stability in HepG2 cells. The residual amount of insulin 115 (86%) and HM12470 (97%) were higher than that of insulin (20%) and HM12460A (78%) in conditioned media. These results suggest that insulin 115 and HM12470 with relatively lower receptor affinity could induce less receptor mediated clearance (RMC) when receptor binding affinity is correlated with RMC. Lastly, the serum stability of Insulin 115 and HM12470 was evaluated, and both insulin 115 (59%) and HM12470 (70%) showed higher % residual levels than insulin (8%) and HM12460A (49%) after prolonged incubation in human serum.

Therefore, our results suggest that the long-acting properties of insulin 115 and HM12470 are based on combined effects of both reduced in vitro activity, less internalization, and improved stability in the cellular/serum compartments.

BACKGROUND

- **LAPSInsulin 115 is composed of insulin 115 and human IgG Fc portion conjugated via a non-peptidyl linker.**
- **Insulin 115 is designed to attenuate receptor mediated clearance and protease resistance.**
- **Aim of this study is to investigate underlying mechanisms of long acting properties of LAPSInsulin 115 based on insulin/insulin receptor destiny.**

Destiny of insulin/insulin receptor



1. Insulin binds to its receptor
2. Insulin receptor phosphorylation
3. Insulin & receptor internalization
4. Insulin degradation

Investigating points

1. How strongly does LAPSInsulin 115 bind to its receptor?
2. How well does LAPSInsulin 115 activate its receptor?
3. What fraction of receptors are internalized?
4. How stable is LAPSInsulin 115 in serum/cellular level?

METHODS

- Receptor binding affinity was measured by SPA (scintillation proximity assay). Insulin and hIR-B were loaded into picoplate-96 before WGA PVT SPA bead and 250 pM of ^{125}I-Insulin were added to each picoplate-96 well. After a four-hour incubation, radioactivity was measured using a scintillation counter. Binding affinity (IC_{50}) of insulin was determined in competition with ^{125}I-Insulin. Insulin receptor was prepared by membrane isolation from CHO cells which overexpress hIR-B.
- Receptor phosphorylation was measured in hIR-B overexpressing CHO cells. Cells were stimulated with insulin for 10 min and then lysed. Phosphorylated insulin receptor (Y1150/1151) in cell lysate was measured using Phospho-Insulin Receptor β Sandwich ELISA Kit (Cell Signaling).
- Receptor internalization was determined by measuring the remaining amount of receptor in 3T3-L1 adipocytes. Cells were pretreated with 1 μM of insulin at 37°C for 24 h. Cell surface-bound ligands were removed by acidic wash using PBS (pH 3.0) and the remaining cell-surface receptors were detected by binding of 100 pM of ^{125}I-Insulin at 4°C for 4 h.
- Cellular stability of insulin was measured in HepG2 cells. Cells were treated with 500 nM of insulin at 37°C for 0-48 h (insulin/insulin 115) or 0-72 h LAPSInsulin/LAPSInsulin 115). Cells' conditioned media was harvested and residual insulin concentrations were measured using Human Insulin ELISA Kit (Alpco).
- Serum stability was determined in rat and human serum. Insulin was spiked into rat serum or pooled individual human serum specimens at 0.01 mg/mL (insulin/insulin 115) or 1 mg/mL (LAPSInsulin/LAPSInsulin 115) final concentration. After incubating the spiked serum at 37°C for 0-8 days, residual insulin amounts were measured using Human Insulin ELISA Kit (Alpco).
- For the PK of LAPSInsulin 115, 65.1 nmol/kg was subcutaneously injected to SD rats. Serum concentration of test articles were determined using a modified ELISA assay and PK parameters were calculated by a non-compartmental method. In an acute study, 4-hr fasting blood glucose level was measured every day after s.c administration of test articles in db/db mice.

RESULTS

In vitro properties of insulin 115 and LAPSInsulin 115

Table 1. Relative *in vitro* properties comparison of insulin 115 and LAPSInsulin vs insulin and LAPSInsulin

Test materials	Insulins		LAPSInsulins	
	Insulin	Insulin 115	LAPSInsulin	LAPSInsulin 115
Receptor binding affinity[1] (%IC_{50} *vs* insulin)	100	86	2.6	1.7
Receptor phosphorylation[2] (% EC_{50} *vs* insulin)	100	63	5.6	2.0
Receptor internalization[3] (% internalized *vs* insulin)	100	74	30	20

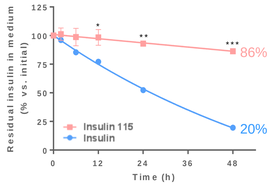
[1] Receptor binding affinity was conducted in IR-B/CHO by SPA method.
[2] Receptor phosphorylation was done in IR-B/CHO.
[3] Receptor internalization was measured by residual surface receptor by ^{125}I-Insulin in 3T3-L1 cell.
% internalized =(initial-residual)$_{sample}$ / (initial-residual)$_{insulin}$ * 100

➢ LAPSInsulin 115 had lower affinity for the human insulin receptor-B than insulin and LAPSInsulin. Similar results were observed when comparing receptor phosphorylation and internalization.

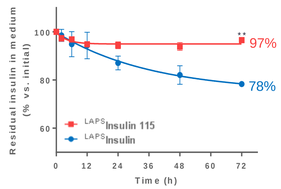
Stability of insulin 115 and LAPSInsulin 115 in a cell environment

Figure 1. Cellular stability of insulin 115 and LAPSInsulin vs insulin and LAPSInsulin

(a) **Insulin 115 *vs* Insulin** in HepG2 cell



(b) **LAPSInsulin 115 *vs* LAPSInsulin** in HepG2 cell

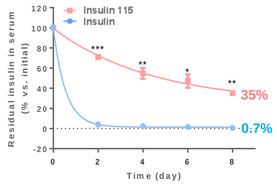


➢ Insulin 115 and LAPSInsulin 115 were more stable than insulin or LAPSInsulin in conditioned media of HepG2 cells. After LAPS-conjugation, the stability improved compared with the stability of peptide alone.

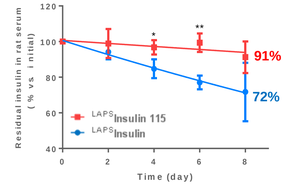
Serum stability of insulin 115 and LAPSInsulin 115

Figure 2. Serum stability of insulin 115 and LAPSInsulin 115 vs insulin and LAPSInsulin

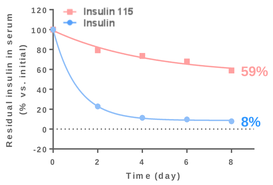
(a) **Insulin 115 *vs* Insulin** in **rat** serum



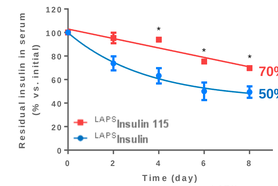
(b) **LAPSInsulin 115 *vs* LAPSInsulin** in **rat** serum



(c) **Insulin 115 vs insulin** in **human** serum



(d) **LAPSInsulin 115 *vs* LAPSInsulin** in **human** serum

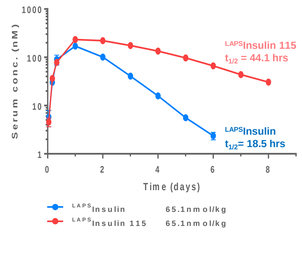


➢ Insulin 115 and LAPSInsulin 115 are more stable than insulin or LAPSInsulin in rat/human serum, respectively.

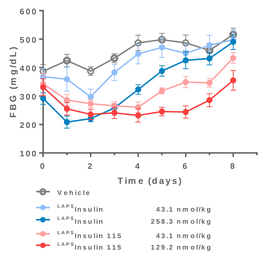
Prolonged PK/PD properties of LAPSInsulin 115

Figure 3. Prolonged PK/PD of LAPSInsulin 115 vs LAPSInsulin

(a) **PK of LAPSInsulin 115 *vs* LAPSInsulin** in SD rats (n=3)



(b) **PD of LAPSInsulin 115 vs LAPSInsulin** in *db/db* mice (n=7, 4-hr FBG)



➢ LAPSInsulin 115 showed 2.4 fold extended $t_{1/2}$ in SD rats *vs* LAPSInsulin which has a once weekly profile in *db/db* mice. LAPSInsulin 115 showed prolonged glucose lowering effect compared to LAPSInsulin at the same dose and at a even at much lower dose in *db/db* mice.

CONCLUSIONS

- **Insulin 115 and LAPSInsulin 115 have lower receptor binding affinity to hIR-B vs insulin and LAPSInsulin. Lower receptor binding affinity of insulin 115 and LAPSInsulin 115 may trigger less receptor phosphorylation and internalization than insulin.**
- **Insulin 115 and LAPSInsulin 115 shows higher cellular stability. The improved cellular stability of insulin 115 and LAPSInsulin 115 may be derived from weaker receptor binding affinity as well as improved serum stability.**
- **Taken together, the long-acting properties of insulin 115 and LAPSInsulin 115 may be associated with the combined effects of both reduced *in vitro* activity and improved stability in the cellular/serum compartments.**

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Cells chronically exposed to the novel weekly insulin HM12470 are protected against desensitization of the insulin signaling cascade

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Abstract

The novel basal insulin HM12470 consists of an insulin analog (Insulin 115) conjugated to the human aglycosylated F_C fragment via a small PEG linker and is developed for once-weekly administration. In the present study, we mimic chronic exposure to HM12470 *in vitro* to analyze the insulin-induced desensitization of target cells.

To establish an *in vitro* model of chronic insulin stimulation, human coronary artery smooth muscle cells (hCASMC) were used in light of their high insulin sensitivity and a good tolerance towards long-term treatment. Cells were exposed for 5 days to 500 nM regular insulin (reg. Ins) or HM12470 and allowed to recover for up to 48 h. After 5 days of chronic exposure, insulin receptor (IR) protein abundance was significantly decreased in both HM12470 and reg. Ins treated cells by 42 and 78 %, respectively. However, in the HM12470 exposed cells, IR levels recovered completely within 48 h in contrast to reg. Ins. Interestingly, under these conditions IR mRNA levels were not altered, indicating a more efficient translational and/or post-translational recovery of IR in HM12470 treated cells. The most prominent effect was observed at the signaling level, where the insulin stimulated Akt phosphorylation dropped down tremendously in both HM12470 (to 28%) and reg. Ins (to 12%) treated cells. However, total Akt levels were not altered. Insulin signaling in the HM12470 treated cells was almost completely restored after 48 h recovery (72%), whereas the reg. insulin treated cells showed no significant improvement under these conditions.

In conclusion, cells chronically exposed to the basal insulin HM12470 showed an improved recovery flexibility of the insulin signaling cascade probably due to changes of IR translation or processing. Thus, HM12470 does not irreversibly desensitize target cells under chronic exposure, and therefore represents an excellent candidate for a weekly insulin.

HM12470 exhibits a prolonged PK and PD profile compared to ^{LAPS}Insulin

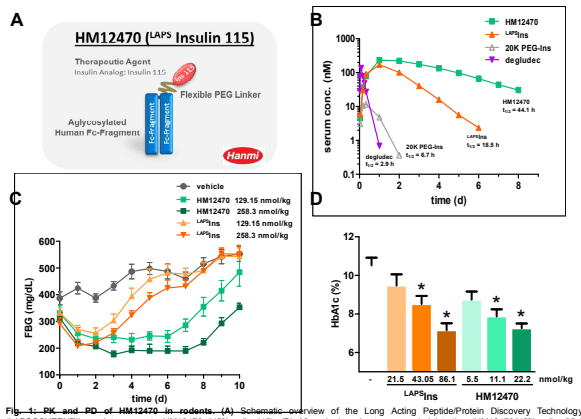


Fig. 1: PK and PD of HM12470 in rodents. (A) Schematic overview of the Long Acting Peptide/Protein Discovery Technology (LAPSCOVERY™) used to generate HM12470 (^{LAPS}Insulin 115). **(B)** After single subcutaneous administration (HM12470/^{LAPS}Insulin: 65.1 nmol/kg; 20K PEG-Insulin: 65.1 nmol/kg; insulin degludec: 55.8 nmol/kg) blood samples were collected at indicated time points, followed by analyzing serum concentrations of the different insulin molecules by ELISA (n=3-5; mean±S.E.M.). **(C)** After a single subcutaneous administration of HM12470 or ^{LAPS}Insulin to $^{db/db}$mice blood samples were collected at indicated time points after 4 h fasting, and blood glucose levels were measured with a One Touch Ultra Glucometer (n=7; mean±S.E.M.). **(D)** HM12470 and ^{LAPS}Insulin were subcutaneously administrated every other day (Q2D) to $^{db/db}$mice. Blood samples were collected after 4 weeks of repeated dosing and HbA1c was determined by DCA vantage analyzer (n=6; mean±S.E.M.; *p<0.05 compared to vehicle). Ins, insulin; 20K PEG-Ins, 20 kDa PEG-Insulin; reg., regular.

After long-term stimulation HM12470 has a similar mitogenic potency compared to regular insulin

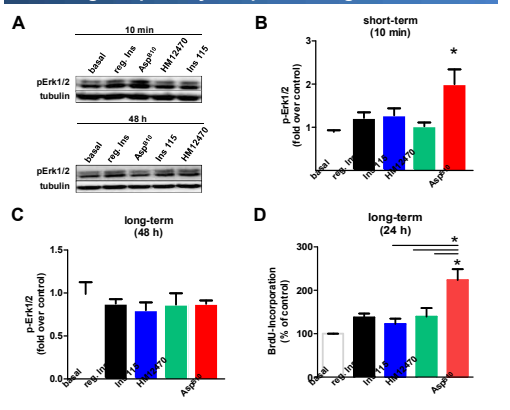


Fig. 2: Effect of HM12470 on the MAPK pathway.(A-C) Human coronary artery smooth muscle cells (hCASMC) were treated short-term (10 min) or long-term (48 h) with 100 nM of the indicated insulins. Erk1/2 phosphorylation was assessed by Western blot analysis as described before. **(A)** Representative Western blots and **(B,C)** respective quantifications are shown. Data are tubulin normalized (n=5-6; mean±S.E.M.; p<0.05 compared to basal). **(D)** Proliferation of hCASMC was determined by measuring the incorporation of BrdU into DNA. Cells were treated with 100 nM of the indicated for 24 h **(d)**. Data are expressed relative to the basal control value taken as 100% (n=6; mean±S.E.M.; *p<0.05 compared to basal or as indicated). Ins, insulin; reg. Ins, regular insulin; BrdU, Bromodeoxyuridine.

After long-term stimulation HM12470 has a similar metabolic potency compared to regular insulin

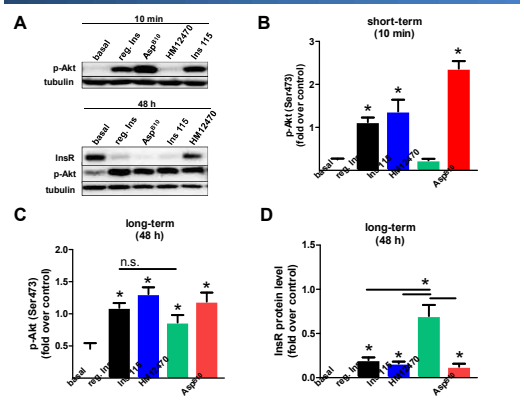


Fig. 3: Effect of HM12470 on the Akt pathway. (A-D) Human coronary artery smooth muscle cells (hCASMC) were treated short-term (10 min) or long-term (48 h) with 100 nM of the indicated insulins. Akt(Ser473) phosphorylation and insulin receptor protein abundance was assessed by Western blot analysis as described before. **(A)** Representative Western blots and **(B-D)** respective quantifications are shown. Data are tubulin normalized (n=5-6; mean±S.E.M.; p<0.05 compared to basal). Ins, insulin; reg. Ins, regular insulin;.

Improved insulin signaling after chronic exposure to HM12470 compared to regular insulin

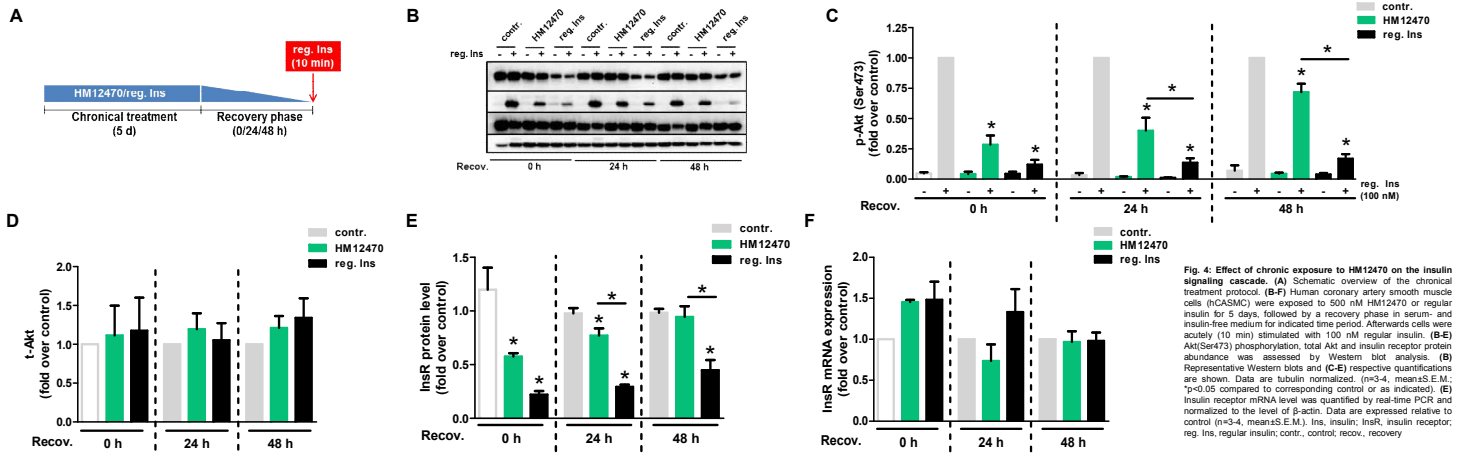
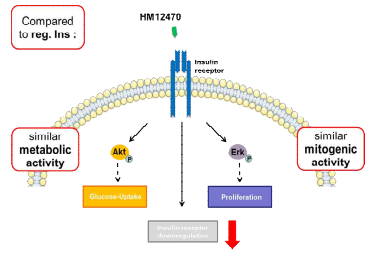


Fig. 4: Effect of chronic exposure to HM12470 on the insulin signaling cascade. (A) Schematic overview of the chronical treatment protocol. **(B-F)** Human coronary artery smooth muscle cells (hCASMC) were exposed to 500 nM HM12470 or regular insulin for 5 days, followed by a recovery phase in serum- and insulin-free medium for indicated time period. Afterwards cells were acutely (10 min) stimulated with 100 nM regular insulin. **(B-E)** Akt(Ser473) phosphorylation, total Akt and insulin receptor protein abundance was assessed by Western blot analysis. **(B)** Representative Western blots and **(C-E)** respective quantifications are shown. Data are tubulin normalized. (n=3-4, mean±S.E.M.; *p<0.05 compared to corresponding control or as indicated). **(E)** Insulin receptor mRNA level was quantified by real-time PCR and normalized to the level of β-actin. Data are expressed relative to control (n=3-4, mean±S.E.M.). Ins, insulin; InsR, insulin receptor; reg. Ins, regular insulin; contr., control; recov., recovery

Summary & conclusion

HM12470 displays…

- Substantially **prolonged pharmacokinetic** and **pharmacodynamic** profile
- **No** increased **mitogenic potency** compared to regular insulin
- **Similar metabolic potency** compared to regular insulin after long-term stimulation
- **Decreased** insulin receptor **downregulation**
- **Improved insulin signaling** under chronic conditions



HM12470 exhibits a unique action profile and thereby represents an excellent candidate for once-weekly administration

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