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Journal

Biochemical and Biophysical Research Communications, 475(3)

ISSN

0006-291X

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Publication Date

2016-07-01

DOI

10.1016/j.bbrc.2016.04.149

Peer reviewed

HHS Public Access

Author manuscript

Biochem Biophys Res Commun. Author manuscript; available in PMC 2016 July 01.

Published in final edited form as:

Biochem Biophys Res Commun. 2016 July 1; 475(3): 295–300. doi:10.1016/j.bbrc.2016.04.149.

A bitter pill for type 2 diabetes? The activation of bitter taste receptor TAS2R38 can stimulate GLP-1 release from enteroendocrine L-cells

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Abstract

The bitter taste receptor TAS2R38 is a G protein coupled receptor (GPCR) that has been found in many extra-oral locations like the gastrointestinal (GI) system, respiratory system, and brain, though its function at these locations is only beginning to be understood. To probe the receptor's potential metabolic role, immunohistochemistry of human ileum tissues was performed, which showed that the receptor was co-localized with glucagon-like peptide 1 (GLP-1) in L-cells. In a previous study, we had modeled the structure of this receptor for its many taste-variant haplotypes (Tan et al. 2011), including the taster haplotype PAV. The structure of this haplotype was then used in a virtual ligand screening pipeline using a collection of ~2.5 million purchasable molecules from the ZINC database. Three compounds (Z7, Z3, Z1) were purchased from the top hits and tested along with PTU (known TAS2R38 agonist) in *in vitro* and *in vivo* assays. The dose-

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2016.04.149.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.04.149.

response study of the effect of PTU and Z7 on GLP-1 release using wild-type and TAS2R38 knockout HuTu-80 cells showed that the receptor TAS2R38 plays a major role in GLP-1 release due to these molecules. *In vivo* studies of PTU and the three compounds showed that they each increase GLP-1 release. PTU was also chemical linked to cellulose to slow its absorption and when tested *in vivo*, it showed an enhanced and prolonged GLP-1 release. These results suggest that the GI lumen location of TAS2R38 on the L-cell makes it a relatively safe drug target as systemic absorption is not needed for a TAS2R38 agonist drug to effect GLP-1 release.

Keywords

GLP-1; Diabetes; Enteroendocrine L-cell; GPCR structure prediction; Virtual ligand screening; Gut receptors

1. Introduction

The surface of GI tract epithelium is endowed with molecular sensing machinery that detects dietary constituents and gut microbial metabolites [1⁻4]. Many types of enteroendocrine cells have been identified and are classified for the most part by their specific contents of endocrine transmitters. Key examples include enteroendocrine I-cells containing cholecystokinin (CCK); and L-cells containing glucagon-like peptide-1 (GLP-1) and peptide tyrosine tyrosine (PYY). The release of these agents into the blood results from interaction of sensors on the cells' lumenal surface to nutrient or environmental factors in the contents of the intestine. Each has specific and necessary functions on gastrointestinal tract responses including local and systemic metabolism.

GLP-1 is derived from the transcription product of the proglucagon gene. The biologically active forms of GLP-1 are: GLP-1-(7–37) and GLP-1-(7–36)NH_2 [5]. Once in the circulation, GLP-1 has a half-life of under 2 min due to rapid degradation by the enzyme dipeptidyl peptidase-4 (DPP4) [6,7]. It is a potent antihyperglycemic hormone, inducing glucose-dependent stimulation of insulin secretion while suppressing glucagon secretion. Such glucose-dependent action is particularly attractive because when the plasma glucose concentration is in the normal fasting range, GLP-1 no longer stimulates insulin to cause hypoglycemia. GLP-1 restores the glucose sensitivity of pancreatic β-cells, using a mechanism involving the increased expression of GLUT2 and glucokinase. GLP-1 also inhibits pancreatic β-cell apoptosis, stimulates the proliferation and differentiation of insulin-secreting β-cells, and inhibits gastric secretion and motility [5,8,9]. This delays gastric emptying which promotes satiety and weight loss. In fact, GLP-1 analogs as well as inhibitors of endogenous GLP-1 degradation have been developed that demonstrate efficacy for treatment of type-II diabetes mellitus, which is the type associated with obesity [5,9]. Not only have the analogs been demonstrated to significantly improve insulin secretion and glucose control, they have been found to decrease gastric emptying and increase satiety resulting in weight loss-benefits [10]. L-cells also release two circulating forms of PYY: PYY1-36 and PYY3-36 [11,12]. The latter form is considered the predominant one in both fasted and fed states and is produced by the cleavage of the N-terminal Tyr-Pro residues from PYY1-36 by peptidase enzyme DPP4. PYY inhibits food intake via PYY-2 receptors

expressed in neurons of the arcuate nucleus of the hypothalamus [13]. Other actions of PYY include slowing of gastric emptying and slowing small intestine motility.

Numerous studies have used animal and human models to probe the fundamental mechanistic roles of nutrient sensing receptors in the gut [14–25]. These studies have identified taste receptors (sweet, umami, and bitter) as well as fatty acid receptors (activated by a broad range of chain lengths in the fatty acids) in various enteroendocrine cells. Sweet and umami taste receptors are most likely sensing/tasting [15] energy nutrients and amino acids in the food, whereas the bitter taste receptors are potentially sensing/tasting any harmful and toxic constituents in the food. Once these food components are sensed, several metabolic pathways are activated. In the case of bitter sensing components, pathways that slow down gastric emptying and food absorption are likely activated. Many of these associations are not very well characterized especially in the context of human physiology and are being slowly uncovered. Several studies have shown that release of GLP-1 or other hormones like PYY, CCK, and ghrelin can be effected by activating one of these sensors [20,26–33]. These associations are critical to understand the detailed role of these gustatory gut sensors in food digestion.

Physiologic roles for the peptide hormones released from the enteroendocrine cells lining the lumen of the GI tract have been known for some time, but the mechanisms underlying the “sensing” and secretion of the hormones by intestinal contents were not known. Findings are now emerging that taste receptors, previously thought to be restricted to the tongue epithelium, are also present in the stomach, small intestine and colon. Several enteroendocrine cell types express TAS2R-family bitter taste receptors and T1R2/3 sweet taste receptors. In fact, the enteroendocrine cells secrete GLP-1, peptide YY (PYY) or CCK in response to bitter ligands like phenylthiocarbamide (PTC) and denatonium, which activate bitter taste receptors TAS2R38 and TAS2R47 respectively [20,25,32–35].

To understand the nutrient mediated signaling of L-cells in the context of peptide hormone release, we focused on a bitter taste receptor TAS2R38 to investigate its mechanistic role in the release of the GLP-1 hormone from L-cells. This understanding will advance novel therapeutic avenues for targeting type 2 diabetes, specifically using bitter (but safe) components of food that can target bitter taste receptors like TAS2R38. In the long term, these advances are also expected to show that some bitter constituents of foods are functional and therapeutically beneficial.

In the current study, we first investigated if the bitter taste receptor TAS2R38 is co-localized with GLP-1 in the L-cells using human GI tissue samples from the Cedars-Sinai Biobank. Then we tested if using TAS2R38 ligands can cause GLP-1 release from L-cells. Both PTU and PTC are agonists for this receptor, and we used PTU as a reference ligand for all studies. We performed an *in silico* ligand screening using our previously published structure for the taster haplotype PAV of this receptor [36] to identify novel agonists for this receptor that can be used to probe its signaling and also serve as lead compounds as potential therapeutics. This identified three novel compounds which were tested along with PTU in *in vitro* and *in vivo* assays measuring GLP-1 release. The *in vivo* studies also involved the use of PTU conjugated with cellulose to see if reducing PTU absorption, thereby prolonging its presence

in the lumen, will cause prolonged GLP-1 release or not. This is important because the target receptor TAS2R38 is located in the gut and any potential therapeutic targeting of that receptor should be gut-restricted to minimize potential side-effects coming from systemic exposure of the drug.

2. Materials and methods

The human ileum tissues from Cedars-Sinai Biobank using IRB protocol 34332 were analyzed by using immunohistochemistry (IHC) methods for co-localization of TAS2R38 with GLP-1 to see if the receptor was present in the L-cells. In a parallel study, the previously determined structure of the taster haplotype PAV of TAS2R38 [36] was used in a structure-based virtual ligand screening of purchasable compounds from the ZINC database [37]. Three of the top hit molecules were purchased and tested in *in vitro* assays utilizing HuTu-80 cells and *in vivo* assays for their potential of GLP-1 release. One of the molecules was also tested with TAS2R38 knockout cells. The methods and materials used for these studies are described below.

2.1. Immunohistochemistry

The experiments were designed to determine if the TAS2R38 receptor is expressed on the human enteroendocrine L-cells. To identify if it is co-localized with GLP-1 on the native L-cells, we performed IHC using previously validated GPCR and GLP-1 antibodies by double immune-staining on human GI tissues. The antibodies used were: TAS2R38 [rabbit polyclonal (H: ab65509, Abcam)] and GLP-1 [goat polyclonal (sc-26637, Santa Cruz Biotechnology)]. The numbers of cells staining GLP-1 or TAS2R38, or both were counted visually.

2.2. Virtual ligand screening

We used the PTU-bound PAV conformation predicted previously [36] in a virtual ligand screen (VLS) study using the DOCK Blaster server [38] which has access to several compound libraries including one with ~2 million commercially available compounds from the ZINC database [39]. For each ligand molecule, this server docks multiple ligand conformations corresponding to its internal torsional degrees of freedom into a putative binding site provided by the user and ranks the molecules by a scoring function. We took the top 500 hits from this server and prioritized them using a more accurate scoring function based on an all-atom Dreiding force field [40] to select top 200 diverse small molecules corresponding to different chemical scaffolds. We selected top 15 molecules from this list and purchased three of them for further studies.

2.3. PTU-cellulose synthesis

The synthesis reaction for PTU-cellulose conjugate molecule is summarized in Fig. 2. PTU with a carboxyl group substituted for a methyl group was synthesized by the condensation reactions starting with 7-ethoxy-5,7-dioxoheptanoic acid and thiourea. Then, the carboxyl-substituted PTU was conjugated with the hydroxyl groups of cellulose using *N, N′*-diisopropylcarbodiimide (DIC) and 4-dimethylaminopyridine (DMAP) in the DMSO. The structure of the product was confirmed by using IR spectroscopic methods.

2.4. In vitro studies

2.4.1. GLP-1 release—HuTu-80 cells (HTB-40, ATCC) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 4.5 g/L glucose, supplemented with 10% FBS and PSG antibiotic mix and maintained at 10% CO_2 and 37 °C. For experiments, these cells were seeded in 6-well plates or 100-mm^2 tissue culture dishes, propagated to confluence (5–7 days) and arrested in serum-free media overnight prior to treatments. Prior to the experiment, spent media was replaced with OPTI-MEM (Gibco, Life Technologies, Grand Island, NY) containing Halt Protease and Phosphatase Inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL) and individual wells dosed for 30 min. For the experiments, we determined dose dependent GLP-1 release from the HuTu-80 cells into the media. The media from cells was collected and frozen for subsequent peptide hormone release measurement using a Luminex MAGPIX metabolism multiplexing assay kit (HMHMAG-34 K) following the manufacturer's instructions.

2.4.2. Inhibition of GPCR expression—In order to determine the specific GPCR mediating the response of a particular ligand, we performed *in vitro* RNA silencing of TAS2R38. RNA silencing was performed using a commercial kit (FlexiTube siRNA, Qiagen) consisting of 4 predesigned oligonucleotides that specifically target each gene transcript. Each kit also had accompanying controls and quantifying assays for detection of gene knockdown. Cultured HuTu-80 cells were transfected with single and mixed oligonucleotide siRNAs for TAS2R38 using HiPerFect transfection reagent (Qiagen) or Lipofectamine 2000 (Life Tech) according to the procedure recommended by the manufacturer. Cells were maintained in the basal medium for up to 48 h. Gene knockdown was evaluated by RT-qPCR and Western blot by suitable antibody to ensure that the inhibition occurred. In the parallel set of transfected HuTu-80 cells, effective dose of specific ligand was used to stimulate GLP-1 release. Dose-response curve of the ligand with and without siRNA treatment was generated.

2.5. In vivo studies

The ligands being tested were administered by gastric gavage to pathogen-free, 6-to 8-week-old male BALB/c mice: predicted ligands, PTU-cellulose conjugate; PTU at the same amount calculated to be in the PTU–cellulose; or cellulose alone. Retro-orbital blood was obtained and GLP-1 was measured at regular intervals.

3. Results and discussion

The use of immunohistochemistry methods on human GI tissue from ileum and colon using the Cedars-Sinai Medical Center BioBank and its Microscopy Core identified many cells co-localized with TAS2R38 and GLP-1. We considered cells staining with antibody to GLP-1 to be L-cells. An example staining with ileum tissue is shown in Fig. 1, where panel D shows GLP-1 co-localized with TAS2R38, which is seen predominantly on the surface of the cell and in higher levels facing the lumen. In addition, visual counting of stained cells covering ileum and colon tissue showed that ~8% of ileum L-cells and ~12% of colon L-cells had staining for TAS2R38. The detailed numbers are shown in Table S1. These should

be considered as qualitative numbers as it was challenging to get a quantitative count of co-localization due to the small surface area of the apical and lumen facing surface of the pyramidal shaped L-cells.

In a previous study we had modeled the structures of many taste haplotypes of the TAS2R38 receptor [36], which included the structures for the taster haplotype PAV, the non-taster haplotype AVI, and intermediate haplotypes (AAI and PVV). The virtual ligand screening using the structure of PAV haplotype led to the identification of ~25 molecules as top hits. Three of these compounds (Z32767960, Z737342178, and Z146157286) were purchased and tested further *in vitro* and *in vivo* assays. Their structures are shown in Fig. 2 and they will be referred to as Z7, Z3, and Z1 respectively in the remainder of the text. Fig. 2 also includes structures for PTU and the synthesis steps for PTU-cellulose.

The dose-response study of the effect of PTU and Z7 on GLP-1 release using wild-type and TAS2R38 knockout HuTu-80 cells was carried out. As shown in Fig. 3, PTU and compound Z7 caused GLP-1 release, which were right-shifted in cells with knock down for TAS2R38. Figs. S1, S2, and S3 show the receptor expression and knockdown in HuTu-80 cells using a TAS2R38 receptor siRNA. These results strongly suggest that PTU and Z7 ligands interact with this receptor. The fact that greater concentrations of the ligands were able to cause GLP-1 release with decreased receptor expression indicates that either these ligands also interact with other receptors to cause GLP-1 release or that only a small fraction of receptors are necessary to provide a full response. The former is consistent with the knowledge compiled on bitter taste receptors and their ligands about the promiscuity of these receptors and their ligands [41]. The fact that Z7 molecule caused GLP-1 responses provides a strong validation of our computational modeling methods for discovering novel ligands. These preliminary data also suggest that HuTu-80 cell's GLP-1 release is sensitive and specific to some bitter ligands making these cells ideal models for a fast screening of new compounds.

In vivo studies of the three predicted compounds along with PTU and PTU-cellulose conjugate on postprandial GLP-1 release are shown in Fig. 4. The data showed that all three predicted compounds (Z7, Z3, Z1) can cause GLP-1 release in animals. In addition, they all showed higher GLP-1 levels than PTU. These results show that we are able to cause GLP-1 release with an agonist to TAS2R38 by gastric gavage administration. The GLP-1 release by three predicted molecules (Z7, Z3, Z1) validates our modeling method for identifying ligands for a specific receptor.

The PTU-cellulose conjugate was tested to see if the GLP-1 release can be sustained by slowing the absorption of PTU from the GI tract. Because the target receptor is accessible from the lumen, the potential drug molecules don't need to be systemically distributed for their pharmacological action. The results in Fig. 4 demonstrated that both PTU and the PTU-cellulose caused GLP-1 release into the blood, and the increase with the PTU-cellulose was greater in magnitude and longer in duration than the PTU alone. The cellulose alone had no effect. PTU-cellulose data shows that functionalizing PTU to reduce absorption can enhance the GLP-1 release, suggesting that gut restriction of potential drugs can be used to control GLP-1 release, in addition to minimizing side-effects arising from systemic circulation. Of

note, in these studies, the three predicted molecules were not conjugated to cellulose, but an optimized small molecule could be functionalized to keep it in the intestinal lumen.

These data indicate that activating TAS2R38 causes release of the gut peptide hormone GLP-1, making TAS2R38 a novel diabetes target as agents that increase GLP-1 levels and GLP-1 analogs are effective in diabetes management. However, the subcellular signaling pathways mediating gut peptides' release have not been fully explored, in terms of differences in $hTAS2R38_{PAV}$ and $hTAS2\text{-}R38_{AVI}$ haplotypes, due in part to technical challenges in handling enteroendocrine cells. In addition, as mentioned before, several other nutrient receptors (sweet taste, fatty acid, peptide receptors) have also been found [14] in enteroendocrine cells that release the gut peptides. These data suggest a complex set of signaling cascades that can modulate the release of gut peptides and potentially cause metabolic effects. This also makes the nutrient receptors very attractive therapeutic targets for metabolic diseases with abnormal gut peptide signaling. From the context of TAS2R38 described in this study, bitter (but safe) components of food that can activate this receptor can become promising therapeutic candidates. The results for TAS2R38 receptor presented in this study open the possibility to screen and identify novel "bitter" molecules that can activate this receptor and cause desirable metabolic effects especially for diabetes resolution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

RA acknowledges support in part from Materials and Process Simulation Center at Caltech when this project started and from start-up funds provided by Cedars-Sinai Medical Center. SJP acknowledges partial support for this project from NIH P01CA163200 and the Department of Veterans Affairs.

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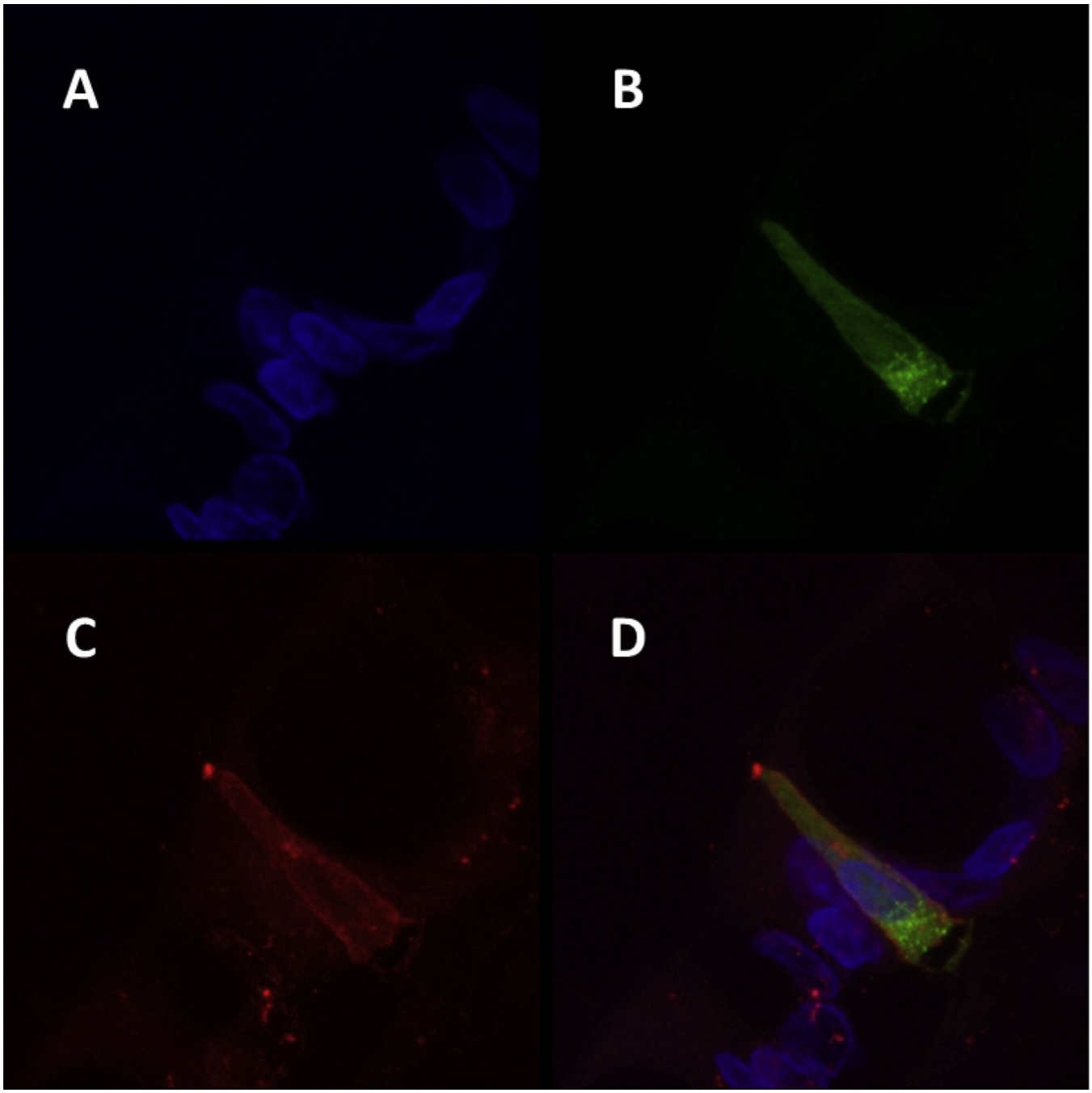


Fig. 1.

Immunohistochemical localization of TAS2R38 in human L-cells using ileum tissues from Cedars-Sinai Biobank. Antibodies used are mentioned in the text. **A**. Nuclear staining with DAPI; **B**. GLP-1 antibody staining; **C**. TAS2R38 antibody staining; **D**. Overlay of DAPI, GLP-1, and TAS2R38 stains to show co-localization.

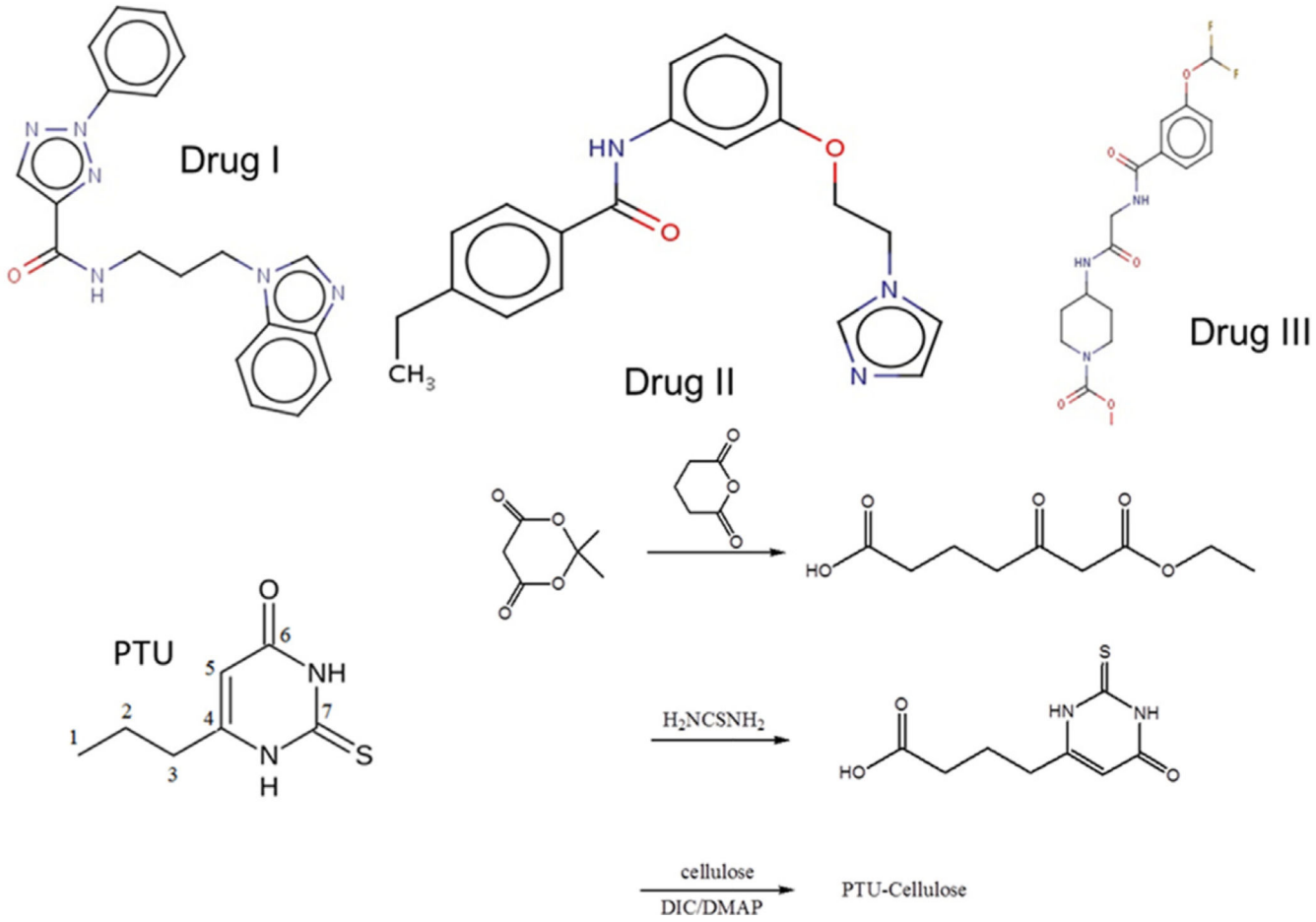


Fig. 2.

Structures of three ligands [Drug I (Z146157286 or Z1), Drug II (Z737342178 or Z7), and Drug III (Z32767960 or Z3)] predicted from virtual ligand screening, PTU, and the synthesis of PTU-Cellulose conjugate.

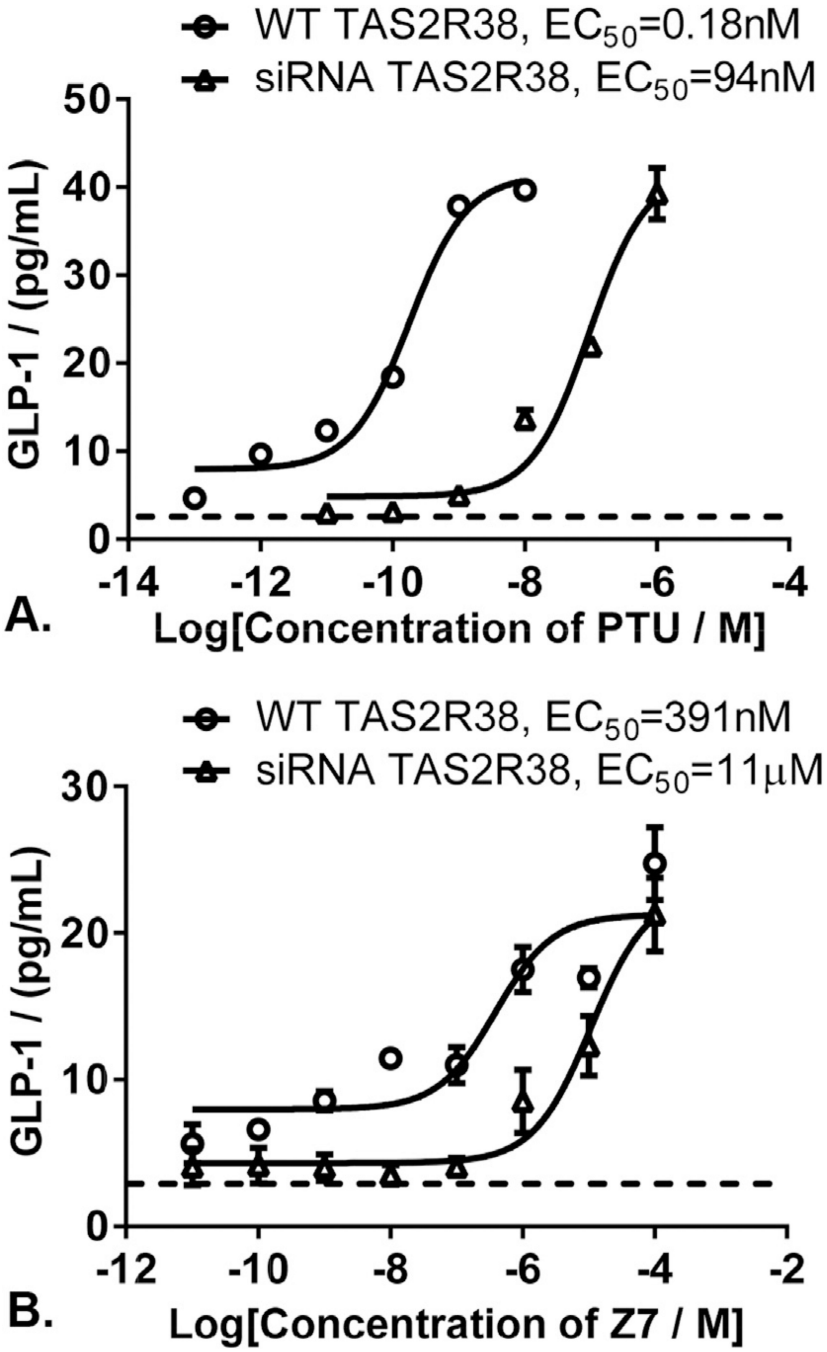
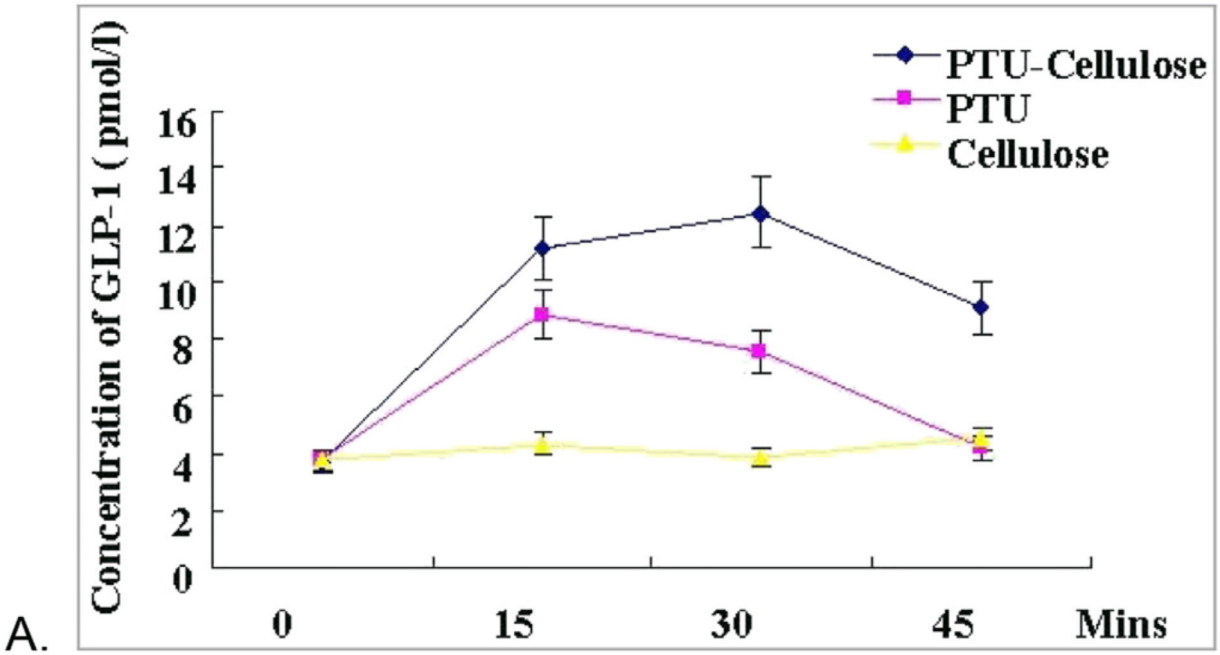


Fig. 3.

Stimulation of GLP-1 release by known TAS2R38 ligand PTU (**A**) and new predicted ligand Z7 (**B**) in cultured HuTu-80 cells, which were untreated or treated with vehicle and indicated concentrations for 30 min. Conditioned media were collected and frozen until GLP-1 measurement. Values are mean ± SE, N = 2(PTU), N = 3(Z7). GLP-1 is measured using Luminex assay. The dashed line refers to the background GLP-1 level observed without ligand.



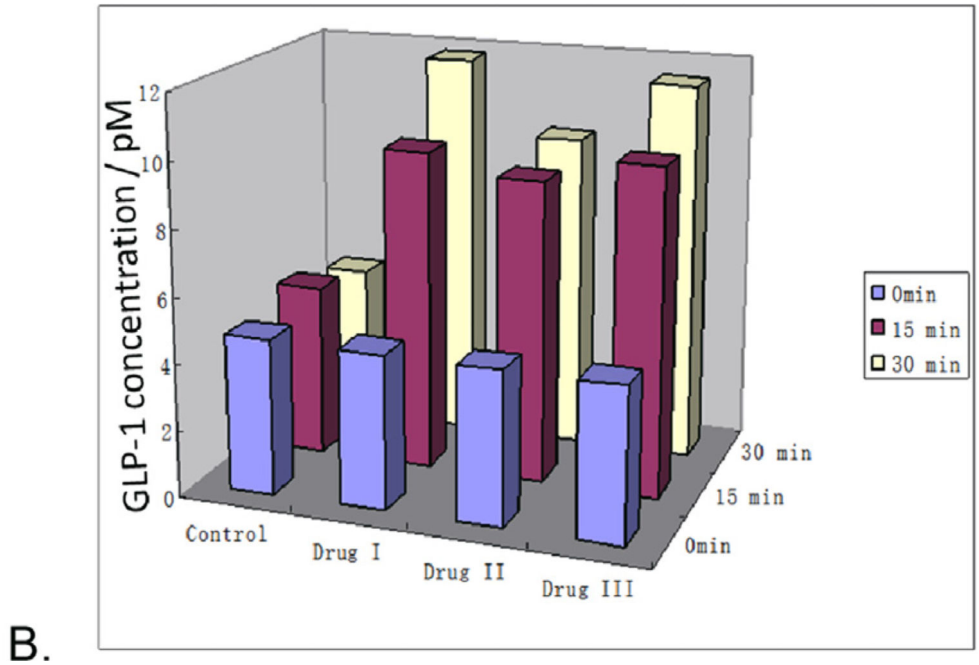


Fig. 4.

A: Average GLP-1 release in seven mice treated with PTU-Cellulose, PTU or Cellulose. The PTU-cellulose dose was 5 g/kg body weight; PTU was 200 mg/kg body weight; and cellulose was 5 g/kg body weight in Male C57Bl/6 mice (N = 3, $p < 0.005$). Blood was drawn at times indicated and GLP-1 measured in serum from the blood using the EGLP-35 K GLP-1 (Active) ELISA kit (Millipore, MA); **B: Average GLP-1 (pM) in six mice given one of the three predicted molecules** Drug I (Z1), Drug II (Z7), and Drug III (Z3). The standard deviations are: 0.7 pM, 1.9 pM, 2.6 pM at three time points 0 min, 15 min, 30 min

respectively for Drug I; 0.7 pM, 1.1 pM, 1.8pM for Drug II; and 0.7 pM, 0.9 pM, 1.4 pM for Drug III ($p < 0.005$ between drugs and control; $p < 0.001$ between time points).