Synthesis of alamandine glycoside analogs as new drug candidates to antagonize the MrgD receptor for pain relief

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Abstract

Two series of putatively brain-penetrant alamandine glycosides have been prepared for screening against the MrgD receptor. The first series retains the initial six residues of the alamandine sequence (ARVYIHP) as the “peptide message,” replacing the C-terminal proline (P) with several serine (S) glycosides at the C-terminus to produce “glycoside addresses.” In the second series, steric bulk was altered to modify the “peptide message”—the N-terminal alanine (A) residue was substituted with glycine (G); D-alanine (a); nor-valine (norV); D-nor-valine (D-norV); valine (V); and D-nor-valine (v), keeping the C-terminal serine-beta-D-glucoside (S-Glc) “glycoside address” constant. All the peptides and glycopeptides were synthesized as their C-terminal amides. The purity of native alamandine and its eleven selected derivatives were each confirmed using analytical HPLC. Also, the molecular weight and chemical composition were confirmed using mass spectroscopy. The MrgD receptor expression was evaluated in rationally chosen human cell lines, A549 and HEK 293. Both cell lines showed the presence of the MrgD receptor around 35 kDa, as confirmed by western blot analysis. The effect of varying concentrations of some alamandine derivatives on cell viability was evaluated on HEK 293 and A549 cell lines.

Graphical abstract
Keywords Renin angiotensin system · Alamandine · Cell culture · MrgD receptor · Pain · Antagonism

Introduction

The Renin-angiotensin system (RAS) is the central control web for the maintenance of fluid and electrolyte balance (K+/Na+), vascular resistance and plays an essential role in the formation of new blood vessels [1]. RAS also plays a vital role in atherogenesis [2], high blood pressure, and other pathological conditions [3]. Important vasoactive controls are mediated by the principal angiotensin receptors AT1 and AT2, which are agonized by the proteolytic cleavage products of Angiotensin I (Ang I), including Ang II, Ang III, Ang (1–7), Ang (1–9) and Ang IV [4, 5]. Angiotensin-Converting Enzymes 1 and 2 (ACE1, ACE2) and Neutral Endopeptidase (NEP) play important roles in these regulatory pathways [6]. It is now clear that there are more receptors and ligands involving a series of deca- to arboxylated angiotensins that have an alanine at the N-terminus instead of an aspartate residue, as represented by the heptapeptide alamandine, which may be regarded as the Ang (1–7) analog Ang-A (where A is the abbreviation for alanine) [7]. Centrally located G protein-coupled receptors (GPCR) such as Mas and the “Mas-Related GPCR receptors (MrgD or TGR7) are believed to play important, although largely undetermined roles in the CNS [2, 8, 9]. Although it seems that Ang (1–7) and alamandine act through different receptors, the biological activities of these peptides are similar, probably because of the high similarity in their structure [2, 10–12], and the in vivo activity of the alamandines may be related to the peptideas ACE1, AC2, or NEP. A potential application of the Ang (1–7)/Mas receptor axis is cancer-induced bone pain, as suggested by Forte et al. [13]. Another study by Hay et al. [14] suggests a novel role for Ang-(1–7) as a potential therapeutic agent for cognitive impairment results of heart disease. The novel glycosylated Ang-(1–7) peptide (PNA5) showed effects of inhibition of vascular contributions to cognitive impairment and dementia/heart failure (VCID/HF)-induced inflammation and rescues impaired cognitive function as suggested by Hay et al. [15]. Alamandine, through its receptor MrgD, is effective in reversing hyperhomocysteinemia-induced vascular dysfunction and, therefore, may reduce the development of atherosclerosis [16]. Due to the bioactivities of these compounds (Ang (1–7) and PNA5) and peptides in general, more attention increased to formulating them to be delivered to the upper (nose to the brain) and lower (inhailation to lungs) respiratory tract [17, 18].

The treatment of moderate to severe pain relies mainly on opioid agonists, which produce analgesic effects and several side effects. These effects include addiction liability, urinary retention, the development of tolerance, gastrointestinal effects, and respiratory depression [19]. In this study, our focus was to synthesize alamandine derivatives for exploring an antinociceptive axis that could be an alternative to opioids based on Alamandine-MrgD agonism. Mas-related genes are a family of GPCRs genetically related to the Mas receptor [12]; hence, they are termed Mas-related GPCR receptors. These newly recognized receptors have been divided into subfamilies according to sequence homology and according to single genes (MrgA, MrgB, MrgC, etc.). In mice, these receptors have undergone clonal expansion to provide a relatively large number of variants (MrgE, MrgF, MrgG, Mrg) that do not seem to be represented in humans or other mammals. MrgD is widely expressed in sensory neurons in the dorsal root spinal ganglia (DRG) in all mammalian species examined. Also, it is located in other organs but with lower expression levels, such as the brain, testes, heart, lung, gastrointestinal tract, etc. [12]. It is hypothesized that the “resting state” of MrgD is tonically active, and its natural ligands should be inverse agonists, which inhibit their activity [20]. Moreover, it was hypothesized that Mrgs did not evolve to recognize endogenous ligands but to cause itching or pain to warn an organism of detrimental substances released to its skin by toxic plants or animals or by parasitic infections. The support for this hypothesis: (1) Their high expression in sensory neurons and mast cells in the skin, the main cellular components of the itch pathway, (2) Their relative affinities for itch-inducing substances, and (3) Their evolutionary appearance in tetrapods together with arms and legs that allow for scratching [20]. Alamandine and non-proteogenic amino acid β-alanine are two known agonists for MrgD; β-alanine can internalize MrgD, inducing intracellular calcium influx and inhibiting cAMP production in the Chinese hamster ovary [21]. The effect in calcium influx suggested the connection of the MrgD with the G-protein α subunit (Gq). The cAMP suppression suggested an interaction between MrgD and an inhibitory regulative G-protein (Gi) [20, 21]. Furthermore, activation of MrgD by β-alanine also suppresses KCNQ/M-type potassium channels, increasing neuron excitability by the Go and phospholipase C (PLC) pathway. In other words, β-alanine-induced activation of MrgD results in robust inhibition of KCNQ2/3 currents and increased phasic neuron excitability [22, 23]. The expression of MgrD was reported in association with several pathologies, e.g., irritable bowel syndrome (IBS) [23] and several clinical cancers, specifically lung cancers, which show high expression.
Therefore, it has been suggested that there are many essential functions for the MrgD receptor since it is involved in pain pathways, sensitivity to thermal and mechanical stimuli, and tumorigenic activity [24]. For example, Bautzova et al. [25] demonstrated that 5-oxoeicosa-tetraenoic acid; an n-6 polyunsaturated fatty acid metabolite, which is selectively increased in colonic tissues from patients with IBS, induced hypersensitivity in an aspect dependent on the MrgD involved in the alteration of sensation to mechanical stimuli leading pain symptoms associated with IBS [25].

All the above suggests that blocking MrgD through antagonists can play a role in producing antinociception effects and neuropathic pain modulation. Alamandine is an MrgD agonist. Hence in this project, we focused on this agonist by synthesizing two sets of glycosylated alamandine peptide derivatives. The first set of glycopeptides was produced by changing the N-terminus amino acid to change the steric bulk while keeping the serine-Glucoside(ser-Glc) as the sugar moiety. In the second set, we synthesized the native alamandine, the native almandine without the proline residue, besides the other four derivatives by introducing different sugar moieties on the C-terminus of the peptides. We hypothesized that varying the N-terminus amino acid of the alamandine would increase the steric demand of the N-terminal amino acid to convert alamandine from an agonist to an antagonist to block the MrgD receptor. Furthermore, changing the sugar moiety may alter the binding affinity of the derivatives to the receptor. In addition, glycosylation is one of the most promising strategies to enhance membrane penetration, increase peptide stability, and enhance the bioavailability of peptides possessing a covalently attached sugar moiety as part of the peptide molecular structure [26]. Peptide glycosylation is known to lead to many improvements in stability and drug delivery, including targeting specific organs, enhancing biodistribution, facilitating the active transport by targeting glucose transporters, improving pharmacokinetic profiles, modulating pharmacodynamic responses, protecting amino acid side-chain oxidation, and stabilizing the physical properties (i.e., precipitation, aggregation, and thermal & kinetic denaturation) of peptides [27–30].

There are two objectives in this study. The first is the use of glycosylation to produce alamandine derivatives with improved physiochemical properties required for drug candidates. The second objective is to expand the potential use of these derivatives by varying the N-terminus amino acid and the sugar moiety at C-terminus to antagonize the MrgD receptor for pain relief. Furthermore, the MrgD receptor might be a novel anticancer drug therapy target, particularly for lung adenocarcinomas, as MrgD enhances tumorigenesis and is highly expressed in lung cancer [24]. Thus, the native alamandine and its eleven targeted derivatives were synthesized using published synthetic methods of solid-phase peptide synthesis (SPPS) [31] with high purity and confirmed molecular weight and chemical composition. The MrgD receptor expression was evaluated in rationally chosen human cell lines, A549 and HEK 293. Both cell lines showed the presence of the MrgD receptor around 35 kDa, as confirmed by western blot analysis. Furthermore, the effect of varying concentrations of the Alamandin glycopeptides derivatives on cell viability was evaluated on noncancer human cells HEK 293 and the human lung cancer cell line, A549.

**Results and discussion**

**Sugar moieties synthesis and solid phase peptide synthesis (SPPS)**

The three sugar moieties, ser-Glucoside (Ser-Glc), ser-Lactoside (Ser-Lact), and ser-Cellobiosed (Ser-Cell), were synthesized with purities that reached more than 95%. Figure 1 shows a representative analytical HPLC chromatogram of one of these carbohydrates, which is Ser-Glc. Following the sugar moieties preparation, a successful synthesis of native alamandine and all alamandine derivatives was performed. The preparative HPLC was applied to purify the alamandine derivatives crudes (Fig. 2), producing compounds with more than 98% purities, as confirmed by analytical HPLC with a retention time around 8.00 min (Fig. 3). Chemical atomic composition and molecular
weights for all compounds were performed after purification using mass spectroscopy (Fig. 4 and Table 1) and demonstrated the success of synthesizing the targeted compounds. Figure 5 shows the chemical structures of the synthesized compounds.

Our primary objective is to develop alamandine structures to antagonize the MrgD receptor. This receptor’s expression was found in DRG and co-localized with vanilloid receptor-1 (an essential receptor for pain and heat sensation). Furthermore, the genetic ablation of MrgD expressing neurons reduces behavioral sensitivity to noxious mechanical stimuli in mice [24]. And a hypothetical mode of action of β-alanine- MrgD activation would promote primary nociceptive excitability [22]. As alamandine is one of the few known agonists of MrgD, then trying to develop a derivative of this agonist by doing the above modifications in the native alamandine structure could change the ligand-receptor binding mode and affinity. It may cause antagonism of the MrgD receptor, which could be one of the antinociception players.

On the other hand, adding the carbohydrates to the peptide’s C-terminus would improve the penetration of
cellular barriers, such as the blood–brain barrier [26]. If the sugar’s attachment is in the appropriate position with optimum type and number, this is expected to increase hydrophilicity [28]. Moreover, glycosylation will enhance the transport of the glycopeptide throughout the body in vivo as the glycopeptide can “hop off” the membrane as the sugar moiety can provide kinetic assistance in pulling the membrane-bound glycopeptide away from the membrane [29]. In other words, a glycopeptide has two states; firstly, the membrane-bound conformations that enhance endocytosis/exocytosis, and secondly, a water-soluble random coil that promotes membrane hopping. A balance between these two states is desired so that the glycopeptide can achieve biodistribution and activity in vivo [32, 33].

Expression of MrgD and cell viability of alamandine glycopeptides

Since the expression of the MrgD receptor is variable in different cell types, its expression was tested utilizing western blot using two human cell lines (A549 and HEK 293). Based on the results, both cell lines showed the presence of the MrgD receptor around 35 kDa (Fig. 6a); this is in good agreement with the literature that demonstrated MrgD expression in both cell lines [11, 34, 35]. Cell viability as a function of glycopeptide concentration was performed to test the biocompatibility of the alamandine derivatives on human cells. A549 and HEK 293 were incubated with a selected alamandine derivative (compounds # 1 to 6) for 96 h. The tested alamandine derivatives

![Mass Spectrometry/ESI positive ionization for alamandine derivatives (Compounds# 1 to 6)](image_url)
with varying concentrations over a wide range of up to 1 mg/mL did not exert any significant cytotoxic effect or anomaly in cell morphology (Fig. 6b). A549 and HEK 293 cells proliferation were not significantly changed, and the results demonstrated the biocompatibility of the derivatives on the tested human cells up to a concentration of 1 mg/mL. Thus, none of the tested derivatives is a potential anticancer for the used cell lines. Nishimura et al. [24] showed that no report has revealed that beta-alanine promotes cancer development. Therefore, there might be another MrgD

![Table 1](image)

| Compound # | Chemical formula | Exact mass (g/mol)a | Molecular weight (g/mol)a | Single charged ion ([MH]+b) | Double charged ion ([M + 2H]+b) |
|------------|------------------|--------------------|--------------------------|----------------------------|-------------------------------|
| 1          | C_{33}H_{69}N_{13}O_{14} | 991.50869          | 992.10200                 | 992.492161                 | 496.749719                     |
| 2          | C_{44}H_{71}N_{13}O_{14} | 1005.52434         | 1006.12900                | 1006.507811                | 503.757544                     |
| 3          | C_{46}H_{73}N_{13}O_{14} | 1033.55564         | 1034.18300                | 1034.539111                | 517.773194                     |
| 4          | C_{46}H_{73}N_{13}O_{14} | 1033.55564         | 1034.18300                | 1034.539111                | 517.773194                     |
| 5          | C_{46}H_{75}N_{13}O_{14} | 1033.55564         | 1034.18300                | 1034.539111                | 517.773194                     |
| 6          | C_{46}H_{75}N_{13}O_{14} | 1033.55564         | 1034.18300                | 1034.539111                | 517.773194                     |
| 7          | C_{55}H_{56}N_{12}O_{7} | 756.43949          | 756.91000                 | 757.446769                 | 379.227023                     |
| 8          | C_{46}H_{73}N_{13}O_{8} | 853.49226          | 854.02700                 | 854.499532                 | 427.753404                     |
| 9          | C_{49}H_{78}N_{14}O_{15} | 1102.57711         | 1103.24600                | 1103.584384                | 552.295830                     |
| 10         | C_{44}H_{71}N_{13}O_{14} | 1005.52434         | 1006.12900                | 1006.531620                | 503.769448                     |
| 11         | C_{50}H_{81}N_{13}O_{19} | 1167.57717         | 1168.27000                | 1168.584444                | 584.795860                     |
| 12         | C_{50}H_{81}N_{13}O_{19} | 1167.57717         | 1168.27000                | 1168.584444                | 584.795860                     |

*aAs calculated by ChemDraw Professional 16 software

*bMass spectroscopy analysis

![Fig. 5](image)

Fig. 5 Chemical structures of native alamandine (compound# 8) and its synthesized derivatives
ligand contributing to cancer development through MrgD. These suggest that MrgD is a robust potential target in cancer therapy, and its antagonists would provide promising anticancer treatment [24]. Accordingly, we will continue to work on testing the remaining derivatives in terms of cytotoxicity thus, for potential anticancer therapeutics. Moreover, we will modify the derivatives structures to achieve the desired level of antagonistic and anticancer effects depending on the results.

**Conclusion and future directions**

In this research, we describe for the first time the synthesis of a group of alamandine derivatives to block the activity of the MrgD receptor. Our rationale for this approach is the involvement of this receptor in itching and pain pathways, in addition to its overexpression in several pathological states and cancer. Also, we demonstrate the first steps toward testing these derivatives in human cell lines, specifically lung cell lines such as A549. The cells that showed expression of MrgD will be used in further studies to check each derivative’s activity as an antagonist. Also, the effect of the derivatives downstream from the MrgD signaling pathway and the affinity of those derivatives should be evaluated.

A parallel approach is to test the Almandine derivatives’ efficiency as anticancer agents since several studies confirm the overexpression of the MrgD receptor in cancer cells. Moreover, this high expression in cancer tissues and cells

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**Fig. 6** a Western blot analysis to show the expression of MrgD in A549 and HEK 293 cells, respectively. b Cell viability of A549 and HEK 293 human cells as a function of the concentrations of alamandine derivatives (1–6) for 96 h to determine biocompatibility.

X-axes and Y-axes represent the concentration of the glycopeptide’s derivatives and the relative cell viability of A549 and HEK 293. Data are mean ± SEM of at least three separate experiments.
may cause the promotion of tumor growth. So, we tested the cytotoxicity of one set of the alamandine derivatives in two human cell lines (A549 and HEK 293), which express the MrgD receptor. The cell viability results reveal that the tested alamandine derivatives are safe up to 1 mg/mL for these two cell lines; this gave a primary indication that these derivatives are not anticancer agents. However, as part of this project, we will check the MrgD expression in more human cell lines, specifically the cancer cells, to test the derivatives activities as MrgD antagonists using the potential cells besides studying the cytotoxicity of these derivatives in the same cells.

**Materials and methods**

**Materials**

Sugar moieties and solid phase peptide synthesis (SPPS) reagents

Lactose and Fmoc-D-norvaline-OH were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Cellobiose was obtained from BIOSNTH (Itasca, IL, USA). H-Ser-OBzl.HCL was obtained from SENN Chemicals (Dielsdorf, Zürich, Switzerland). Indium (III) bromide (InBr3) was obtained from Acros organics (New Jersey, USA). Anhydrous sodium acetate was obtained from AMRESCO Inc. (Solon, Ohio, USA). Fmoc-OSuc, 6-Chloro-1-hydroxybenzotriazole (CTHOBt), Fmoc-L-His (Trt)-OH, Fmoc-L-Ile-OH, Fmoc-L-Tyr(Bu)-OH, Fmoc-L-Val-OH, Fmoc-L-Ala-OH, Fmoc-L-Ser-OH, N-Methylmorpholine (MMP), and 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) were obtained from Chem-Impex INT'L INC (Wood Dale, IL, USA). Fmoc-Gly-OH was obtained from Advanced ChemTech (Louisville, KY, USA). Fmoc-Pro-OH, Fmoc-Nva-OH, 2-(1H-benzo-triazol-1-yl)-1,3,3,3-tetramethyluronium hexafluorophosphate, and N. N-Diisopropylcarbodiimide (DIC) were obtained from AAPPTec (Louisville, KY, USA). Anisole was obtained from Aldrich Chemical Co., INC (Milwaukee, WI, USA). Palladium activated carbon and N, N-Diisopropyllethylamine were obtained from Sigma-Aldrich, Co (St. Louis, MO, USA). N-methylpyrrolidone (NMP) was obtained from VWR Chemicals BDH (Radnor, PA, USA). Dichloromethane and ethyl acetate were obtained from fisher chemicals:(Fair Lawn, NJ, USA). N. N-Dimethylformamide (DMF), chloroform, sodium bicarbonate, sodium sulfate, and acetic anhydride were from EMD Millipore Corporation, an Affiliate of Merck. (Darmstadt, Germany). beta-D-Glucose pentaacetate, piperidine, and hydrazine monohydrate were obtained from Alfa Aesar (Ward Hill, MA, USA). Tri-fluoroacetic acid (TFA) and Triethylsilane (TES) were obtained from Oakwood chemical (Columbia, South Carolina, USA). Ether Anhydrous, Hexane, Hydrochloric Acid (HCL), and Acetonitrile were obtained from avantor—J.T. Baker’ (Radnor, PA, USA).

**In vitro cell culture assays (cell viability experiments) reagents**

Cell viability assay MTS phenazine methosulfate (PMS) (Promega, Madison, WI, USA). SynergyÔ HT Multi-detection Microplate Reader (BioTek, Winooski, VT, USA).

Cell culture and media A549 and HEK 293 were obtained from the Experimental Mouse Shared Resource Laboratory, Cancer Center at The University of Arizona (Tucson, AZ, USA). F-12K Medium (Catalog No. 30-2004) and Eagle’s Minimum Essential Medium (Catalog No. 30-2003) (ATCC, Manassas, VA, USA), fetal bovine serum (FBS) (Cat# F4135-500ML) was obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany and/or its affiliates).

Western blot reagents

Bio-Rad Protein Assay Dye Reagent (Cat#G000001) and −12% or 12% gradient polyacrylamide SDS-PAGE were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Anti-GPCR TGR7 antibody [EPR10597] (ab155999) from Abcam(Cambridge, MA, USA) and β-actin(sc-8432) was purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Secondary antibody mouse IgG and rabbit IgG antibodies tagged with horse-radish peroxidase (Cat#1706516 and #1706515 Bio-Rad Laboratories, Inc., Hercules, CA, USA). Chemiluminescence substrate kit, Cat#32106, was purchased from Thermo Fisher Scientific (Rockford, IL, USA).

**Methods**

Sugar moieties synthesis

The glycosides were synthesized via glycosylation reactions of Fmoc protected serine and the corresponding sugar peracetates. In the case of the Ser-Glc (Fig. 7 and Table 2) and serine cellobioside (ser-Cell) moieties, Fmoc-Ser-OH and the corresponding peracetylated sugars were refluxed in CHCl3 in the presence of InBr3 and stirred overnight using an oil bath at 60 °C. It took around 1 h to dissolve InBr3.

![Fig. 7 Equation representing the Ser-Glc preparation](image-url)
entirely, and the color of the solution started turning from clear to dark green. After all the starting Fmoc-Ser-OH was consumed, the solvent was evaporated and co-evaporated two times with ethyl acetate to obtain a dark brownish thick slurry (oil). The oil was washed with water to remove InBr3, followed by alternating sodium bicarbonate and water washes. The water washes were acidified to pH 2 using 1 M HCl. A white precipitate appeared and was filtered; then, the filtrate was washed with water and dried by sodium sulfate (Na2SO4). This solution was evaporated to an oil and then co-evaporated with CH3OH to obtain a brownish solid, which was dissolved in CH3OH. Seed crystals were added into the solution and kept overnight, and white powdery crystals were formed, collected by filtration, and washed with CH3OH several times, and the crystals were dried to obtain white powder. The serine lactoside (ser-Lact) was synthesized with Fmoc-L-serine-OBn and lactose peracetate under the same reaction conditions. The glycoside was purified via column chromatography using ethyl acetate/hexane.

**Solid-phase peptide synthesis (SPPS)/alamandine derivatives assembly and purification**

The most common methods for the synthesis of glycopeptides (N- or O-linked) strategies are based on Fmoc-protection and SPPS [36], which were used to assemble the Almandine derivatives. For N-linked glycopeptide synthesis, the convergent method is principally used by the amino acid condensation, which conjugates the glycosylamine-free Asp residue on a peptide [30]. In this study, the native alamandine (8) and eleven derivatives were prepared. The sequences were chosen to serve two goals; first, to vary the N-terminus amino acid to evaluate the effect of changing the steric bulk of the initial residue on receptor binding and efficacy. Secondly, adding a sugar moiety and varying its type on the C-terminus to examine the impact on stability and transport of the glycosides (Table 3).

Alamandine derivatives were synthesized using Rink amide resin (substitution: 0.5 mmol/g) (Scheme 1a) to provide the C-terminal amides. The side-chain-protected amino acids used in the synthesis were: Fmoc-His (Tri), Fmoc-Ile, Fmoc-Tyr (But), Fmoc-Val-OH, Fmoc-Arg(pbf), Fmoc-L-Ala-OH, Fmoc-Gly-OH, Fmoc-D-Ala-OH, Fmoc-Pro-OH, Fmoc-Nva-OH, Fmoc-D-Nva-OH, and Fmoc-D-Val-OH. The coupling of Fmoc-Ser (Glc, Lact, Cell)-OH (1.5 eq compared to resin) was done manually using 1.5 eq of 6-Chloro-1-hydroxybenzotriazole (ClHOBt) and 1.5 eq of N,N′-diisopropylcarbodiimide (DIC) in a 1:1 mixture of DMF and NMP (Scheme 1b). The hydroxyl groups of the sugar moieties used were masked as acetates, which were removed "on resin" with aqueous hydrazine.

The Fmoc-protected amino acids His, Ile, Tyr, Val, Arg, Ala, and Pro were coupled automatically using Peptide Synthesizer “Prelude” (from Gyros Protein Technologies) by adding 3.0 eq Fmoc-AA compared to resin using 3.0 eq of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate and 12 eq of NMP in DMF (Scheme 1c). Coupling of the expensive Fmoc-protected amino acids (Fmoc-D-Ala-OH, Fmoc-Nva-OH, Fmoc-D-Nva-OH, and Fmoc-D-Val-OH) was done manually by adding 2.5 eq of each Fmoc-AA-OH, 2.5 eq of CIHOBt, and 2.5 eq DIC in NMEP(NMeMorpholine) (Scheme 1d).

The Fmoc groups were removed using a mixture of 2% piperidine and 2% diaza-1,3-bicyclo[5.4.0]-undecane (DBU) in DMF for 10 min agitation with argon bubbling. The last Fmoc deprotection and the acetyl protecting groups of the sugar moiety were removed by (1:1, hydrazine (H2N-HN2·H2O): NMP (N-methyl pyrrolidone) with argon agitation for 4 h. The synthetic derivatives were cleaved from the resin with a peptide cleavage cocktail (90% TFA, 10% DCM, 2% H2O, 3% TES, 0.5% anisole) which simultaneously removed the side chain protecting groups. The crude glycopeptides were precipitated in cold ether, dissolved in a minimal amount of distilled water, and followed by

| Table 2 Example of general calculations for Fmoc-protected Ser-Glc preparation |
|-----------------|-----------------|-------------------|-----------------|-----------------|-----------------|
| Material        | Molecular weight | Mass (g) | mmol | Eq.* | Volume (mL) |
| Fmoc-Ser-OH     | 327.3            | 16.3     | 50   | 1.0  | -             |
| β-Glc(OAc)5     | 390.3            | 58.5     | 150  | 3.0  | -             |
| InBr3           | 354.3            | 3.54     | 10   | 0.20 | -             |
| CHCl3           | -                | -        | -    | -    | 75            |

Eq.* equivalent

| Table 3 Sequences of alamandine derivatives |
|-----------------|-----------------|
| Compound        | Amino Acid Sequence |
| 1               | GRVYIH-Ser (Glc) |
| 2               | D-ARVYIH-Ser (Glc) |
| 3               | nor-VRVYIH-Ser (Glc) |
| 4               | D-nor-VRVYIH-Ser (Glc) |
| 5               | VRVYIH-Ser (Glc) |
| 6               | D-VRVYIH-Ser (Glc) |
| 7               | ARVYIH |
| 8               | ARVYIH-P |
| 9               | ARVYIH-Ser (Glc) |
| 10              | ARVYIH-Ser (Lact) |
| 11              | ARVYIH-Ser (Cell) |
| 12              | ARVYIH-Ser (Cell) |
lyophilization. Purification of a quantity of each crude was accomplished by RP-HPLC using a Gilson system with a UV detector (at 280 nm) on a preparative C18 Phenomenex (5 µm, 100 Å, 250 × 21.9 mm) column with gradient program mobile phase of (A: 5–80% ACN) vs. (B: 0.1% TFA in H2O) over 60 min to give the glycopeptides in pure form. Then, assessing the purity by analytical HPLC (Inspire C18 5 µm 250 mm × 4.6 mm column) on a Varian LC with a diode array detector system (at 280 nm) employing the same gradient for 15 min. The pure fractions obtained from preparative HPLC purification were frozen at −80 °C and then lyophilized to afford the pure peptides as white and fluffy solids with an unoptimized (5–8)% yield range (not the whole crudes quantities were purified). Analytical RP-HPLC and high-resolution mass spectrometry confirmed the purity and homogeneity of all derivatives.

In vitro cell viability

In 96-well plates, the A549 and HEK 293 cells were plated at a concentration of 7500 cells/well and incubated overnight after peptide treatment at various concentrations over an exposure time of 96 h. In vitro cell viability was determined using 0.33 mg/mL MTS dye in the presence of 25 µM PMS per the manufacturer’s protocol (Promega, Madison, WI, USA) [37, 38]. The absorbance was measured at a wavelength of 590 nm using a SynergyÔ HT Multi-detection Microplate Reader (BioTek, Winooski, VT, USA).

Cell culture and media

The human lung carcinoma cell line (A549) and human embryonic kidney 293 (HEK 293) cell line were cultured in F-12K Medium and Eagle’s Minimum Essential Medium, respectively, with 15% heat-inactivated FBS. All cells were maintained in a humidified incubator at 37 °C and 5% CO2. All cell lines were tested negative for mycoplasma contamination. Cell’s authentication was completed for all cell lines.

Western blotting

After 24 h of seeding the cells, whole-cell extracts were prepared as described previously [38]. Protein concentration was measured by using Bio-Rad Protein Assay Dye Reagent as previously described [39]. Proteins were resolved by either 4–12% or 12% gradient polyacrylamide SDS-PAGE, as described previously [38]. The primary antibodies used were recombinant Anti-GPCR TGR7 antibody [EPR10597] from Abcam (Cambridge, MA, USA) and β-actin (dilution 1:300) Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Secondary antibodies, either mouse or rabbit IgG antibodies tagged with horseradish peroxidase, were used as (dilution 1:1000). An enhanced chemiluminescence substrate was used for detection.

Statistical analysis

All data are presented as the standard error of the mean (±SEM) of at least three independent experiments (n = 3). A Student’s t test was performed where applicable to test the significant difference between the two treatment groups. The difference between groups was considered to be statistically significant when \( p < 0.05 \). GraphPad Prism V.8 (GraphPad Software, Inc.) software was utilized for all data analysis.
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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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