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RESEARCH ARTICLE

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Antimicrobial, cytotoxic, and insulin-releasing activities of the amphibian host-defense peptide ocellatin-3N and its L-lysine-substituted analogs

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The host-defense peptide ocellatin-3N (GIFDVLKNLAKGVITSLAS.NH_2), first isolated from the Caribbean frog *Leptodactylus nesiotus*, inhibited growth of clinically relevant Gram-positive and Gram-negative bacteria as well as a strain of the major emerging yeast pathogen *Candida parapsilosis*. Increasing cationicity while maintaining amphipathicity by the substitution Asp^4→Lys increased potency against the microorganisms by between 4- and 16-fold (MIC ≤3 μM) compared with the naturally occurring peptide. The substitution Ala^{18}→Lys and the double substitution Asp^4→Lys and Ala^{18}→Lys had less effects on potency. The [D4K] analog also showed 2.5- to 4-fold greater cytotoxic potency against non-small-cell lung adenocarcinoma A549 cells, breast adenocarcinoma MDA-MB-231 cells, and colorectal adenocarcinoma HT-29 cells (LC_{50} values in the range of 12–20 μM) compared with ocellatin-3N but was less hemolytic to mouse erythrocytes. However, the peptide showed no selectivity for tumor-derived cells [$LC_{50} = 20$ μM for human umbilical vein endothelial cells (HUVECs)]. Ocellatin-3N and [D4K]ocellatin-3N stimulated the release of insulin from BRIN-BD11 clonal β-cells at concentrations ≥1 nM, and [A18K]ocellatin-3N, at concentrations ≥0.1 nM. No peptide stimulated the release of lactate dehydrogenase at concentrations up to 3 μM, indicating that plasma membrane integrity had been preserved. The three peptides produced an increase in intracellular [Ca^{2+}] in BRIN-BD11 cells when incubated at a concentration of 1 μM. In view of its high insulinotropic potency and relatively low hemolytic activity, the [A18K] ocellatin analog may represent a template for the design of agents with therapeutic potential for the treatment of patients with type 2 diabetes.

KEYWORDS
antimicrobial peptide, cytotoxicity, diabetes, frog skin, insulin release, Leptodactylidae

Abbreviations: LDH, lactate dehydrogenase; OCN-3N, ocellatin-3N; RBC, red blood cells; T2DM, type 2 diabetes mellitus.

1 | INTRODUCTION

Ever since the pioneering studies of Erspamer and co-workers beginning in the 1950s,[1] it has been known that frog skin secretions contain compounds with a quite remarkably diverse spectrum of biological activities.[2,3] From a therapeutic standpoint, host-defense peptides, particularly those isolated from frogs belonging to the extensive families Hylidae, Pipidae, and Ranidae, that possess the ability to inhibit the growth of clinically relevant pathogenic bacteria and fungi have received the most attention.[4] Such peptides are believed to be a component of the frog's system of innate immunity, and they may also possess the ability to permeabilize mammalian cells. It has been proposed that they act synergistically with myotropic peptides in the secretions, such as bradykinins and tachykinins to constitute a defense against predators.[5] It is now appreciated that many frog skin peptides that were first identified on the basis of their cytotoxic actions are in fact multifunctional and may display other properties with potential clinical applications such as antioxidant,[6] wound-healing[7] and cytokine-mediated immunomodulatory properties,[8] and the ability to produce tumor regression in animal models of cancer.[9] In addition, a number of frog skin peptides display a range of potential antidiabetic properties such as the ability to stimulate release of insulin from BRIN-BD11 clonal β-cells and to protect the cells against cytokine-induced apoptosis[10] as well as lowering blood glucose and increasing insulin sensitivity when administered to mice with obesity and degenerative diabetes.[11]

The subfamily Leptodactylinae within the extensive family Leptodactylidae currently contains 116 well-characterized taxa distributed in four genera [*Adenomera* (29 species), *Hydrolaetare* (three species), *Leptodactylus* (83 species) and *Lithodytes* (one species)].[12] Skin secretions from several species of frogs belonging to the genus *Leptodactylus* contain structurally related host-defense peptides with antimicrobial activity that have been termed ocellatins according to a generally accepted nomenclature.[13] These peptides are cationic and adopt a amphipathic α-helical conformation in a membrane mimetic solvent such as 50% trifluoroethanol-water.[14] In addition to the ocellatins, conformationally flexible glycine/leucine-rich plasticins, which lack antimicrobial activity, have been isolated from the skin secretions of *Leptodactylus pentadactylus*[15] and *Leptodactylus laticeps*.[16]

A previous study identified multiple ocellatin peptides in norepinephrine-stimulated skin secretion from the Caribbean frogs *Leptodactylus insularum* and *Leptodactylus nesiotus*.[17] In common with ocellatins from other *Leptodactylus* species, these peptides displayed only relatively weak growth inhibitory activity against Gram-negative bacteria but ocellain-3N (GIFDVLKNLAKGVITSLAS.NH_2) from *L. nesiotus* was also active against Gram-positive bacteria. The aim of the present study was to determine whether ocellatin-3N and analogs with increased cationicity that maintain the amphipathic helical character of the peptide show therapeutic potential as antimicrobial agents for treatment of patients infected with antibiotic resistant microorganisms. In addition, their potential for development into anticancer agents and antidiabetic agents for treatment of patients with type 2 diabetes mellitus (T2DM) was evaluated.

2 | MATERIALS AND METHODS

2.1 | Synthetic peptides

Ocellatin-3N, [D4K]ocellatin-3N, [A18K]ocellatin-3N, and [D4K,A18K]ocellatin-3N were supplied by Synpeptide Co. Ltd. (Shanghai, China) at a purity greater than 98%. Confirmation of their identity was provided by electrospray mass spectrometry and their purity by HPLC. The primary structures of the peptides, their molecular charges at pH 7, and the grand average of hydropathy (GRAVY), defined as the sum of the hydropathy values of all the amino acids calculated using the hydrophobicity scale of Kyte and Doolittle[18] divided by the sequence length, are shown in Table 1.

2.2 | Antimicrobial assays

Reference strains of microorganisms were purchased from the American Type Culture Collection (Rockville, MD, USA). Peptides were dissolved in 0.1% trifluoracetic acid in water and stored as a 1 mM stock solution at −20°C. Minimum inhibitory concentrations (MICs) of the peptides were determined in the serial concentration range 0.19 to 50 μM by standard microdilution assays under the conditions mandated by the Clinical Laboratory and Standards Institute[19,20] as previously described.[21] The following clinically relevant strains of microorganisms were investigated—Gram-negative bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Acinetobacter baumannii* ATCC 19606; Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Bacillus megaterium* BM11, and the opportunist yeast pathogen *Candida parapsilosis* ATCC 22019. MIC was defined as the lowest concentration able to totally inhibit microbial growth, and MICs are reported as the modal values of three independent experiments.

2.3 | Cytotoxicity assays

Synthetic peptides in the concentration range 8–128 μM were incubated for 60 min at 37°C with washed erythrocytes (2×10^7 cells) taken from male NIH male Swiss mice (Harlan Ltd., Bicester, UK) as previously described.[22] The LC_{50} value was taken as the mean concentration of peptide producing 50% hemolysis in three independent experiments. All procedures involving animals were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 and EU Directive 2010/63EU for animal experiments.

Cytotoxicities against A549 human non-small cell lung adenocarcinoma cells, MDA-MB-231 human breast adenocarcinoma cells, HT-29 human colorectal adenocarcinoma cells, and HUVEC human umbilical vein endothelial cells were measured as previously described.[22] The effects of the peptides (1–100 μM) on cell viability following a 24 h incubation were determined by measurement of ATP concentrations using a CellTiter-Glo Luminescent Cell Viability

TABLE 1 Primary structures and physicochemical properties of the ocellatin-3N (OCN-3N) peptides used in this study

Peptide	Primary structure	Charge	GRAVY
OCN-3N	GIFDVLKNLAKGVITSLAS.NH_2	+2	0.91
[D4K]OCN-3N	GIFKVLKNLAKGVITSLAS.NH_2	+4	0.89
[A18K]OCN-3N	GIFDVLKNLAKGVITSLKS.NH_2	+3	0.61
[D4K,A18K]OCN-3N	GIFKVLKNLAKGVITSLKS.NH_2	+5	0.59

Note: Charge refers to the net charge at pH 7.0, and GRAVY represents 'grand average of hydropathy' determined using the hydrophobicity scales of Kyte and Doolittle.[18]

assay (Promega Corporation, Madison, WI, USA). The LC_{50} value, calculated using GraphPad Prism version 8.3.1 (GraphPad Software, San Diego, CA, USA) was taken as the mean concentration of peptide producing 50% cell death in a minimum of three independent experiments.

2.4 | Insulin-releasing activities

BRIN-BD11 clonal β-cells[23] were seeded into 24-well plates and allowed to attach during overnight incubation at 37°C. Incubations with the synthetic ocellatin peptides (10^{-10}–3×10^{-6} M; $n = 8$) were carried out for 20 min at 37°C in Krebs-Ringer bicarbonate (KRB) buffer supplemented with 5.6 mM glucose as previously described.[24] After incubation, aliquots of cell supernatant were removed for insulin radioimmunoassay.[25] Control incubations were carried out in parallel with the well-established insulin stimulatory agents, alanine (10 mM), and exendin-4 (10^{-6} M).

Effects of the peptides (10^{-10}–3×10^{-6} M; $n = 4$) on the rate of lactate dehydrogenase (LDH) release from BRIN-BD11 cells were determined using a CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Southampton, UK) according to the manufacturer's instructions as previously described.[25]

2.5 | Effects on intracellular Ca^{2+} concentrations and membrane potential

Effects of ocellatin-3N (1 μM), [Lys^4]ocellatin-3N (1 μM), and [Lys^{18}] ocellatin-3N (1 μM) on intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were determined fluorimetrically with monolayers of BRIN-BD11 cells using a FLIPR Calcium 5 Assay Kit (Molecular Devices, Sunnyvale, CA, USA) according to the manufacturer's recommended protocols as previously described.[26] Changes in membrane potential in response to incubation with the three ocellatin-3N peptides (1 μM) were determined fluorimetrically with monolayers of BRIN-BD11 cells using a FLIPR Membrane Potential Assay Kit (Molecular Devices).[26] Data were acquired using a FlexStation scanning fluorimeter with integrated fluid transfer workstation (Molecular Devices). The cells were incubated at 37°C for 300 s with test peptides. Control incubations in the presence of 5.6 mM glucose alone, 5.6 mM glucose containing 10 mM alanine and 5.6 mM glucose containing 30 mM KCl were also carried out.

2.6 | Statistical analysis

Data were compared using unpaired Student's *t*-test (nonparametric, with two-tailed *p* values and 95% confidence interval) and one-way ANOVA with Bonferroni post-hoc test wherever applicable. Area under the curve (AUC) analysis was carried out using the trapezoidal rule with baseline correction. Values are presented as mean ± standard error of mean (SEM). Results are considered to be significantly different if $p < 0.05$.

3 | RESULTS

3.1 | Antimicrobial activities

Ocellatin-3N showed broad-spectrum antimicrobial activity inhibiting the growth of reference strains of Gram-positive bacteria (*S. aureus*, *S. epidermidis*, and *B. megaterium*), Gram-negative bacteria (*E. coli*, *P. aeruginosa*, and *A. baumannii*), and the yeast pathogen *C. parapsilosis* (Table 2). MIC values were in the range 1–25 μM. The potency of the [D4K] analog against the different microorganisms was between 4- and 16-fold greater the corresponding values with the naturally occurring peptide with MIC values ≤3 μM. The effects on potency of the substitution Ala^{18}→Lys in the [A18K] analog was less pronounced but the [D4K,A18K] peptide was between 4- and 8-fold more potent than ocellatin-3N against *S. epidermidis*, *B. megaterium*, *P. aeruginosa*, *A. baumannii*, and *C. parapsilosis*.

3.2 | Cytotoxic activities

The effects of increasing concentrations of the synthetic ocellatin-3N peptides on the viability of A549 human non-small cell lung adenocarcinoma cells, MDA-MB-231 human breast adenocarcinoma cells, HT-29 human colorectal adenocarcinoma cells, and HUVEC human umbilical vein endothelial cells are summarized in Table 3. [Lys^4]ocellatin-3N was the most cytotoxic of the four peptides against the tumor-derived cells with LC_{50} values between 3- and 4-fold less than the native peptide (Figure 1). Death of all cell types was extremely rapid (<5 min) at the highest concentration tested (100 μM). However, the potential of the analog for development into a therapeutically valuable anticancer agent was seriously limited by the fact that the peptide was also strongly

TABLE 2 Minimum inhibitory concentrations, in micromol per liter, of synthetic replicates of ocellatin-N (OCN-3N) and its lysine-substituted analogs against reference strains of Gram-positive and Gram-negative bacteria and a yeast

Microorganism	OCN-3N	[D4K]OCN-3N	[A18K]OCN-3N	[D4K,A18K]OCN-3N
Gram-positive				
S. aureus ATCC 25923	12.5	3.12	25	6.25
S. epidermidis ATCC 12228	6.25	1.56	6.25	1.56
B. megaterium BM11	0.78	<0.19	0.19	<0.19
Gram-negative				
E. coli ATCC 25922	6.25	1.56	6.25	6.25
P. aeruginosa ATCC 27853	25	1.56	25	3.12
A. baumannii ATCC 19606	6.25	1.56	1.56	1.56
Yeast				
C. parapsilosis ATCC 22019	25	3.12	25	3.12

Cell	OCN-3N	[D4K]OCN-3N	[A18K]OCN-3N	[D4K,A18K]OCN-3N
A549	35 ± 1	12 ± 1	30 ± 1	25 ± 1
MDA-MB-231	51 ± 16	15 ± 1	69 ± 18	31 ± 1
HT-29	69 ± 10	20 ± 1	59 ± 4	46 ± 12
HUVEC	48 ± 9	20 ± 3	56 ± 8	28 ± 4
RBC	56 ± 7	107 ± 8	>128	>128

Note: Data show mean LC_{50} values (μM) ± SEM.

TABLE 3 Cytotoxicities of ocellatin-3N (OCN-3N) peptides against A549 lung adenocarcinoma cells, MDA-MB-231 breast adenocarcinoma cells, HT-29 colorectal adenocarcinoma cells, HUVEC umbilical vein endothelial cells, and mouse red blood cells (RBC)

cytotoxic to non-neoplastic HUVECs. The effects on cytotoxic potency of the substitution Ala^{18}→Lys was less pronounced. While increasing cationicity increased activity against both the tumor-derived cells and HUVECs, the lysine-containing analogs were less hemolytic against mouse erythrocytes compared with the naturally occurring peptide (Table 3).

3.3 | Insulin-releasing activities

In the absence of added peptide, the rate of insulin release from BRIN-BD11 clonal β-cells in the presence of 5.6 mM glucose was 1.0 ± 0.1 ng/20 min/10^6 cells. Both ocellatin-3N (Figure 2A) and [Lys^4]ocellatin-3N (Figure 2B) produced a concentration-dependent stimulation in the rate of insulin release from the β-cells with a significant ($p < 0.05$–$p < 0.001$) increase over the basal rate in the presence of 5.6 mM glucose at a concentration of 10^{-9} M. [Lys^{18}]ocellatin-3N was the most potent peptide producing a significant ($p < 0.001$) increase in the rate of insulin release at a concentration of 10^{-10} M (Figure 2C). The analog produced an approximately twofold increase in the rate of insulin release at a concentration of 3×10^{-6} M, which was not significantly different from the increase produced by 10 mM alanine but less than that produced by 10^{-6} M exendin-4. No peptide at concentrations in the range 10^{-10} M to 3×10^{-6} M produced a significant increase in the rate of release of the cytosolic enzyme LDH demonstrating that the integrity of the plasma membrane had not been compromised (data not shown).

3.4 | Effects on intracellular [Ca^{2+}] concentrations and membrane potential

In control incubations of BRIN-BD11 cells, the established insulin secretogogue alanine (10 mM) caused a marked and immediate rise in [Ca^{2+}]i concentrations (Figure 3), and a depolarizing concentration of KCl (30 mM) produced a rapid and sustained increase in membrane potential (Figure 4). Ocellatin-3N (Figure 3A), [Lys^4]ocellatin-3N (Figure 3B), and [Lys^{18}]ocellatin-3N (Figure 3C), at a concentration of 1 μM, produced significant ($p < 0.01$–$p < 0.001$) increases in [Ca^{2+}]i compared with 5.6 mM glucose alone. The time-course of the increase is shown in panel A and the integrated response in panel B. In contrast, incubation of BRIN-BD11 cells with the three ocellatin-3N peptides produced no significant rise in membrane potential under the same experimental conditions (Figure 4).

4 | DISCUSSION

The worldwide emergence of pathogenic bacteria and fungi resistant to commonly used antibiotics has necessitated an urgent search for new types of microbial agents. The relative potencies of frog skin host defense peptides against bacteria and fungi and against mammalian cells are determined by physicochemical characteristics such as cationicity, hydrophobicity, conformation (α-helicity), and amphipathicity.[27] The bacterial cell membrane is associated with a greater negative charge than the plasma membrane of mammalian cells due to a higher

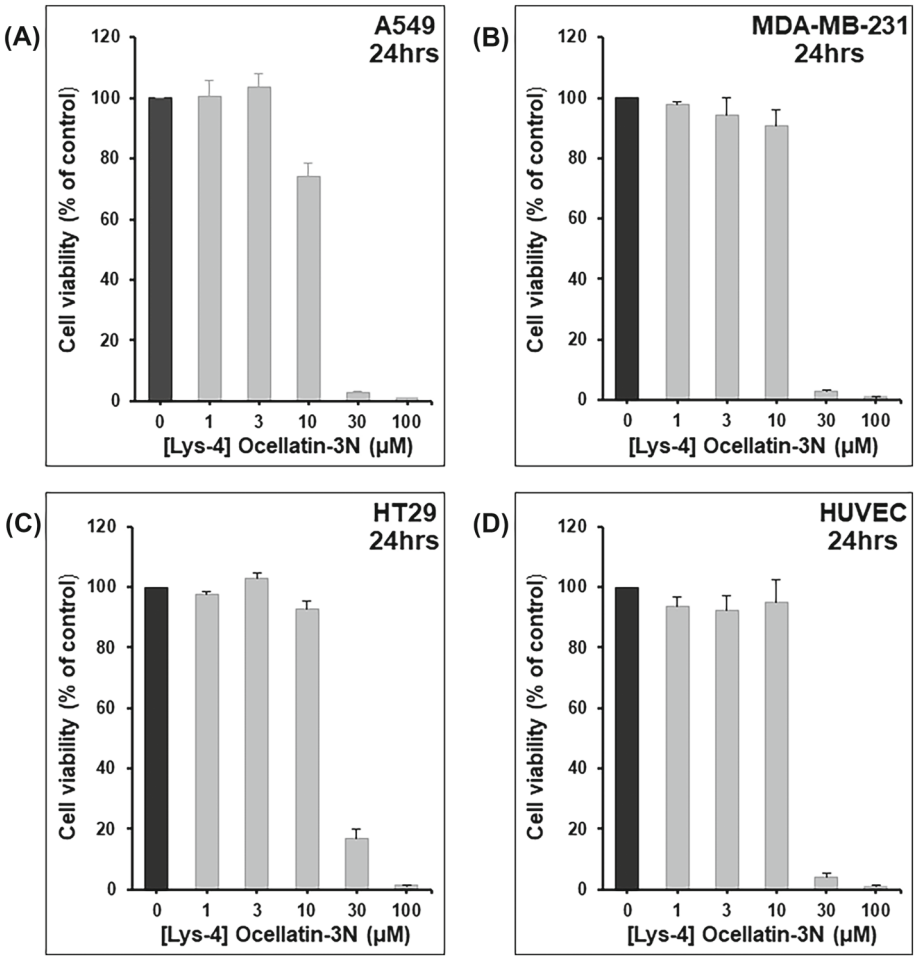

FIGURE 1 Effects of [Lys^4] ocellatin-3N on the viability of (A) A549 non-small cell lung adenocarcinoma cells, (B) MDA-MB-231 breast adenocarcinoma cells, (C) HT-29 colorectal adenocarcinoma cells and (D) HUVEC umbilical vein endothelial cells after 24 h exposure. All experiments were repeated at least three times. Data points show mean ± SEM.

proportion of anionic phospholipids so that an increase in peptide cationicity, while maintaining amphipathicty, should enhance antimicrobial potency without increasing toxicity against mammalian cells.[28] Studies with the naturally occurring amphipathic α-helical peptides such as magainin-2,[29] pseudin-2,[30] and alyteserin-2[31] have demonstrated that increasing the positive charge on the peptides does indeed produce an increase in antimicrobial activity until a limit is reached, whereupon further increase in cationicity does not result in any further increase in potency.

A previous study using the AGADIR prediction algorithm[32] has shown that ocellatin-3N shows a strong propensity to adopt α-helical conformations between residues 4–11 and 13–18 in a membrane-mimetic environment.[17] The substitution Asp^4→Lys in ocellatin-3N increased the molecular charge at pH 7 from +2 to +4 while maintaining the integrity of the predicted α-helical domain (Table 1) and so, as expected, resulted in a marked increase in antimicrobial potency against all microorganisms tested (Table 2). The effects on growth inhibition of *B. megaterium*, a Gram-positive bacterium implicated in rare cases of endocarditis following bacteremia,[33] *P. aeruginosa*, a Gram-negative bacterium strongly implicated in pulmonary infections among immunocompromised and hospitalized patients,[34] and *C. parapsilosis*, a major emerging yeast pathogen causing severe infections in neonates and patients in intensive care units,[35] are particularly pronounced. The high potency of [D4K]ocellatin-3N against *A. baumannii* and *P. aeruginosa* was also encouraging as the World Health Organization has described antibiotic resistance among these pathogens as 'critical'. The substitution Ala^{18}→Lys increases the molecular charge of ocellatin-3N from +2 to +3 but concomitantly reduces hydrophobicity with the result that the effect on antimicrobial potency was less marked. Similarly, the double substitution Asp^4→Lys and Ala^{18}→Lys, increasing the molecular charge to +5, did not raise antimicrobial potency relative to that of the [DK4] analog, suggesting that a charge of +4 is optimum.

The use of conventional chemotherapeutic agents for the treatment of cancer is often restricted by toxic side effects and the development of multi-drug resistance by tumor cells so that there is a constant need to develop new anticancer agents. Certain cationic antimicrobial peptides have shown therapeutic potential by their abilities to kill cancer cells not only by a membrane-lytic mechanism but also by inducing apoptosis and acting as inhibitors of angiogenesis.[36] In common with other frog skin peptides previously investigated such as caerulein precursor fragment (CPF) peptides from *Xenopus* sp., dermaseptins and phylloseptins from *Phyllomedusa* sp., and aureins from *Litoria* sp.,[37] the ocellatin-3N peptides displayed cytotoxic activity against a range of human tumor-derived cell lines (Table 3). Increasing cationicity by the substitution Asp^4→Lys led to 2.5- to 4-fold

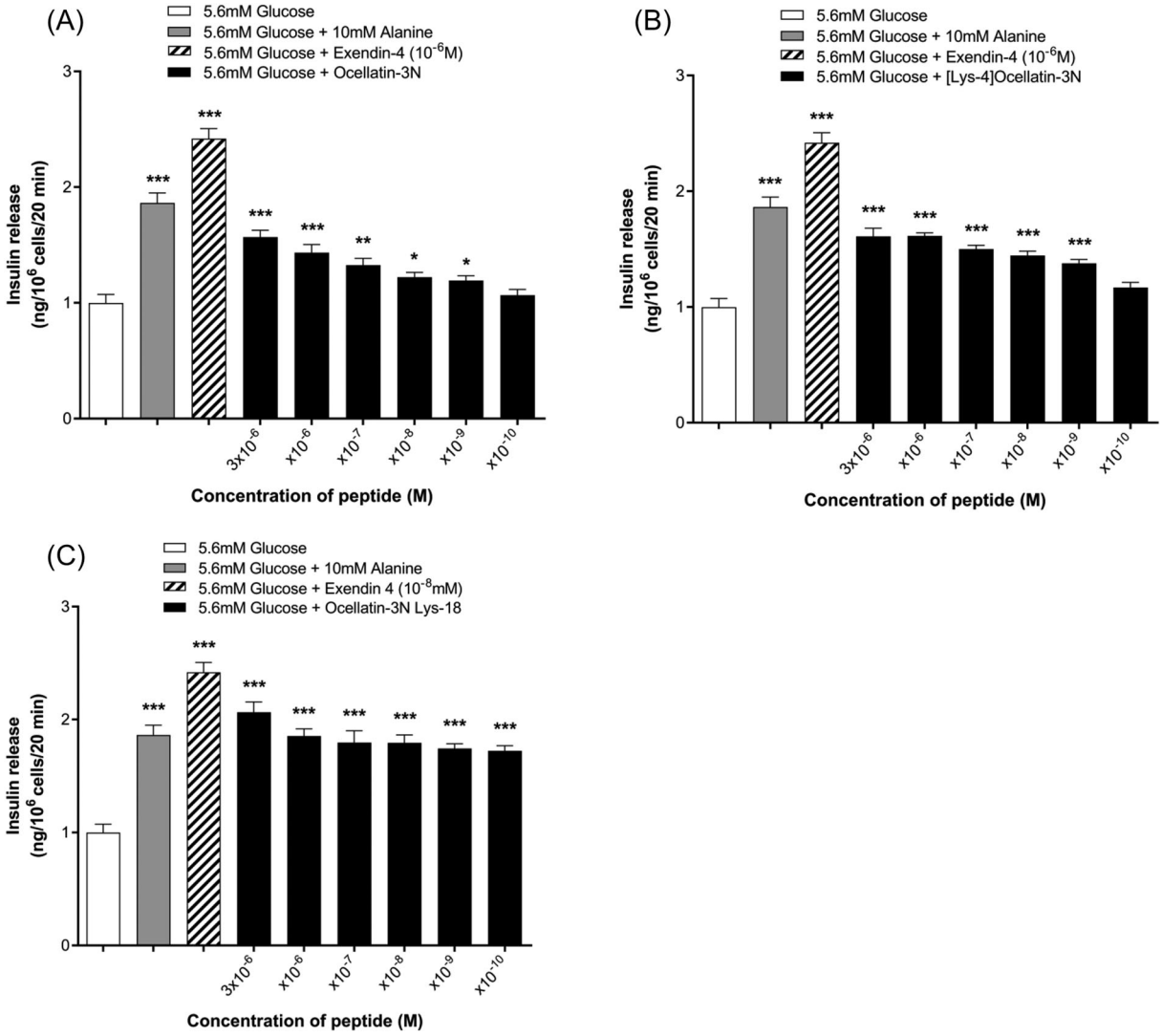

FIGURE 2 Effects of (A) ocellatin-3N, (B) [Lys^4]ocellatin-3N, and (C) [Lys^{18}]ocellatin-3N on the rate of release of insulin from BRIN-BD11 clonal β-cells compared with alanine (10 mM) and exendin-4 (10^{-6} M). Values show mean ± SEM ($n = 8$). ***$p < 0.001$, **$p < 0.01$. *$p < 0.05$ compared with 5.5 mM glucose alone

increases in potency against tumor-derived cells but a twofold decrease in hemolytic activity against mouse erythrocytes. However, the analog showed no selectivity for neoplastic cells. The LC_{50} value obtained with non-neoplastic HUVECs (20 μM) was comparable to the values obtained with the tumor-derived cells (12–20 μM), which discourages further efforts to transform the naturally occurring peptide into analogs with therapeutic potential as anticancer agents.

A possible explanation for the contrasting effects of lysine-substitutions on cytotoxity against tumor cells and erythrocytes resides in the fact that the plasma membrane of cancer cells is generally associated with a greater negative charge compared with erythrocytes because of a higher proportion of anionic phospholipids such as phosphatidylserine, O-glycosylated mucins, sialic acid-containing glycolipids, and heparan sulfate proteoglycans.[38] In contrast, the erythrocyte plasma membrane contains primarily zwitterionic phospholipids so that increasing the cationicity of a cell-penetrating peptide should promote interaction with tumor cells thereby enhancing anti-tumor potency relative to hemolytic activity. The reduced hemolytic activity of the [A18K] and [D4K,A18K] analogs relative to the [D4K] analog may be a consequence of the lower hydrophobicity of the peptides (Table 1). Studies with model peptides have demonstrated a direct correlation between increased hydrophobicity and increased hemolytic activity.[39]

The global pandemic of T2DM has stimulated the search for naturally occurring compounds with therapeutic potential for development into agents for the treatment of patients with this disease. Impaired glucose-induced insulin release is a feature of long-standing T2DM.[40]

Incubation of ocellatin-3N with BRIN-BD11 rat clonal β-cells resulted in a dose-dependent increase in the rate of insulin releases at concentrations that did not affect the integrity of the plasma membrane. The threshold concentration (minimum concentration producing a significant increase) was the same (10^{-9} M) for the [Lys^4] analog

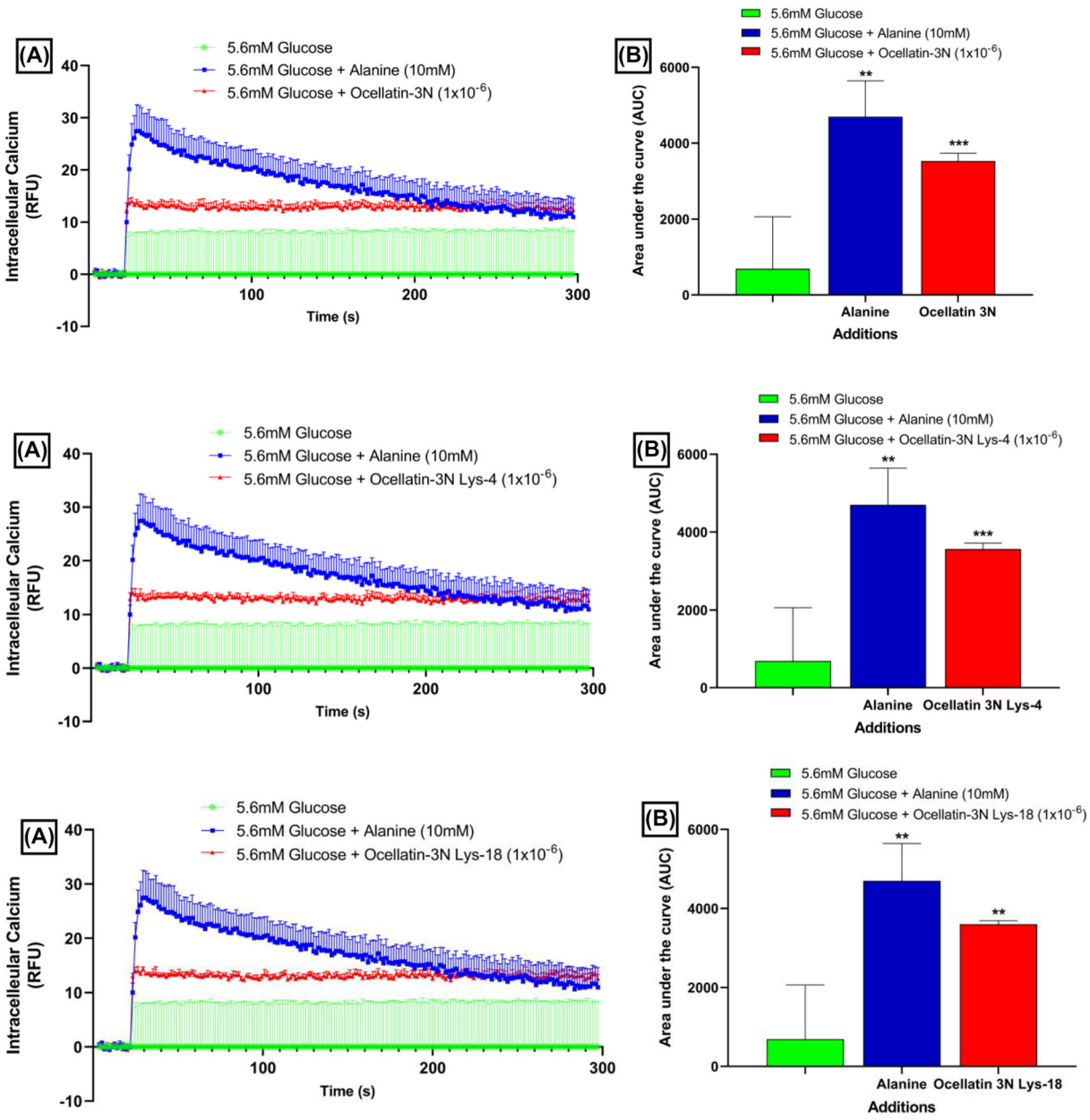

FIGURE 3 Effects of ocellatin-3N (1 μM), [Lys^4]ocellatin-3N (1 μM), [Lys^{18}]ocellatin-3N (1 μM), and alanine (10 mM) on (A) intracellular calcium ion concentrations $[Ca^{2+}]_i$ in BRIN-BD11 cells expressed as relative fluorescence units, RFUs and (B) the integrated response (area under the curve, AUC). Values show mean ± SEM ($n = 6$). $^{**}p < 0.05$ and $^{***}p < 0.001$ compared with 5.6 mM glucose alone

as for the naturally occurring peptide but the [Lys^{18}] analog was more potent (threshold concentration 10^{-10} M). Structure–activity relationships with respect to the insulin-releasing activities of frog skin host-defense peptides are incompletely understood. Analogs of hymenochirin-1B, alyteserin-2a, pseudin-2, and brevinin-2-related peptide with amino acid substitutions that increased cationicity displayed greater incretin activity than the native peptides but the tryptophan-containing [A5W], [L8W], and [I10W] analogs of tigerinin-1R also produced a greater increase in the rate of insulin release compared with the underivatized peptide.[3,41]

Insulin secretion from pancreatic β-cells involves the integration and interaction of multiple external and internal stimuli. Numerous steps are involved in physiological regulation of insulin secretion by glucose, including GLUT-2-mediated transport into β-cells, increase in ATP concentration, closure of K_{ATP} channels with resulting depolarization of the cell membrane, opening of L-type Ca channels, and Ca^{2+} influx leading to exocytosis.[42] Several peptides with the ability to stimulate the rate of release of insulin from BRIN-BD11 clonal β-cells have been identified in skin secretions of frogs but appear to differ in their mechanism of action. For example, stimulation of insulin release by alyteserin-2a, CPF-SE1, PGLa-AM1, tigerinin-1R, esculentin-1a (1-21).NH_2, and esculentin-2CHa is associated with an increase in $[Ca^{2+}]$i, whereas stimulation by frenatin-2D, hymenochirin-1B, pseudin-2, and temporin-A does not involve such an increase in

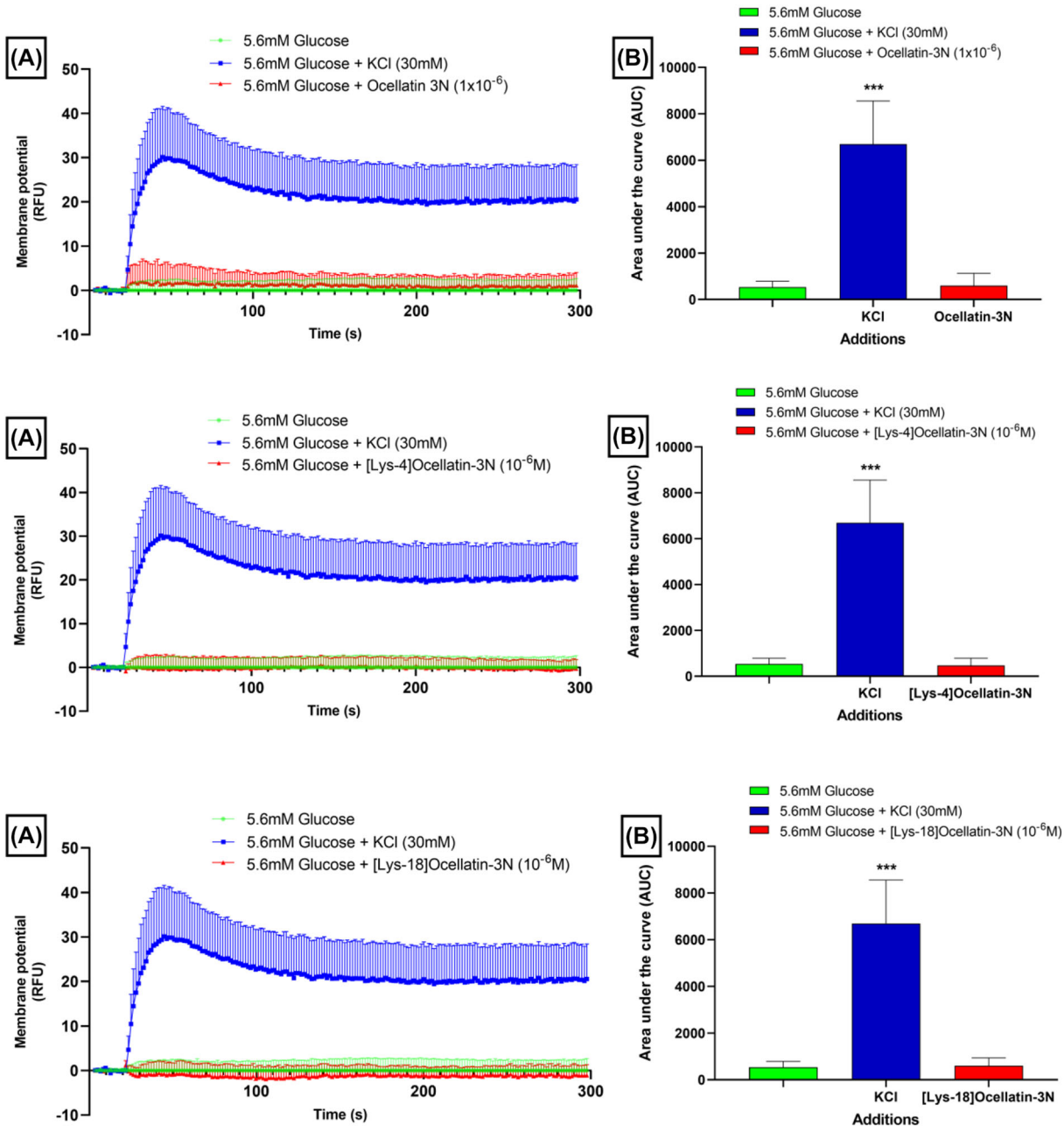

FIGURE 4 Effects of ocellatin-3N (1 μM), [Lys^4]ocellatin-3N (1 μM), [Lys^{18}]ocellatin-3N (1 μM), and KCl (30 mM) on (A) membrane potential in BRIN-BD11 cells expressed as relative fluorescence units, RFUs and (B) the integrated response (area under the curve, AUC). Values show mean ± SEM ($n = 6$). ***$p < 0.001$ compared with 5.6 mM glucose alone

$[Ca^{2+}]i$.[3,41] Incubation of BRIN-BD11 cells with the ocellatin-3N peptides investigated in this study resulted in an increase in $[Ca^{2+}]_i$ without any significant change in membrane potential. This suggests that the mechanism of action of the peptides is not mediated by the K_{ATP} channel pathway but involves mobilization of intracellular stores of Ca^{2+} such as the endoplasmic reticulum.[43] Incubation of BRIN-BD11 cells with the [P5K] analog of hymenochirin-1B[44] and frenatin-2D[10] increased the intracellular cAMP concentration concomitant with insulin release, and downregulation of the protein kinase A pathway by forskolin abolished the insulinotropic activity of the peptides. Future studies will address the possible role of the protein kinase A and protein kinase C pathways in mediating the insulin-releasing activities of the ocellatin-3N peptides.

5 | CONCLUSION

The results from this study may have clinical relevance in terms of development of new therapeutic agents. Although lack of selectivity likely precludes the possibility of transforming ocellatin-3N into an

anticancer agent, the peptide shows potential for development into a compound for use in therapy for T2DM. Several efficacious peptide drugs based upon the structures of the incretin peptides' glucagon-like peptide-1 and glucose-dependent insulinotropic peptide, such as liraglutide, semaglutide, and tirzepatide, are now available in clinical practice for treatment of patients with obesity-related T2DM but their use in some individuals is precluded because of unacceptable side effects.[45] Consequently, new antidiabetic agents are always needed, particularly those whose mechanism of action is different from those of existing drug classes. In view of its potent in vitro insulinotropic activity and low hemolytic activity, the therapeutic potential of [A18K]ocellatin-3N as a template for design of antidiabetic drugs is worthy of further investigation. Future studies will address the synthesis of long-acting, peptidase-resistant analogs and investigate their potential as antidiabetic drugs (effects on circulating glucose, insulin resistance, and β-cell proliferation) in vivo in animal models of T2DM such as the *db*/*db* mouse and the high fat-fed mouse. Individuals with T2DM are more susceptible to infectious diseases, particularly bacterial infections of the lower extremities and urinary tract and superficial fungal infections such as oral candidiasis.[46] Consequently, the potent, broad-spectrum antimicrobial activity of the cationic insulin-releasing ocellatin-3N analogs may represent an additional therapeutic advantage, not possessed by existing antidiabetic drugs, in protecting patients against such infections.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Yasser H. A. Abdel-Wahab, Milena Mechkarska, and J. Michael Conlon conceived and designed the study. Lauren Hunter performed the insulin-release assays, Bruno Casciaro the antimicrobial assays, and Samir Attoub the cytotoxicity assays. J. Michael Conlon wrote the manuscript. All authors analyzed and interpreted the data and have approved the final submission.

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