Engineering Protein Venoms as Self-Assembling CXCR4-Targeted Cytotoxic Nanoparticles

Naroa Serna, Olivia Cano-Garrido, Laura Sánchez-García, Mireia Pesarrodona, Ugutz Unzueta, Alejandro Sánchez-Chardi, Ramon Mangues, Esther Vázquez,* and Antonio Villaverde*

Protein venoms are effective cytotoxic molecules that when conveniently targeted to tumoral markers can be exploited as promising anticancer drugs. Here, it is explored whether the structurally unrelated melittin, gomesin, and CLIP71 could be functionally active when engineered, in form of GFP fusions, as self-assembling multimeric nanoparticles. Incorporated in modular constructs including a C-terminal polyhistidine tag and an N-terminal peptidic ligand of the cytokine receptor CXCR4 (overexpressed in more than 20 human neoplasias), these venoms are well produced in recombinant bacteria as proteolytically stable regular nanoparticles ranging between 12 and 35 nm. Being highly fluorescent, these materials selectively penetrate, label, and kill CXCR4* tumor cells in a CXCR4-dependent fashion. The obtained data support the concept of recombinant venoms as promising drugs, through the precise formulation as tumor-targeted nanomaterials for selective theragnostic applications in CXCR4* cancers.

Molecular therapies of cancer are based on cytotoxic drugs, most of them being small molecular weight chemicals that are administered systemically devoid of cell and tissue selectivity.[1] However, cytotoxic proteins are especially promising as new generation antitumoral agents.[2] This is in part since, as versatile macromolecules, polypeptides can be extensively engineered. Genetic fusion allows gaining additional functions such as self-assembling at the nanoscale, cell targeting and fluorescent emission, which might be useful for imaging purposes. This can be achieved by the construction of modular proteins that combine protein segments from different origins and with different biological activities.[3] Among such functions, the oligomerization within nanoscale dimensions and the multivalent display of cell surface peptidic ligands[4] are highly desirable to favor the enhanced retention and permeability (EPR) effect and a proper tumor biodistribution, and to stimulate the intracellular delivery in target tissues through cooperative cell binding. The incorporation of C-terminal polyhistidine peptides to modular proteins represents a simple strategy to promote robust self-assembling, as divalent metal and non-metal cations in the media, acting as molecular cross-linkers, induce protein clustering[5] and regular oligomerization in form of stable nanoparticles.[6,7] An N-terminal cationic region in the building blocks is also required for oligomerization.[6,7]

Among cytotoxic proteins, venoms developed for animal predation and defense are particularly appealing as cytotoxic drugs,[8,9,10] and the possibility to engineer venoms as}

DOI: 10.1002/ppsc.202000040
self-assembling tumor-targeted nanoparticles would open a wide spectrum of potential new clinical applications. In this context, we have explored such approach by submitting two structurally and functionally unrelated venoms and one venom-like synthetic protein (namely melittin, gomesin and CLIP71) to modular protein engineering for the construction of CXCR4-targeted nanoparticles (Figure 1A). The CXCR4 peptidic ligand T22[7,11] has been incorporated as a ligand in those modular proteins, in which the venom domains result fused to GFP. Melittin is a pore-forming peptide from the Western honey bee *Apis mellifera* that shows antitumoral and antimetastatic activities linked to induction of apoptosis, inhibition of calmodulin binding activity and suppression of the Rac1-dependent pathway.[10] Gomesin is an antimicrobial peptide isolated from the Brazilian tarantula *Acanthoscurria gomesiana*. Apart from its intrinsic antimicrobial activities gomesin shows potent cytotoxic effects on cancer cells and a generic antitumoral activity.[12] The membrane-disrupting peptide CLIP71,[13] also showing antitumoral activities, is currently in clinical trials as an anticancer drug component (https://clinicaltrials.gov/ct2/show/NCT01485848).

These peptides are structurally divergent, and despite having been explored in recombinant and synthetic forms,[9,10,14] and with a recognized potential as anticancer drugs,[10,15,16] how they would perform in supramolecular complexes as multifunctional proteins has been never explored. For that, we constructed venom fusion proteins with GFP as a fluorescent marker (Figure 1B) to enable potential theragnostic uses of the resulting materials. Furin cleavage sites, previously shown as efficient in modular proteins,[17] were incorporated in the intersect of the functional modules to ensure the release of functional peptides once internalized in target cells. T22, a cationic CXCR4-targeted peptide, has been also included to confer cancer cell targeting. The cytokine receptor CXCR4 is overexpressed in more than 20 human malignancies in which CXCR4 levels are associated with aggressiveness and poor prognosis,[18] including the main health conditions colorectal cancer, pancreatic cancer, breast cancer, ovarian cancer, prostatic cancer, lung adenocarcinoma, leukemia, lymphoma, and melanoma.[19] For some neoplasias, such as the diffuse large B-cell lymphoma, the levels of CXCR4 overexpression are 50–200 times the levels in normal lymphocytes of lymph nodes.[20] In particular, T22 promotes an excellent biodistribution of associated proteins upon systemic administration in mouse models of human (CXCR4+ colorectal cancer, where injected proteins accumulate in primary tumor and metastatic foci.[7,21] The peptide also promotes an excellent targeting in diffuse large B-cell lymphoma mouse models.[22] The fusion proteins were all produced in *Escherichia coli* as full-length protein forms (Figure 1C), equally stable than the related protein T22-GFP-H6 (previously used as nanoscale drug carrier in tumor-targeted chemotherapy).[7] Interestingly, all these modular proteins, even containing very short peptides as core...
elements, efficiently self-assembled as regular nanoparticles ranging from 12 to 35 nm (Figure 2A), similar in morphology to those formed by T22-GFP-H6 (Figure 2A,B). GFP-H6, devoid of any N-terminal cationic peptide able to assist in the nanoparticle formation, is unable to form supramolecular structures (Figure 2A).

The participation of divalent cations in maintaining the oligomeric architecture of T22-GFP-H6, through the coordination with the C-terminal His tail, is predicted to keep the CXCR4-ligand T22 in a solvent-exposed accommodation. To confirm the surface-display of T22 on the venom nanoparticles and its availability for molecular interactions, these materials were studied regarding their potential internalization into cultured CXCR4+ HeLa cells. Since the generated nanoparticles were fluorescent (Figure 3A), monitoring the protein uptake by cells was experimentally feasible, upon a harsh trypsin treatment especially designed to inactivate any GFP material externally attached to cells. For that, the total green fluorescent emission of the materials was corrected by the specific emission of the proteins to render values representative of protein amounts. As observed, CLIP71- and gomesin-based nanoparticles penetrated into HeLa cells more efficiently than the parental T22-GFP-H6 nanoparticles (Figure 3B). Melittin oligomers, in contrast, were engulfed by cells at levels similar to those described for T22-GFP-H6 (Figure 3B). Despite the high cell penetrability shown by nanostructured gomesin and CLIP71, such uptake was CXCR4-dependent. The pre-addition of AMD3100, a CXCR4 antagonist that competes with T22 for CXCR4 binding, dramatically blocked the increase of intracellular fluorescence linked to these materials (Figure 3C). On the contrary, the penetrability of melittin was essentially unspecific, since it was unaffected by the T22 competitor. Finally, HeLa cell death promoted by targeted nanoparticles

Figure 2. Nanostructural characterization of venom-based proteins. A) Hydrodynamic size distribution of venom-based nanoparticles (mean ± SE). The parental T22-GFP-H6 and the unassembled GFP-H6 proteins are included here for size comparison. All proteins were in solution in their respective storage buffers. B) FESEM images of randomly selected fields showing the ultrastructural morphology of nanoparticles. Bars indicate 20 nm.

Figure 3. Biological characterization of venom-based nanoparticles. A) Fluorescence emission spectra of GFP-containing proteins. B) Cell penetrability of venom-based nanoparticles and the parental T22-GFP-H6 in cultured CXCR4+ HeLa cells, 2 h after exposure. The intensity of intracellular fluorescence is corrected by specific fluorescence, thus representing protein amounts. C) Specificity of CXCR4-mediated internalization of nanoparticles as determined by the inhibition mediated by the CXCR4 antagonist AMD3100. D) Cell death induced by venom-based nanoparticles (at 4 × 10⁻⁶ M) over the CXCR4+ HeLa cell line. Significant differences between relevant data pairs are indicated as $p < 0.01$. Venom names (namely MEL, GOM and CLIP71) are used to abbreviate the fusion proteins indicated in Figure 1.
was confirmed by cell viability analyses at increasing exposure times. While gomesin and CLIP71 were clearly cytotoxic, melittin did not show any significant deleterious effect on cultured cells (Figure 3D).

To explore the dose-response toxicity of the nanoparticles up to higher concentrations, we exposed target cells to unusual forms of these proteins, namely bacterial inclusion bodies (IBs). These are nontoxic functional amyloids formed by protein clusters in the recombinant bacteria, accompanying the soluble protein versions.[26] Such protein complexes, when formed by self-assembling proteins, are able to release assembled nanoparticles to the media, for both local and systemic administration in different pathologies.[27,28] These released nanoparticles fully keep the targeting properties conferred by fused homing peptides displayed on such oligomers, as recently demonstrated.[28] In this particular format, that allows reaching high concentrations, toxins act already at 24 h of exposure (Figure 4), and melittin, at such high doses, is highly functional. CLIP71 is still, in this innovative format, the most cytotoxic agent among those tested (Figure 4). Probably, venom-based IBs are able to execute their action through the protein in both packaged and released form, what might account for the faster action compared to data in Figure 3.

In summary, short peptidic natural venoms such as gomesin, melittin and the synthetic cytotoxic peptide CLIP71 can be engineered to constitute self-assembling building blocks of tumor-targeted nanoparticles, thus expanding the spectrum of therapeutic utilities of venoms and related peptides. Gaining nanostructure is expected to enhance the intrinsically antitumor properties of these peptides[28] and generically, the effectiveness of any drug intended for targeted delivery.[29,30] This is because the nanoscale size allows the material fully exploiting the EPR effect, while it also prevents renal filtration.[31] Apparently, this combination of factors ensures reaching the proper biodistribution intrinsically defined by the homing peptide that is necessary but not sufficient for tumor accumulation.[12] The nanoformulation, dimerization and other particular presentations of venom peptides have been described as positive influences of their therapeutic values.[33] However, the targeting effect here provided by T22, namely CXCR4 binding, but also its multiple surface display on oligomeric nanoparticles, are expected to offer additional values over the mere nanoscale size, such as for instance the mimicking of the cooperative attachment of viral particles to target cells.[34] On the other hand, we have proved here that these cytotoxic stretches can be engineered as intricate modular proteins with complex functionalities to generate self-assembled, self-delivered anti-cancer nanorods in absence of any external vehicle,[35] as previously shown for full-length protein toxins.[36] Interestingly, venom peptides can be accompanied by fluorescent proteins, which might offer an interesting additional benefit regarding theragnostic applications or for image-assisted surgery.[35] Gomesin and CLIP71 fully keep their functionalities in their accommodation sites, namely the carboxy terminus of the fusion proteins. However, the proximity of melittin to the amino terminus, even not precluding nanoparticle formation, prevents the specific CXCR4-dependent cell penetration mediated by T22 (Figure 3). This is probably caused by a disturbed capability and specificity of the whole macromolecular complex in its interaction with the receptor, by the presence of the foreign peptide placed in close vicinity to the cationic T22. In this particular construct, the nanoparticle lacked the ability to specifically interact with CXCR4+. T22, in close vicinity of highly structured protein domains might have its ability to interact with CXCR4 importantly reduced, in agreement with previous data.[36] The position of such venom in any modular construct intended for targeted delivery should be then accurately explored, to keep the intended selectivity. Cell penetration of the present construct might occur by a direct insertion of melittin molecules into the cell membranes. Indeed, the melittin domain might have a residual cytotoxic activity against cells in the media due to its pore-forming action, that could be executed externally irrespective of cell penetration (Figure 3D). Such membrane activity would be not expected, by itself, to cause lack of CXCR4 selectivity in the penetration of the materials, since the biological activity of the successful CLIP71 construct also relies on membrane disruption[31] and the protein kills the cells via its precise binding to CXCR4 (Figure 3C).

All these data fully support the structural robustness of the self-assembling platform presented here and they also point out

![Figure 4](https://www.advancedsciencenews.com/otherimages/fig4.png)

**Figure 4.** Biological characterization of venom-based nanoparticles. Dose-response cell death of cultured CXCR4\(^+\) HeLa cells 24 h (left) and 72 h (right) after exposure to protein nanoparticles, presented in IB format,[30] as insoluble, protein-releasing material. Nonfunctional IBs formed by the related protein T22-GFP-H6 are used as a control. Untreated HeLa cells are represented by a red discontinuous line. The standard error is represented by a black line. Mann–Whitney test revealed significant differences between untreated and treated cultures. The level of significance difference is indicated by superscripts (*p < 0.05).
The need for a proper design of the modular scheme when preparing multifunctional proteins intended for molecular medicine and addressed to precision therapies. Since the impact of the module distribution in fusion proteins appears to be higher than previously expected, the activities of functional domains might be enhanced or diminished according to their position in the fusion. Irrespective of the modular arrangement, that would require special attention during protein design, functional recruitment is revealed here as a promising approach to organize protein venoms as stable and highly efficient protein materials. The resulting supramolecular complexes show promise and potential as protein-only nanomedicines for the precision treatment of cancer. Among other cytotoxic proteins, venoms are of particular interest since they exhibit variate and multiple anticancer activities. In addition to causing cell death by pore formation and cell permeabilization, other cell alterations have been described. Melittin induces cell cycle alterations, promotes cell proliferation and/or growth inhibition and triggers apoptotic and necrotic cell death through several mechanisms, including the activation of caspases and matrix metalloproteinases. Gomesin affects programmed cell death through the expression levels of cell cycle proteins, by the generation of reactive oxygen species (ROS) and changes in the intracellular levels of Ca²⁺. Regarding CLIP71, several mechanisms of action have been suggested, namely induction of apoptosis via upregulation of death effectors, by mitochondrial membrane permeabilization or changes in the mitochondrial membrane potential.

In a general context, the development of cytotoxic proteins (venoms and others), that are tumor-targeted through fused homing peptides and that self-organize as regular multimeric nanoparticles, is a step further toward the emerging concept of self-delivered, vehicle-free nanoscale drugs. In conventional nanoconjugates, nanocarriers, being therapeutically inert, demand the major fraction of material and production resources and pose severe concerns regarding side toxicities linked to the used nanomaterials. In contrast, protein only drugs are intrinsically biocompatible, and the environmental release (being a matter of concern, for instance, in the case of metal and other type of nanoparticles), would be strictly prevented by cell or media proteases, expected to degrade the administered proteins upon the execution of their biological activities over target cells. Note also that conventional untargeted cytotoxic drugs, secreted by urine, are also a matter of environmental concern. Also, as in the case of the engineered venoms presented here, protein-only drugs can be adapted through simple genetic engineering to self-organize as nanoscale functional entities without chemical conjugation, and to result in true self-delivered, self-targeted drugs for precision medicines in absence of heterologous carrier materials. The presentation of these protein drugs as secretory materials (Figure 4) would benefit from additional benefits, such as faster biological effect and the possibility to increase protein doses, at least upon local administration.

**Experimental Section**

*Protein Design, Production, and Purification*: The engineered fusion proteins were designed in house and produced by GeneArt (Invitrogen, Thermo Fisher Scientific). The proteins were produced in plasmid-bearing *E. coli* Origami B cells (BL21, Omp T-, Lon-, TrxB-, Gor-, Novagen), cultured in 2 L shake flasks with 500 mL of LB medium with 100 µg mL⁻¹ ampicillin, 15 µg mL⁻¹ kanamycin, and 12.5 µg mL⁻¹ tetracycline at 37 °C. Recombinant gene expression was induced at an OD₅₅₀ around 0.5–0.7 upon the addition of 0.1 × 10⁻³ M isopropyl-β-D-thiogalactopyranoside (IPTG) and subsequent overnight culture at 20 °C. Then, cells were harvested by centrifugation at 5000 g for 15 min at 4 °C. All proteins were purified by His-tag affinity chromatography using HisTrap HP 1 mL columns (GE Healthcare) by AKTA purifier FPLC (GE Healthcare). For that, bacterial cells were resuspended in wash buffer (20 × 10⁻³ M Tris-HCl, 500 × 10⁻³ M NaCl, 10 × 10⁻³ M imidazol, pH 8.0) in presence of EDTA-free protease inhibitor (Complete EDTA-Free, Roche). Cell disruption was performed by French press (Thermo FA-078A) at 1100 psi and lysates were centrifuged for 45 min (15 000 g at 4 °C) to obtain the soluble cell fraction. Soluble samples were loaded onto the column and after a washing step with 10 column volumes of Wash buffer, the proteins were eluted by a linear gradient of 20 × 10⁻³ M Tris-HCl, 500 × 10⁻³ M NaCl, 100 × 10⁻³ M imidazol, pH 7.0 buffer for T22-FCS-MEL-FCS-GFP-H6 and T22-GFP-H6-FCS-CLIP71 and by 20 × 10⁻³ M Tris-HCl, 500 × 10⁻³ M NaCl, 1 M imidazol, pH 8.0 buffer for T22-GFP-H6-FCS-GOM. Purified fractions were collected and analyzed by TGL stain-free gels (Bio Rad) and Western Blotting with anti-His monoclonal antibody (Santa Cruz Biotechnology Inc.) to observe the protein of interest. Proteins were finally dialyzed against sodium bicarbonate buffer with salt (333 × 10⁻³ M NaHCO₃, 166 × 10⁻³ M NaCl, pH 7.5) overnight at 4 °C. Protein venom nanoparticles, in form of functional IBs, were purified by a protocol for proteins with high tendency to become soluble. The amount of protein was quantified by Western Blotting using an Anti-His monoclonal antibody (Genscript). The amount of recombinant protein was finely estimated by comparison with a GFP-H6 calibration curve. Protein integrity and purity were checked by mass spectrometry (MALDI-TOF) and final protein amounts quantified by Bradford’s assay.

**Fluorescence Determination**: The fluorescence emission spectrum of the fusion proteins was determined in a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies) using an excitation wavelength of 450 nm. Protein samples were diluted in the corresponding storage buffer to 1 mg mL⁻¹ in a final volume of 100 µL.

**Cell Culture and Internalization Studies**: Cultured CCR4⁺ HeLa cell line (ATCC-CCL-2) was used to explore the internalization and specific uptake of nanoparticles. Cells were cultured in Minimum Essential Medium α (Gibco) supplemented with 10% fetal calf serum (Gibco), and incubated at 37 °C and 5% CO₂ in a humidified atmosphere. For internalization studies, cells were cultured on 24-well plates at 6 × 10⁵ cells/well for 24 h until reaching 70% confluence and then, nanoparticles were added at different concentrations (0.025, 1, and 4 × 10⁻⁶ M) to the cell culture in the presence of Optipro medium (Gibco) during 2 h before the flow cytometry analysis. Cells were detached from the plate with 1 mg mL⁻¹ Trypsin (Gibco) for 15 min and then, analyzed on a FACSCanto system (Becton Dickinson) using a 15 W air-cooled argon-ion laser at 488 nm excitation. GFP fluorescence emission was measured with a detector D (530/30 nm band pass filter). Additionally, specific internalization...
through CXCR4 receptor was proved using AMD3100 (octahydrochloride hydrate, Sigma-Aldrich) which inhibits CXCR4-T22 interaction. For that, proteins were incubated at 0.025 $\times$ 10$^{-4}$ m for 2 h in presence of AMD3100 at 1:10 ratio and cell samples were analyzed on the FACS Canto system. Experiments were performed in duplicates. 1 $\times$ 10$^6$ m of protein is estimated to be equivalent to 1.16 $\times$ 10$^{10}$ nanoparticles $\mu$L$^{-1}$, considering 12 monomers per particle as revealed by a recent refining over previous in silico models.\(^{[2]}\)

**Cytotoxicity Analyses:** The CellTiter-Glo Luminescent Cell Viability Assay (Promega) was used to determine the cytotoxicity of protein nanoparticles. Cells were plated in opaque-walled 96-well plates at 3500 cells/well in Minimum Essential Medium $\alpha$ (Gibco) supplemented with 10% fetal calf serum (Gibco) for 24 h at 37 °C until reaching 70% confluence. Then, cells were incubated in presence of protein during 24, 48, and 72 h at 37 °C. For extended time experiments, namely lasting 96 h in confluence. Then, cells were incubated in presence of protein during 24, 48, and 72 h at 37 °C. For extended time experiments, namely lasting 96 h.

**Statistical Analyses:** Quantitative values were expressed as mean ± standard error (SE) of the mean. Data were transformed when necessary and checked for normality and homogeneity of variances with Shapiro–Wilk and Levene tests, respectively. Pairwise divergences of internalization and cell death were evaluated using Student’s t-tests. Differences between groups were considered significant at $p < 0.01$ and $p < 0.05$ and are indicated as $\dagger$ or $\ddagger$ respectively, in the figures. Pairwise divergences of internalization and cell death were evaluated using Student’s t-tests. All statistical analyses were performed with SPSS (IBM SPSS Statistics v. 23.0).

**Acknowledgements**

The authors are indebted to Agencia Estatal de Investigación (AEI) and to Fondo Europeo de Desarrollo Regional (FEDER) (Grant BIO2016-76063-R, AEI/FEDER, UE) to A.V., AGAUR (2017SGR-229) to A.V. and 2017SGR-65 GRC to RM; CIBER-BBN (project NANOPROTHER) granted to AV and CIBER-BBN project 4NanoMets to R.M.; ISCIII (PI15/00272 co-founding FEDER) to E.V. and ISCIII (Co-founding FEDER), PIE15/00028 and PI18/00650 to R.M., and to EU COST (PI15/00272 co-funding FEDER) to E.V. and ISCIII (Co-founding FEDER) to A.V., AGAUR (2017SGR-229) to A.V. and R.M. hold a patent on the therapeutic portfolio (u18-nanotoxicology-unit/). Electron microscopy studies were performed by the Multiblub Plater Reader VICTOR3 (PerkinElmer). Experimental using IBS was done following this same protocol, adjusting times and doses. Experiments were performed in triplicates.

**Conflict of Interest**

A.V., E.V., N.S., L.S.-G., U.U. and R.M. hold a patent on the therapeutic use of nanostructured cytotoxic proteins.

**Keywords**

cytotoxic nanoparticles, functional materials, protein venoms, self-assembled nanoparticles

Received: February 6, 2020
Revised: March 27, 2020
Published online: May 25, 2020

[1] a) B. Gustavsson, G. Carlsson, D. Machover, N. Petrelli, A. Roth, H. J. Schmoll, K. M. Tveit, F. Gibson, Clin. Colorectal Cancer 2015, 14, 1; b) M. A. Socransky, I. Bondarenko, N. A. Karaseva, A. M. Makhson, I. Vynnychenko, I. Okamoto, J. K. Hon, V. Hirsh, P. Bhat, H. Zhang, J. L. Iglesias, M. R. Schenkel, J. Clin. Oncol. 2012, 30, 2055; c) M. Joerger, Cancer Chemother. Pharmacol. 2016, 77, 221;
d) M. S. Lee, E. C. Dees, A. Z. Wang, Oncology 2017, 31, 198.

[2] a) L. Li, J. Huang, Y. Lin, Toxins 2018, 10, 346; b) C. C. Liu, D. J. Hao, Q. Zhang, J. An, J. J. Zhao, B. Chen, L. L. Zhang, H. Yang, Cancer Chemother. Pharmacol. 2016, 78, 1113; c) X. Y. Zhang, P. Y. Zhang, Oncol. Lett. 2016, 12, 3683; d) Y. Q. Yan, J. Xie, J. F. Wang, Z. F. Shi, X. Zhang, Y. P. Du, X. C. Zhao, Exp. Biol. Med. 2018, 243, 645.

[3] N. Serna, L. Sanchez-Garcia, U. Unzueta, R. Diaz, E. Vazquez, R. Mangues, A. Villaverde, Trends Biotechnol. 2018, 36, 318.

[4] a) J. Shi, P. W. Kantoff, R. Wooster, O. C. Farokhzad, Nat. Rev. Cancer 2017, 17, 20; b) U. Unzueta, M. V. Cepedes, E. Vazquez, N. Ferrer-Miralles, R. Mangues, A. Villaverde, Trends Biotechnol. 2015, 33, 253; c) N. Olov, S. Bagheri-Khoulenjani, H. Mirzadeh, J. Biomed. Mater. Res., Part A 2018, 106, 2272.

[5] a) W. Huang, P. Hao, J. Qin, S. Luo, T. Zhang, B. Peng, H. Chen, X. Zan, Acta Biomater. 2019, 90, 441; b) T. Y. Chen, W. J. Cheng, J. C. Horng, H. Y. Hsu, Colloids Surf., B 2020, 187, 110644.

[6] H. Lopez-Laguna, U. Unzueta, O. Conchillo-Sole, A. Sanchez-Chardi, M. Sarasodona, O. Cano-Carrido, E. Volta, L. Sanchez-Garcia, N. Serna, P. Saccardo, R. Mangues, A. Villaverde, E. Vazquez, Acta Biomater. 2019, 83, 257.

[7] M. V. Cepedes, U. Unzueta, A. Avino, A. Gallardo, P. Alamo, R. Sala, A. Sanchez-Chardi, I. Casanova, M. A. Mangues, A. Lopez-Pousa, R. Eritja, A. Villaverde, E. Vazquez, R. Mangues, EMBO Mol. Med. 2018, 10, e8772.

[8] a) J. R. Prashanth, A. Brust, A. H. Jin, P. F. Alewood, S. Dutertre, R. J. Lewis, Future Med. Chem. 2014, 6, 1659; b) S. S. Pineda, E. A. Undheim, D. B. Rupasinghe, M. P. Ikonomopoulou, G. F. King, Future Med. Chem. 2014, 6, 1699; c) E. Ortiz, G. B. Currola, E. F. Schwartz, L. D. Possani, Toxicoin 2015, 93, 125.

[9] Y. N. Utkin, World J. Biol. Chem. 2015, 6, 28.

[10] J. Chaisakul, W. C. Hodgson, S. Kuruppu, N. Prasongsok, J. Cancer 2016, 7, 1571.

[11] a) H. Tamamura, M. Kuroda, M. Masuda, A. Otaka, S. Funakoshi, H. Nakashima, N. Yamamoto, M. Waki, A. Matsumoto, J. M. Lancelin, D. Kohda, S. Tate, F. Inagaki, N. Fuji, Biochim. Biophys. Acta, Protein Struct. Mole. Enzymol. 1993, 1163, 209; b) H. Tamamura, M. Imai, T. Ishihara, M. Masuda, H. Funakoshi, H. Oyake, T. Murakami, R. Arakaki, H. Nakashima, A. Otaka, T. Ikuba, M. Waki, A. Matsumoto, N. Yamamoto, N. Fujii, Bioorg. Med. Chem. 1996, 4, 1033.

[12] J. D. Tanner, E. Deplazes, R. L. Mancerna, Molecules 2018, 23, 1733.

[13] K. K. Curtis, J. Sarantopoulos, D. W. Northfelt, G. J. Weiss, K. M. Barnhart, J. K. Whisnant, C. Leuschner, H. Alila, M. J. Borad, R. R. Kamanathan, Cancer Chemother. Pharmacol. 2014, 73, 931.

[14] C. Y. Koh, R. M. Kini, Toxicoin 2012, 59, 497.

[15] a) B. Chatterjee, Curr. Top. Med. Chem. 2018, 18, 2555; b) P. G. Ojeda, C. K. Wang, D. J. Craik, Biopolymers 2016, 106, 25; c) C. Safo-Poku, O. Eshun, K. H. Lee, Toxicoin 2016, 122, 109.
