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Review

Snake Venom and 3D Microenvironment Cell Culture: From Production to Drug Development

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Abstract: Snake venoms are a natural biological source of bioactive compounds, mainly composed of proteins and peptides with specific pathophysiological functions. The diversity of protein families found in snake venoms is reflected by the range of targets and toxicological effects observed, and consequently, a wide variety of potential pharmacological activities. In this context, in vitro biomimetic models such as spheroid and organoid systems, which are three-dimensional (3D) cell culture models, enable extensive screening and identification of substances with pharmacological potential and the determination of the mechanisms underlying their activities. In this review we summarize the main findings of 3D microenvironment cell culture as a promising model for snake venom research, from producing snake toxins on venom gland organoids to screening pharmacological active compounds on spheroids for drug development.

Keywords: snake toxins; 3D cell culture; spheroids; organoids

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1. Introduction

Three-dimensional (3D) in vitro models have wide applications in several fields of biology, including cancer research, stem cell research, drug discovery, pharmacological research, etc. The 3D models provide an in-vivo-like microenvironment that better reflects physiological properties, such as cell–cell and cell–matrix interactions, tissue-specific architecture, cell differentiation, cell metabolism, signal transduction, gene and protein expressions, etc., as opposed to traditional two-dimensional (2D) monolayer cell models [1–5]. The 2D cell models are currently the standard technology applied in life science research as these serve as useful tools for the selection and evaluation of a drug candidate in terms of its efficiency and toxicity toward several targets and pathways [6,7]. However, due to the lack of tridimensionality, 2D cell models do not effectively imitate the cell–cell or cell–matrix interactions and the spatial architecture and microenvironments observed in vivo [7]. Therefore, 3D cell culture is emerging as an attractive tool to recapitulate the in vivo architecture and microenvironment of healthy tissue and organs, as well as those of solid tumors, to evaluate the in vivo-like response to drug candidates and obtain better insights into the molecular mechanisms becoming an interesting preclinical model [8–10]. Several 3D cell cultures have become available currently, including scaffold-free models such as spheroids and organoids, as well as scaffold-based technologies such as hydrogels and bioprinting [1,6].

Natural products obtained from animal venoms, especially snake venoms, have demonstrated significant therapeutic potential in this regard. Therefore, due to varied classes of molecules exhibiting a wide range of pharmacological activities, snake venom compounds were used as a design for novel therapeutic agents [11]. A classic example of

drug development is an anti-hypertensive agent, Captopril, the first approved drug that was designed based on the structure of a bradykinin-potentiating peptide isolated from *Bothrops jararaca* venom [12–14].

The traditional 2D in vitro models have been applied extensively for characterizing the biological functions of the constituents from animal venoms, including their anti-inflammatory, immunomodulation, anti-viral, anti-microbial, and anti-cancer activities. However, in recent studies, 3D models have been gaining preference for understanding the functional role of these venoms in biomimetic microenvironments, thereby emerging as important tools for the development of novel drugs. This article summarizes the findings of relevant studies conducted on animal venoms, with a focus on snake venom toxins as potential drug candidates and 3D microenvironment models as an effective platform for such evaluations.

2. Snake Venom Toxins

Snake venoms are complex mixtures of biologically active compounds, mainly comprising proteins and peptides, secreted from specialized venom glands [15,16]. Venomous snake species have relied for millions of years on their venom for hunting to obtain food, evolving in these years to render their venom toxic to a wide range of animals [17,18]. The constituents of these venoms are diverse at all taxonomic and biological levels, from family to populations, and even between gender and age, which could result in unexpected variation in the toxicity and mechanism of action of the compounds obtained from different sources, even if the source species are closely related [16,19]. Snake toxins are capable of selectively recognizing different biological targets and interfering with one or more physiological processes, causing different manifestations, including interruption of nervous system function, blood clotting, hemolysis, local and systemic hemorrhages, tissue necrosis, etc. [11,20,21]. The constituents of snake venoms may be classified into enzyme components and non-enzyme components. The most common enzymatic snake toxins include phospholipase A_2 (PLA2), snake venom metalloproteinases (SVMP), snake venom serine protease (SVSP), and L-amino acid oxidases (LAAO). The non-enzymatic snake venom components include disintegrins (DIS), three-finger toxins (3FTx), Kunitz-type protease inhibitors (KUNs), cysteine-rich secretory proteins (CRISP), C-type lectins (CTL) and bradykinin-potentiating peptides (BPP) [22–25]. However, despite their toxicity, isolated snake toxins may exhibit a wide range of therapeutic effects owing to their diverse and distinct pharmacological activity, high target affinity, and receptor selectivity (Table 1) [22,23].

Table 1. Snake venom compounds and their known toxicological effects illustrate their wide range of potential pharmacological applications.

Snake Venom Toxin Family	Snake Family	Toxicological Effects	Pharmacological Effects	Reviewed by
PLA2	*Viperidae, Elapidae, Colubridae.*	Neurotoxicity, Myotoxicity, Cytotoxicity, Cardiotoxicity, Hemolysis, Edema, Hyperalgesia	Antiinflammatory Analgesic, Antitumoral, Antiangiogenic, Antibacterial, Antiviral	[26,27]
SVMP	*Viperidae, Elapidae, Atractaspididae, Colubridae.*	Hemorrhage, Myonecrosis, Coagulopathy, Tissue Damage	-	[28,29]
SVSP	*Viperidae, Elapidae, Colubridae.*	Hemotoxic, Rupture Capillary Vessels, Pro-coagulant or Anti-coagulant, Fibrinolysis, Platelet Aggregation	Thrombolytic	[30,31]
LAAO	*Viperidae, Elapidae.*	Apoptosis, Hemorrhage, Cytotoxicity, Edema	Antibacterial, Antitumoral, Antiprotozoan, Antiviral	[32]

Table 1. *Cont.*

Snake Venom Toxin Family	Snake Family	Toxicological Effects	Pharmacological Effects	Reviewed by
3FTx	*Elapidae, Colubridae.*	Neurotoxicity, Paralysis	Analgesic	[28]
DIS	*Viperidae, Atractaspididae, Elapidae, Colubridae.*	Inhibit Cell-ECM, Loosen Anchoring Tissue	Antitumor, Anti-platelet	[33,34]
CTL	*Viperidae, Elapidae.*	Induction or inhibition of platelet aggregation	Antimetastatic, Antiangiogenic	[35,36]
CRISP	*Viperidae, Elapidae, Colubridae.*	Myotoxicity, Inhibition of smooth muscle contraction	Antiparasitary	[37,38]
BPP	*Viperidae.*	Hypotension	Anti-hypertensive	[39,40]

PLA2: Phospholipase A_2; SVMP: Snake venom metalloproteinase; SVSP: Snake venom serine protease; LAAO: L-amino acid oxidase; 3FTx: three-finger toxin; DIS: Disintegrin; CTL: C-type lectin; CRISP: Cysteine-rich secretory protein; BPP: Bradykinin-potentiating peptide.

3. Spheroid Technology

Spheroids are cellular aggregates that self-assemble in a scaffold-free manner, thereby preserving cell–cell interactions and the tissue-specific phenotype [1,6,41,42]. Spheroids may be obtained using different strategies, such as liquid overlay, microfluidic-based assembly, magnetic levitation, spinner flasks, the hanging drop method, etc. [1,4,8]. The principle underlying these techniques is to induce spontaneous cellular adherence and assembly by minimizing cellular interaction with the substrate via physical forces, such as gravitational or centrifugal forces, thereby allowing the formation of a compact, well-defined structure [43]. The hanging drop method is the most commonly used technique for preparing spheroids owing to its simplicity and low cost [8]. Spheroids recapitulate the physiological characteristics of tissues and tumors, as this model reproduces the cell–cell and cell–matrix interactions, cellular heterogeneity, the nutrient, metabolite, and oxygen gradients, gene expression, and drug resistance observed in the in vivo conditions [4]. Moreover, the extracellular matrix found in spheroids is synthesized by the cells of the model, without interference from an external hydrogel or scaffold allowing for natural cell–matrix interactions [42,44]. Tumor spheroids resemble the initial avascular aggregates of malignant cells and/or micrometastatic regions in vivo and are, therefore, very useful in cancer research and drug screening [45]. Importantly, when using tumor spheroids, the tumor size is correlated to its function, drug penetration, and transport [41]. While larger spheroids (>400 μm) allow for imitating the oxygen, nutrient, and catabolite gradients and hypoxic regions in the poorly vascularized tumors, smaller spheroids (<200 μm) may be used for drug evaluations [41].

Applications of Spheroids for Evaluating Snake Venom Components

The compound PLA2 derived from different snake venoms has demonstrated the potential for antitumor, antimetastatic, and antiangiogenic effects in vitro in 2D monolayer culture [46–49]. Further, Azevedo and colleagues demonstrated the antitumor and antimetastatic effects of BthTX-II, an Asp49-PLA2 isolated from the venom of *Bothrops jararacussu*, on MDA-MB-231 human triple-negative breast cancer cells using the 3D culture technique [50]. The authors monitored the development of spheroids in Matrigel for 7 days and reported that the presence of different concentrations of BthTX-II (1, 10, and 50 μg/mL) impaired spheroid formation and tumor growth compared to the non-tumorigenic MCF10A cell line [50]. In their recent study, these authors demonstrated the antiangiogenic effect of the BthTX-II molecule by co-culturing MDA-MB-231 spheroids with an HUVEC vessel network on Matrigel. The authors reported that during the interaction between tumor spheroids and endothelial cells, BthTX-II promoted the disruption of the HUVEC ves-

sel network by inhibiting endothelial cell aggregation, exhibiting a complete inhibition of cell co-culture migration and proliferation compared to the control cells cultured in Matrigel [51]. A different study reported the modulatory effect of Crotoxin (CTX), a heterodimeric PLA2 present in the *Crotalus durissus terrificus* venom, during the epithelial–mesenchymal transition (EMT) process. In this study, spheroids composed of non-small-cell lung cancer cells (NSCLC, A549, and Calu-3 cell lines) and normal lung fibroblasts cells (MCR-5 cell line) were utilized to imitate the early tumor–stroma interactions, i.e., to mimic the avascular tumor initiation step [52]. However, unlike BthTX-II, CTX did not interfere with spheroid formation. The use of 12.5 nM of CTX promoted the reduction in the invasion area of the cells that migrated out from the spheroids toward the 3D collagen matrix. Moreover, this effect was observed to be correlated to the decreased protein expression levels of EMT markers, such as N-cadherin, α-SMA, and integrin αv, and was accompanied by decreased secretory levels of MMP-9 and MMP-13 as well as the cytokines and growth factors associated with the EMT process, particularly from the CXCL5/CXCR2 and IL-8/CXCR1/CXCR2 axes [52]. Both of the above-stated studies demonstrated that the antimetastatic and antiangiogenic effects of the snake venom constituent PLA2 were not related to catalytic activity but due to interaction with integrin-mediated signaling pathways [51,52]. Another component from *C. d. terrificus*, a myo-neurotoxin named crotamine (Crot), a cell-penetrating peptide (CPP), was synthesized as an analog (sCrot) [53]. It was demonstrated that sCrot could selectively penetrate a spheroid model composed of melanoma cells as well as other tumor cells in suspension. As opposed to CTX and BthTX-II, which exhibit antitumor effects, sCrot could serve as a model for tumor microenvironment investigations for studying cancer and stromal cell interactions and also as a carrier of antitumor drugs for evaluating novel therapeutics in drug development [53].

Another class of snake venom components is disintegrins, a family of small, non-enzymatic substances containing the arginine-glycine-aspartic acid (RGD) peptide sequence. Disintegrins have been detected in the venoms derived from the *Viperidae*, *Crotalidae*, *Atractaspididae*, *Elapidae*, and *Colubridae* snake families. Disintegrins bind specifically to certain integrins expressed by tumor cells and the endothelial cells in the tumor microenvironment, such as αvβ3, αvβ5, α5β1, and αIIbβ3 [23,54,55]. Swenson and colleagues studied the effect of vicrostatin, a disintegrin synthesized from the natural snake venom disintegrin named contortrostatin, on a spheroid model composed of SKOV3 ovarian cancer cells that was implanted in a mouse model to simulate a condition of solid tumor resistance to chemotherapy [56]. Vicrostatin was observed to promote the highly effective inhibition of ovarian cancer dissemination and progression. In addition, bioluminescence imaging results revealed ~95–98% inhibition of tumor growth [56].

Bhat and colleagues reported the application of P-I metalloproteinases and L-amino acid oxidases (LAAO) derived from the genus *Bothrops* in the spheroids composed of endothelial cells [57]. P-I metalloproteinases belong to the group of SVMPs, which are large multidomain proteins containing a pro-enzyme domain and a conserved zinc-protease domain. SVMPs are classified into three types, P-I to P-III, among which the P-I metalloproteinases contain only one metalloproteinase domain [58]. LAAO is a flavoenzyme that converts the stereospecific L-amino acid into the corresponding alpha-keto acid along with hydrogen peroxide and ammonia as byproducts [23]. In the above-stated study, the P-I metalloproteinases and LAAO toxins were isolated from *Bothrops moojeni* (BmMP-1 and BmLAAO, respectively) and *Bothrops atrox* (BaMP-1 and BaLAAO, respectively), and subsequently evaluated using HUVEC spheroids within a collagen matrix. The P-I metalloproteinases and LAAO from both the *Bothrops* species promoted the modulation of angiogenesis by reducing sprout outgrowth in the 3D spheroid model. The authors, therefore, recommended using these toxins for designing an antiangiogenic strategy for pathological conditions in which angiogenesis is exacerbated, such as tumor growth, metastasis, diabetic retinopathy, and inflammatory diseases [57]. Figure 1 summarizes the main effects of different snake venom toxins applied in the spheroid model.

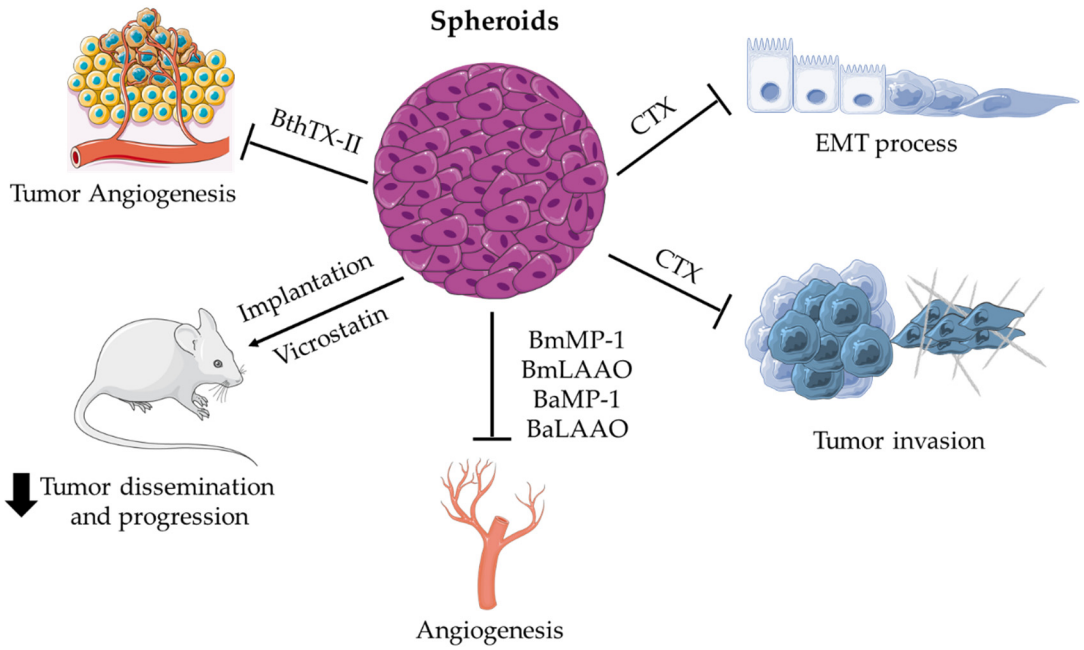

Figure 1. Spheroid applications for the study of snake toxins. This figure was created using Smart-Servier Medical Art (https://smart.servier.com, Accessed on 13 April 2022).

4. Organoid Technology

The term organoid was initially used to refer to a specialized and individual organ-like structure constructed in vitro within 3D gels from small tissue fragments derived from the patients' tissue separated from stroma [1]. Currently, however, the organoid technology involves a variety of tissue culture techniques for preparing self-organizing and self-renewing 3D cultures from embryonic stem cells (ESC) or organ-restricted adult stem cells (ASC) [59]. Both ESC and ASC approaches exploit the seemingly infinite expansion potential of normal stem cells in culture to recapitulate the cell functionality and morphology similar to that exhibited by the native tissue [42]. In order to induce a self-organizing structure, cells are embedded in a hydrogel rich in matrix extracellular proteins, such as Matrigel or Basement Membrane Extract (BME), to simulate the appropriate physiological microenvironment [42,60].

In the context of drug development, organoids serve as a reliable 3D model in a physiologically relevant manner for investigating the pharmacokinetics and toxicity of drug candidates [9]. Since the phenotype, genotype, and metabolic profiles of organoids are highly similar to those of the native tissues, such as cancer tissues, tumor organoids may be utilized as a valuable model for drug candidate screening and understanding the pharmacodynamics of a potential pharmacological drug candidate [1].

Application of Organoids in Snake Venom Research

Recently, an organoid platform with the potential for in vitro snake venom production has been established. Yorick and collaborators reported developing functional snake venom glands from nine different species belonging to two major families of venomous snakes—the *Elapidae* (*Naja pallida*, *Naja annulifera*, *Naja nivea*, *Naja atra*, and *Aspidelaps lubricus cowlesi*) and the *Viperidae* (*Echis ocellatus*, *Deinagkistrodon acutus*, *Crotalus atrox*, and *Bitis arietans*) [61]. The authors dissected both late-stage embryo and adult specimens to obtain the venom gland tissue cells, which were then cultured into mini-organs in long-term culture [61]. First, the venom gland tissue was dissociated to release cells, which were homogenized and then embedded into a 3D environment BME for generating organoids that were allowed to expand (Figure 2) [61–63]. The technology produced organoids that were phenotypically similar to natural venom glands and also exhibited similar functions as these

secreted, functionally active venom components. Interestingly, the venom gland organoids exhibited cellular heterogeneity as different cell types produced different compounds of the venom [61,62]. The authors also used another 3D cell culture technology known as the organ-on-chip technology to assess the biological activity of the organoid-secreted venom peptides by exposing the murine muscle cells to the organoid supernatant. The results revealed that the firing of the muscle cells was terminated, which simulated the paralysis condition observed upon snakebite envenoming [61]. Studies have also reported the in vitro cell suspension culture of secretory cells from *Bothrops jararaca* venom glands for up to 21 days [64–66], with the synthesized toxin exhibiting hemorrhagic activity similar to that of the toxin from the venom glands harvested directly from *B. jararaca* specimens [66,67]. However, due to the lack of representation of tissue complexity, as demonstrated by the organoids, the in vitro cell suspension culture of secretory cells had a short lifespan, as opposed to the organoids model that could be cultured for over 2 years [62]. The above study contributed immensely to the establishment of a platform for venom-based drug development and also to the research on antivenoms.

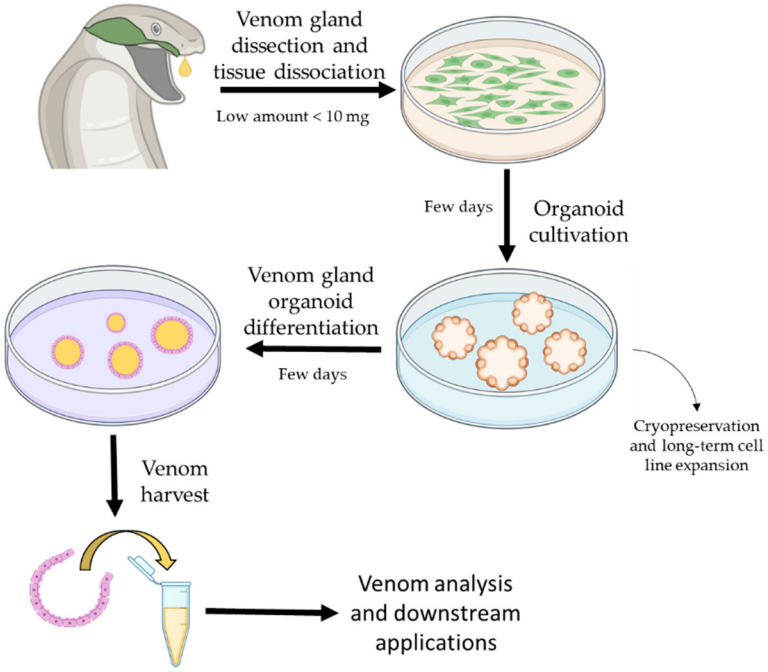

Figure 2. Schematic illustration of the venom gland's organoid generation. Created with BioRender.com.

5. Conclusions

The current challenge is not just the identification of natural substances with potential pharmacological and therapeutic properties but also the establishment of cellular and biomimetic microenvironment platforms, considering the cellular, extracellular matrix, and tissue elements for the expansion of mechanistic studies that require low concentrations of snake venoms, toxins, and other isolated compounds. In this context, 3D cell culture models, such as spheroids and organoids, have demonstrated great potential. The mechanistic studies that were conducted to reveal the effect of snake venom components on normal as well as tumor cells and the interactions of these substances with other elements in the cellular microenvironment, such as stromal cells and the extracellular matrix, have reported promising outcomes. The main advances in the determination of the antitumor activity of venoms and toxins include the unraveling of the mechanisms of action of these substances in relation to each element of the cellular microenvironment as well as the role of autocrine and paracrine mediation in this process. Therefore, the use of a combination of in vitro 3D culture technology and natural substances, such as snake venom components, represents a

promising preclinical strategy for the fields of drug development, cancer target therapy, antivenom research, and tissue replacement.

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