

Marine biomaterials: Biomimetic and pharmacological potential of cultivated *Aplysina aerophoba* marine demosponge

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ARTICLE INFO

Keywords:

Marine biomaterials
Chitin
Cell culture
Tissue engineering
Cardiomyocytes
Aerophysinin-1

ABSTRACT

Marine demosponges of the Verongiida order are considered a gold-mine for bioinspired materials science and marine pharmacology. The aim of this work was to simultaneously isolate selected bromotyrosines and unique chitinous structures from *A. aerophoba* and to propose these molecules and biomaterials for possible application as antibacterial and antitumor compounds and as ready-to-use scaffolds for cultivation of cardiomyocytes, respectively. Among the extracted bromotyrosines, the attention has been focused on aerophysinin-1 that showed interesting unexpected growth inhibition properties for some Gram-negative clinical multi-resistant bacterial strains, such as *A. baumannii* and *K. pneumoniae*, and on aerophysinin-1 and on isofistularin-3 for their anti-tumorigenic activity. For both compounds, the effects are cell line dependent, with significant growth inhibition activity on the neuroblastoma cell line SH-SY5Y by aerophysinin-1 and on breast cancer cell line MCF-7 by isofistularin-3. In this study, we also compared the cultivation of human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) on the *A. aerophoba* chitinous scaffolds, in comparison to chitin structures that were

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<https://doi.org/10.1016/j.msec.2019.110566>

Received 29 August 2019; Received in revised form 28 November 2019; Accepted 15 December 2019

Available online 17 December 2019

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pre-coated with Geltrex™, an extracellular matrix mimetic which is used to enhance iPSC-CM adhesion. The iPSC-CMs on uncoated and pure chitin structures started contracting 24 h after seeding, with comparable behaviour observed on Geltrex-coated cell culture plates, confirming the biocompatibility of the sponge biomaterial with this cell type. The advantage of *A. aerophoba* is that this source organism does not need to be collected in large quantities to supply the necessary amount for further pre-clinical studies before chemical synthesis of the active compounds will be available. A preliminary analysis of marine sponge bioeconomy as a perspective direction for application of biomaterials and secondary bioactive metabolites has been finally performed for the first time.

1. Introduction

Sponges (Porifera) are recognized as the first multicellular organisms which have successfully survived over 600 million years of evolution [1]. The survival strategies of these sessile, feed filtering aquatic organisms are based on two principles: the existence of mechanically rigid biopolymer- or biopolymer/biomaterial-based three dimensional (3D) skeletons and their ability to synthesize a broad diversity of secondary metabolites with antimicrobial as well as antipredator activities. Most sponges belonging to the class Demospongiae possess mechanisms to regenerate their damaged bodies or to develop 3D cell aggregate growth while attached to natural substrates [2]. This phenomenon has been observed in vivo [3] and remains the basic method for aquacultural cultivation of demosponges [4–7]. Regeneration of corresponding tissue-like structures has been observed in both spongin- [5] and chitin-producing [3] keratosan demosponges. Intriguingly, the structural features of spongin- and chitin-based skeletons – 3D micro-architecture, porosity, elasticity, anastomosing morphology – possess excellent biomimetic potential for applications in naturally pre-designed constructs in regenerative medicine and tissue engineering [8–15].

After the discovery of chitin in diverse representatives of demosponges belonging to the Verongiida order [12,16–19], this group of sponges [20] has attracted scientific attention as a renewable potential source of naturally pre-fabricated 3D chitinous scaffolds [21] and bromotyrosines, well known bioactive secondary metabolites [22–24]. Previously bromotyrosines have been extracted from verongioid sponges without any attention to the possibility to also isolate the chitin skeleton. Nowadays, the methodological strategy is based on development of methods for simultaneous isolation of both chitin and bromotyrosines in such way so that the unique 3D structural organization of their chitinous skeletons remains stable and useful for further practical applications, for example, as scaffolds in tissue engineering and regenerative medicine. Recently, one of such express methods using microwave irradiation of the *Aplysina archeri* demosponge combined with stepwise treatment by 1% sodium hydroxide, 20% acetic acid, and 30% hydrogen peroxide has been reported [12]. The procedure does not deacetylate chitin to chitosan and enables the recovery of structurally integral 3D biomaterial. Fibrous and anastomosing skeletons of all verongioid demosponges studied so far [10,11] possess fan-shaped and flat (family Ianthellidae) [25], or cylinder-like (i.e. sponges belonging to the family Aplysinidae) [12] architecture. Both forms remain to be found in the chitinous scaffolds which resemble the shape, size and porosity of the naturally occurring skeletons.

In this study, we focus our attention on chitinous scaffolds from the *Aplysina aerophoba* demosponge [16], especially due to its broad distribution in Europe (Adriatic and Mediterranean Sea) as well as the ability to grow under marine ranching conditions [26] (Fig. 1). The aim was to isolate selected bromotyrosines and chitinous scaffolds simultaneously and to study their applications as antitumor and antibacterial reagents against some clinically relevant strains and as ready-to-use constructs for cultivation of cardiomyocytes, respectively. Our efforts primarily focused on aeroplysinin-1 [27], as one of the most studied bromotyrosines with multi-target activities (i.e. antibiotic, cytostatic, anti-algal, anti-inflammatory, anti-angiogenic, antitumor as

well as endothelial cell redox regulation activity) (see for review [28–30]). For the first time, we plan to calculate economic potential of *A. aerophoba* demosponges as renewable source of biomaterials and bioactive compounds using open access data.

2. Materials and methods

2.1. Sample collection

A. aerophoba was collected in the Adriatic Sea (Kotor Bay, Montenegro) from the marine aquaculture facility (Fig. 1) from the depths of 3–5 m by SCUBA diving. Sponge samples were put in ziplock bags underwater, brought to the laboratory and washed with water to remove salts. Selected specimens were air-dried for 7 days prior to further treatment. The use of chemicals was avoided in the cleaning and preparing procedure of the chitinous scaffolds (Fig. 2) and bromotyrosines (Fig. S1).

2.2. Stereomicroscopy imaging

Images of studied materials were taken with a Keyence VHX-6000 digital optical microscope.

2.3. Isolation of chitinous scaffolds

Samples of *A. aerophoba* sponges (Fig. 2A) were carefully cut with a razor blade to produce square-shaped scaffolds with sizes of about $2 \times 2 \times 2$ cm. The isolation of chitin was performed accordingly to previously reported method [14] with slight modifications. In the first stage, the samples of *A. aerophoba* were treated with methanol using an ultrasonic bath to remove the pigments and cells/tissue from the skeletons. The obtained brown methanol-based extract was used in next steps for isolation of bromotyrosines (for details refer to Section 2.4). Afterwards, cell-free skeletal fibers (Fig. 2C) were placed into plastic boxes with 2.5 M NaOH at 37 °C for 72 h to remove residual proteins and pigments. In the last stage, the samples were demineralized with 20% acetic acid at 37 °C for 6 h and after that washed with distilled water until the pH reached 6.8. This isolation procedure was repeated three times to obtain colourless tubular scaffolds (Figs. 2D,E,F).

2.4. Characterization of bromotyrosines

^1H and ^{13}C NMR spectra were acquired on Agilent VNMRS 400 (Agilent, USA) and Bruker Avance DRX 500 (Bruker, Switzerland) spectrometers using DMSO- d_6 as a solvent and tetramethylsilane as an internal standard. Mass spectra were recorded on an LC-MS instrument with chemical ionization (CI). LC-MS data were recorded on an Agilent 1100 HPLC equipped with a diode-matrix and mass-selective detector Agilent LC/MSD SL. Column: Zorbax SB-C18, 4.6 mm $\times$ 15 mm. Eluent: A, acetonitrile – H_2O with 0.1% of trifluoroacetic acid (TFA; 95:5); B, H_2O with 0.1% of TFA. Flow rate: 1.8 mL/min. Thin layer chromatography (TLC) was performed on Polygram SIL G/UV254 plate (Machery-Nagel, Germany) using a 19:1 mixture of CHCl_3 –MeOH as eluent. Column chromatography has been performed using silica gel 60 (230–400 mesh, Merck, Germany) as the stationary phase. Melting

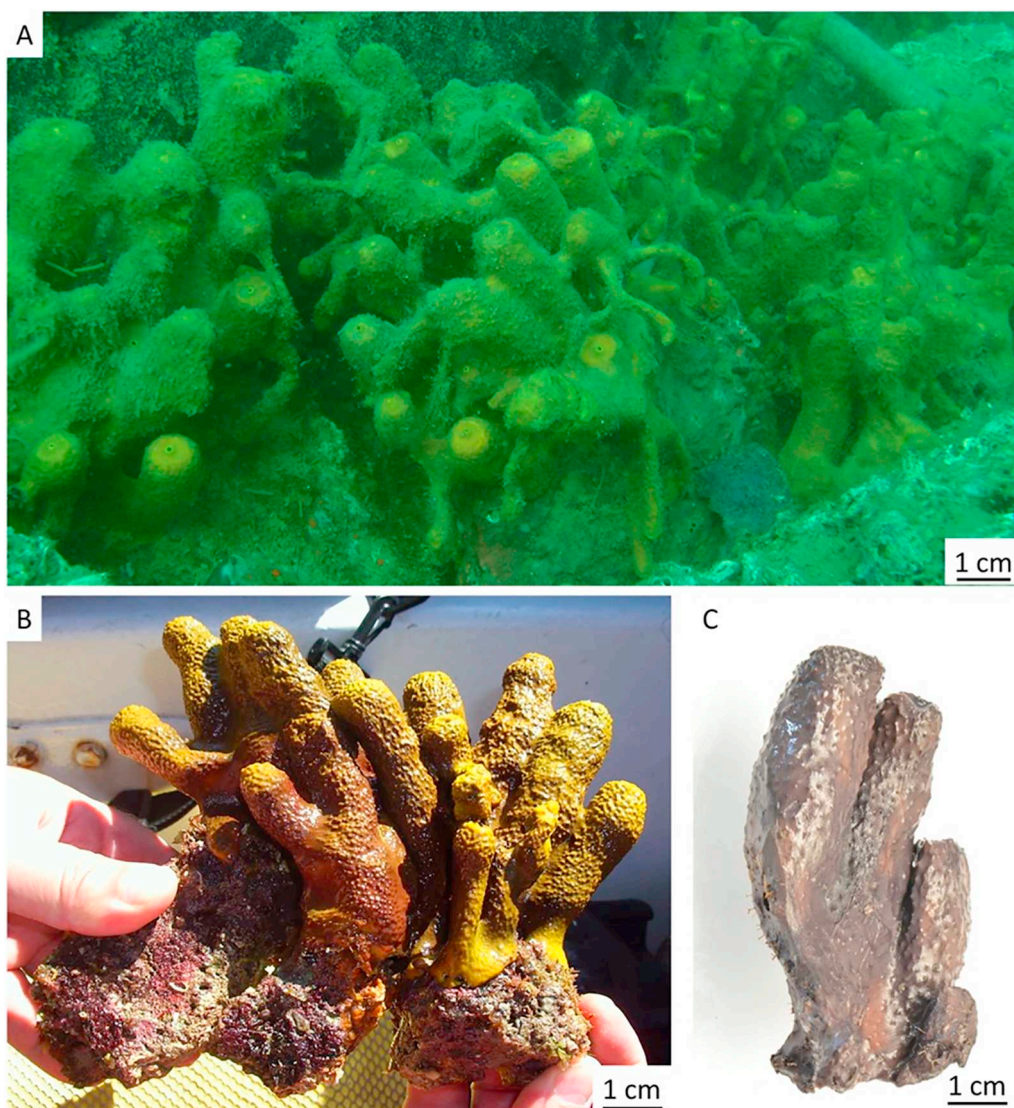


Fig. 1. Underwater image of the colony of *A. aerophoba* demosponges cultivated in Kotor Bay, Montenegro (A). Freshly collected sponges remain yellow (B). After 2 h on air, the colour of the sponge body becomes brown (C). This behaviour is characteristic to *A. aerophoba* and related verongiids. After the drying on air (25–30 °C), the collected sponges lose about 20–40% of their body volume and become black coloured (see Fig. 2A, B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

points were determined using a Boetius melting point apparatus (Boetius Franz Kustner, Germany).

2.5. Isolation of bromotyrosines

The dried *A. aerophoba* sponge (Fig. 2 A, B) (200.0 g) was treated with MeOH (1 L) three times for 36 h, and the extracts were concentrated under reduced pressure to yield 13.5 g of residue (Fig. S1). The residue was then separated on a silica gel column (80 × 5 cm), eluted with a solvent gradient (CHCl₃/MeOH starting from 1:0 to 0:1) and after that divided into 45 fractions. The resulting fractions were pooled together on the basis of TLC. Aeroplysinin-1 (1) (40 mg) was isolated from fraction 10 and was further purified by crystallization from CHCl₃. From 13 to 16 fractions, isofistularin-3 (2) (1.1 g) was obtained. Fractions 34–35 were purified by crystallization with MeOH as solvent, to yield a bisoxazolidinone derivative (3) (35 mg). Fraction 43 was purified with ethanol and it resulted in isolation of *N,N,N*-trimethyl-3,5-dibromotyramine (4) (75 mg).

Aeroplysinin-1 (1) [31,32]. White crystals. Mp = 119–120°C. ¹H NMR (400 MHz, DMSO-*d*₆) 2.78 (s, 1H, CH₂), 3.62 (s, 3H, OCH₃), 3.92

(d, *J* = 8.0 Hz, 1H, CH), 6.13 (s, 1H, OH), 6.19 (d, *J* = 8.0 Hz, 1H, OH), 6.31 (s, 1H, CH); ¹³C NMR (125 MHz, DMSO-*d*₆) 26.4 (CH₂), 59.9 (OCH₃), 73.6 (C), 77.4 (CH), 113.7 (C), 118.6 (C), 119.4 (C), 133.7 (CH), 147.1 (C). MS (CI): *m/z* (%) = 439.8 [M + H]⁺.

Isofistularin-3 (2) [33,34]. White powder. ¹H NMR (400 MHz, DMSO-*d*₆) 3.16–3.41 (m, 4H), 3.45–3.52 (m, 2H), 3.59–3.64 (m, 2H, CH₂), 3.65 (s, 6H, 2OCH₃), 3.81–3.84 (m, 1H, CH₂), 3.87–3.90 (m, 1H, CH₂), 3.92 (d, *J* = 7.2 Hz, 2H, 2CH), 4.05–4.09 (m, 1H, CH), 4.66–4.69 (m, 1H, CH), 5.25–5.31 (m, 1H, OH), 5.71–5.75 (m, 1H, OH), 6.36 (d, *J* = 7.2 Hz, 2H, 2OH), 6.57 (s, 1H, CH), 6.59 (s, 1H, CH), 7.57 (s, 2H, 2CH), 8.37 (t, *J* = 5.6 Hz, 1H, NH), 8.42 (t, *J* = 5.6 Hz, 1H, NH); ¹³C NMR (125 MHz, DMSO-*d*₆) 43.0 (CH₂), 46.8 (CH₂), 59.9 (2OCH₃), 68.5 (CH), 69.7 (CH), 73.8 (2CH), 75.8 (CH₂), 90.7 (C), 113.5 (2C), 117.6 (2C), 121.4 (2C), 130.7 (2CH), 131.4 (CH), 131.5 (CH), 143.1 (C), 147.6 (2C), 151.7 (C), 154.8 (2C), 159.4 (C=O), 159.5 (C=O). The signal of CH₂ overlaps with DMSO. MS (CI): *m/z* (%) = 1115.2 [M + H]⁺.

Bisoxazolidinone derivative (5-[3,5-dibromo-4-(2-oxo-oxazolidin-5-ylmethoxy)phenyl]oxazolidin-2-one) (3) [35]. Colourless crystals. Mp = 215–216°C. ¹H NMR (500 MHz, DMSO-*d*₆)

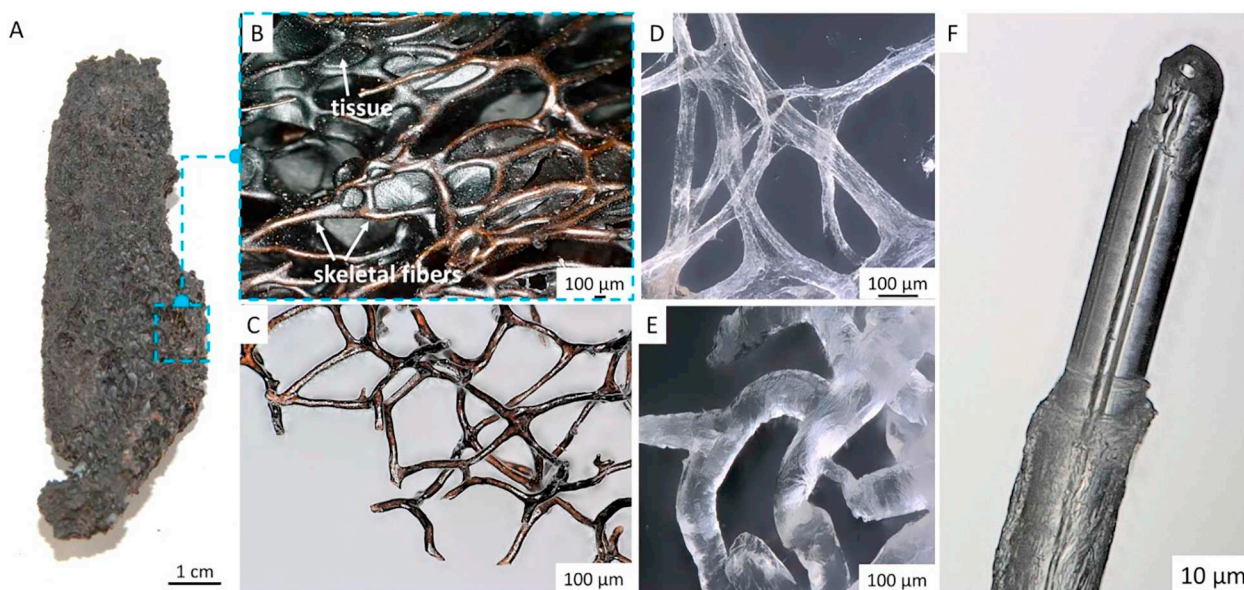


Fig. 2. Selected dried specimens (A, B) of *A. aerophoba* demosponge cultivated under marine ranching conditions (Fig. 1A) have been used for extraction of bromotyrosines (see Fig. 3) and skeletal fibers (C) as a source of three dimensional chitinous scaffolds (D, E). Isolated chitin fibers resemble microtubule morphology (F) and possess high capillary activity against diverse liquids.

3.35–3.37 (m, 1H, CH₂), 3.52 (dd, $J = 5.0, 10.0$ Hz, 1H, CH₂), 3.65 (t, $J = 5.0$ Hz, 1H, CH₂), 3.85 (t, $J = 10.0$ Hz, 1H, CH₂), 4.11–4.16 (m, 2H, OCH₂), 4.92–4.97 (m, 1H, CH), 5.59 (t, $J = 10.0$ Hz, 1H, CH), 7.60 (s, 1H, NH), 7.71 (s, 2H, Ar), 7.75 (s, 1H, NH); ¹³C NMR (125 MHz, DMSO-*d*₆) 41.3 (CH₂), 47.0 (CH₂), 73.1 (OCH₂), 73.6 (CH), 74.4 (CH), 117.6 (2C), 130.4 (2CH), 138.9 (C), 151.7 (C), 158.2 (C=O), 158.5 (C=O). MS (CI): m/z (%) = 437.0 [M + H]⁺.

***N,N,N*-trimethyl-3,5-dibromotyramine (4)** [36]. White crystals. $M_p = 228$ – 229°C . ¹H NMR (400 MHz, DMSO-*d*₆) 2.95–2.98 (m, 2H, CH₂), 3.12 (s, 9H, NCH₃), 3.50–3.53 (m, 2H, CH₂), 7.56 (s, 2H, Ar); ¹³C NMR (125 MHz, DMSO-*d*₆) 27.2 (CH₂), 52.6 (NCH₃), 65.9 (CH₂), 112.5 (2C), 131.1 (C), 133.1 (2CH), 150.1 (C). MS (CI): m/z (%) = 338.0 [M + H]⁺.

2.6. Antimicrobial activity

For the determination of antimicrobial activity of aeropylsinin-1, *Staphylococcus aureus* (ATCC 6538-P) as well as clinical multiresistant strains (Supplementary Table S1), including *Candida albicans*, *Enterobacter cloacae* 40, *Pseudomonas aeruginosa* 89, *Acinetobacter baumannii* 1, *A. baumannii* 130, *Klebsiella pneumoniae* 10, and *K. pneumoniae* 131 from the collection of Department of Microbiology of the National Pirogov Memorial Medical University, Vinnytsya, Ukraine, which were isolated from the patients with wound and burn injuries, were used.

The agar diffusion assay was performed according to standard Kirby-Bauer test [37] using meat peptone agar (MPA) for bacteria and Sabouraud agar for *C. albicans*. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) were determined by standard method of double serial dilutions [38]: to 0.5 mL of liquid nutritive environment, 0.5 mL of 1 mg/mL methanol or DMSO solution of aeropylsinin-1 was added, then double serial dilutions were made. Afterwards, the 0.1 mL of inoculum with 10⁶ colonia-forming units (CFU) of bacteria was added to all solutions with calibrated bacteriological loop. The plates were then incubated for 24 h at 37 °C. The rate of microbial death was estimated with a quantitative suspension test [39]. Briefly, suspension of microorganisms was made in 0.9% solution of sodium chloride and then spread over the sterile MPA for identification of primary CFU number. Simultaneously, with a calibrated bacteriological loop, 0.1 mL of suspension was put to the

solution of aeropylsinin-1 (25 μg/mL) and diluents (water-methanol 3:1). In 15, 30 and 60 min, solutions were spread with the sterile spatula over the MPA and afterwards were incubated for 24 h at 37 °C. The numbers of CFU on MPA were counted and compared to control. The D-rate (time of death of 90% CFU or decreasing of log CFU to 1) was calculated. All the tests were provided with proper control: sterility control of nutritive environment, control of microorganism growth without compound, and control of diluents for antimicrobial activity.

2.7. Cell culture and viability assays

All cell lines were obtained and cultured as indicated in Supplementary Table S2 at 37 °C, 5% CO₂, and 95% humidity. To test whether the cells are mycoplasma free, the MycoAlert Mycoplasma Detection Kit (Lonza, Switzerland) was used. After trypsinization (trypsin/EDTA; 0.05%/0.02%) cells were counted by using counting chambers (Neubauer improved). Experiments were performed antibiotic free after at least one passage after re-cultivation. To determine cytotoxicity, cell viability assays were performed as previously described by us [29]. Briefly, all cell lines (1.75 × 10⁴) were seeded in 96-well plates and incubated for 24 h with different concentrations of the compounds. After 3 h incubation at 37 °C with CellTiter 96® Aqueous One reagent (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega, Mannheim, Germany) the absorption of the whole plate was measured at 490 nm by Anthos htIII plate reader. Cell viability was normalized to the corresponding DMSO control.

2.8. Differentiation and culture of human iPSC-CMs

The chitin scaffolds were autoclaved in PBS (Sigma-Aldrich), cut in fragments (approx. 10 mm × 10 mm) and pre-incubated in DMEM-F12 (Gibco, with L-Glutamine, as uncoated controls) or the extracellular matrix mimetic Geltrex (Gibco, 2 mg/mL in DMEM) for 1–2 h at 37 °C. Human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) were differentiated from two different iPSC lines, which were obtained from healthy donors [40]. The differentiation was performed according to established protocols [40–42] using the modulators CHIR99021 (Merck Millipore) and IWP2 (Merck Millipore). iPSC-CMs were cultured in standard cardiomyocyte medium (RPMI1640 medium (Gibco) with 2% B27 supplement (Gibco)) at 37 °C, 5% CO₂. After

30–35 days, the beating iPSC-CMs were detached using collagenase B (1 mg /mL) for 30–60 min at 37 °C. iPSC-CM layers were carefully transferred into fresh reaction tubes and further digested with 0.25% trypsin/EDTA (Gibco) for 8 min at 37 °C. Afterwards, the cells were resuspended in digestion medium (RPMI1640, 2% B27 supplement, 15% FBS, 2 µM thiazovivin) and chitin scaffolds were incubated with iPSC-CM suspension (total of 1–1.5 million cells) overnight (16–24 h) at 37 °C. The chitin scaffolds were carefully transferred to a transwell plate (Costar, Transwell 6-well plate, 24 mm Insert) and cultured for 20 days. The cell culture medium was exchanged every 2–3 days. Documentation of the cultures was performed using light microscopy (Leica M80, Axiovert100, Leica MC170 HD camera).

2.9. Immunostaining and fluorescence microscopy

After 20 days in culture, chitin scaffolds with cells were carefully washed with PBS (Sigma Aldrich) and incubated in ice-cold methanol (VWR) – acetone (Merck) fixation solution (MeOH/Ac ratio 7:3 v/v) for 10 min at –20 °C. The samples were washed 3 times with PBS at room temperature and incubated in PBS with 1% BSA (Sigma) and 0.1% TritonX-100 (Ferak Berlin) overnight. Next, chitin scaffolds were transferred to 35 mm dishes (MatTek, Glass Bottom Microwell Dishes) with glass bottom. All samples were incubated in a primary antibody mix containing rabbit anti Ki-67 (Abcam, ab833, 1:400) and mouse anti-α-actinin (Sigma-Aldrich A7811, 1:500) in PBS with 1% BSA and 0.1% TritonX-100 overnight at 4 °C. Chitin scaffolds were washed 3 times with PBS at room temperature and incubated in goat anti-mouse-AlexaFluor488 (Life technologies A11001, 1:1000) and goat anti-rabbit-AlexaFluor546 (Life technologies A11035, 1:1000) antibody conjugates for 1 h at room temperature. Hoechst33342 (molecular probes H3570, 1:800) was used to stain cell nuclei. Samples were washed 3 times with PBS and prepared in Fluoromount-G (SouthernBiotech, storage at 4 °C). Respectively, the negative controls were included by incubation of tissue with secondary antibody only. Fluorescence images were obtained using a confocal laser scan microscope (LSM880, Zeiss). To allow 3D reconstructions of the cells on the 3D scaffold, z-stacking of 10–30 images were captured and analyzed (10–30 µm z-range) using Fiji software [43]. Relative quantification of Ki-67-positive cells was performed by manual counting (> 500 cells total) from two independent experiments and different chitin scaffolds.

3. Results and discussion

The isolation of bromotyrosines from the methanolic extract was optimized to estimate their amounts with respect to profitability for their extraction on the large scale in the future. The methanolic extract was evaporated under reduced pressure and the residue was chromatographed on a silica gel column with gradient of CHCl₃/MeOH to obtain aeropylsinin-1 (1), isofistularin-3 (2), and bisoxazolidinone derivative (3) (Fig. S1). These compounds have been previously reported to be present in *A. aerophoba* and other species of the Verongiida order [44–46]. Isofistularin-3 (2) was identified as the major compound in the methanol extract. The halogenated compounds were identified by analysis of the NMR and MS data and comparing their characteristics with the respective data for previously known compounds from sponges. The bromotyrosine derivative bisoxazolidinone (3) was identified by analysis of the 2D NMR spectroscopic data (see Supplementary Material). *N,N,N*-trimethyl-3,5-dibromotyramine (4) was also isolated from the sponge and identified by analysis of NMR spectra and comparison with data obtained for the same compound previously isolated from such verongiids as *Verongula gigantea* and *Aplysinella* sp. [36,46].

Previously, diverse extracts and compounds, obtained from verongioid sponges, were widely investigated for antimicrobial activities [47–49]. However, of the brominated compounds that have been isolated from *A. aerophoba* sponges, it was confirmed that only two of them (aeropylsinin-1 and dienone-1) possess antibacterial activity

[50,51]. Some authors also reported weak or medium antimicrobial activity of isofistularin-3 [50] which is recognized, however, as a perspective epigenetic drug [34]. It is reported that aeropylsinin-1 showed antibiotic activities against *Bacillus cereus*, *B. subtilis*, *S. aureus*, *S. albus*, *Vibrio anguillarum*, *Flexibacter* sp., *Moraxella* sp. and *Escherichia coli* in an agar diffusion assay and did not show any effect on such Gram-negative bacteria as *P. aeruginosa*, or yeasts, including *Saccharomyces cerevisiae* [27,28,50,51]. However, Fulmor et al. [52] previously determined MIC 20–100 µg/mL for both (+)-aeropylsinin-1 and (–)-aeropylsinin-1 against *S. aureus*, *B. subtilis*, *Micrococcus luteus* and *P. aeruginosa*. Other authors also reported on the inhibition properties of aeropylsinin-1 isolated from *Suberia mollis* verongioid demosponge in an agar diffusion test against *S. aureus*, *P. aeruginosa*, and *K. pneumonia* [53] and in a quantitative test for cell viability against *B. subtilis*, *S. lentus*, *Erwinia amylovora*, *P. aeruginosa*, *S. epidermidis* and *Propionibacterium acnes* [54]. Similarly to results that have been reported previously [28,50], the agar diffusion assay in our study showed that both methanol and DMSO solutions of aeropylsinin-1 inhibited growth of *S. aureus* and did not have any activity against *C. albicans*. In contrast to some previously reported data [28,50], aeropylsinin-1 we isolated from *A. aerophoba* also showed growth inhibition properties for such Gram-negative clinical multiresistant strains as *A. baumannii* and *K. pneumoniae* (Supplementary information Table S1), and weak growth inhibition properties against *E. cloacae*. These strains are referred as ESKAPE pathogens [55] and represent the most common causes of intra-hospital infections. For the clinical strains of *A. baumannii*, *E. cloacae* and *K. pneumoniae* used for research here, the MIC and MBC of aeropylsinin-1 varied in range from 5.6 µg/mL to 125 µg/mL (Table 1). Under same experimental conditions, isofistularin-3 has not shown any activity against investigated strains. However, aeropylsinin-1 solution against *P. aeruginosa* 89 had no significant difference in MBC tests in comparison to control. Notably, investigation of the rate of microbial death revealed that 25 µg/mL water-methanol solution of aeropylsinin-1 decreases the D-rate of *P. aeruginosa* 89 almost twice comparing to 1:3 solution of methanol used as a control (19 min against 35 min respectively). In 30 min, aeropylsinin-1 solution leads to 99.9% death of the bacteria studied here (Fig. 3).

In our previous publication [29], we have demonstrated the anti-tumorigenic and anti-metastatic activity of aeropylsinin-1 against pheochromocytoma cells. Here, we aimed to expand these findings also to more common tumor entities to determine a potential target specificity of the anti-tumorigenic activity of aeropylsinin-1 and isofistularin-3 (Fig. 4). Treatment with aeropylsinin-1 increased viability of the breast cancer cell line MCF-7, while the melanoma cell lines (SK-Mel-147, Mel-Juso and Malme-3M), the prostate cancer cell line Du-145, the adrenocortical carcinoma cell line NIC-H295R and the human pheochromocytoma cell line hPheo1 showed no significant effects after 24 h treatment with aeropylsinin-1 up to a concentration of 5 µM (Fig. 4A). Treatment of the neuroblastoma cell line Kelly with 0.1–1 µM aeropylsinin-1 resulted in an increased cell viability, while 5 µM aeropylsinin-1 had no effect. A concentration of 1–5 µM aeropylsinin-1 resulted in a significantly decreased viability of the neuroblastoma cell line SH-SY5Y. These findings indicate a more cell line-dependent effect

Table 1

The MIC and MBC (µg/mL) values of aeropylsinin-1 against clinical strains of Gram-negative bacteria.

Bacteria strains	MIC, µg/mL	MBC, µg/mL
<i>E. cloacae</i> 40	62.5	62.5
<i>A. baumannii</i> 1	31.2	62.5
<i>A. baumannii</i> 130	5.6	11.2
<i>K. pneumoniae</i> 10	62.5	125
<i>K. pneumoniae</i> 131	62.5	125
<i>P. aeruginosa</i> 89	62.5	> 250

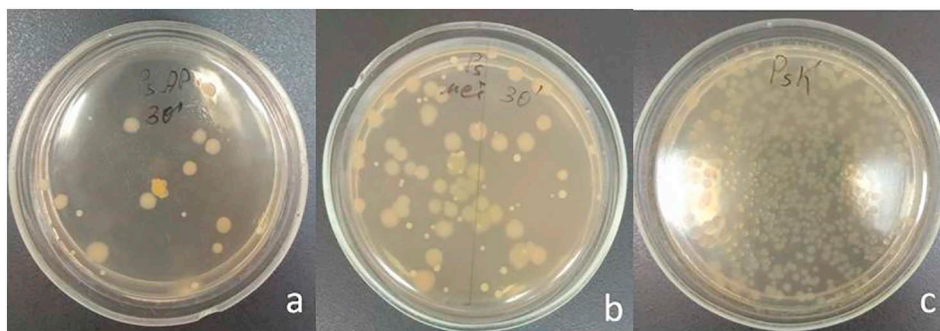


Fig. 3. The rate of microbial death investigation: (a) growth of *P. aeruginosa* 89 after 30 min exposition in 25 µg/mL solution of aeropylsinin-1; (b) growth of *P. aeruginosa* 89 after 30 min exposition in 1:3 methanol-water solution; (c) control.

emphasizing the importance of extended drug screening assays ideally on primary cells of the tumor.

Isofistularin-3 had no influence on the viability of the rat pheochromocytoma cell line PC12 up to a concentration of 100 µM, while viability of mouse pheochromocytoma cell lines, MPC and MTT, was reduced in a high micromolar range ($EC_{50} = 43\text{--}44$ µM) [29]. The human pheochromocytoma cell line hPheo1, the prostate cancer cell line Du-145 as well as the neuroblastoma cell lines SH-SY5Y and Kelly were not significantly affected by the treatment with isofistularin-3 up to a concentration of 25 µM. However, a concentration of 25 µM isofistularin-3 diminished viability of the cell lines MCF-7, NCI-H295R and Malme-3 M significantly. For the Mel-Juso cell line, a concentration of 5 µM of isofistularin-3 was already sufficient to significantly reduce the viability. In contrast to these findings, isofistularin-3 increased the viability of the third melanoma cell line SK-Mel-147. Similar to the results obtained for aeropylsinin-1, the effect of isofistularin-3 seems to

be cell line dependent. This indicates the requirement of a cell line-specific target to cause a reduction of cell viability. These findings emphasize the urgent need to identify specific interaction partners of isofistularin-3 and aeropylsinin-1. In future, identification of specific targets would allow the individualized treatment of suitable patients.

The application of several drugs is often limited by their unspecific toxicity towards the normal tissue and the associated side effects. Here, we examined the cytotoxicity of aeropylsinin-1 and isofistularin-3 on cells of the normal tissue (Fig. 5). Therefore, mouse endothelial cells (MS1) and fibroblasts (3T3) were treated with different concentrations of aeropylsinin-1 or isofistularin-3 up to 50 µM. The half maximal effective concentration of aeropylsinin-1 was determined in a high micromolar range ($EC_{50,MS1} = 18.1$ µM, $EC_{50,3T3} > 20$ µM). MS1 cells showed a higher sensitivity towards aeropylsinin-1 treatment compared to the 3T3 cells. It is well known that aeropylsinin-1 inhibits angiogenesis by diminishing endothelial cell growth, migration and invasion

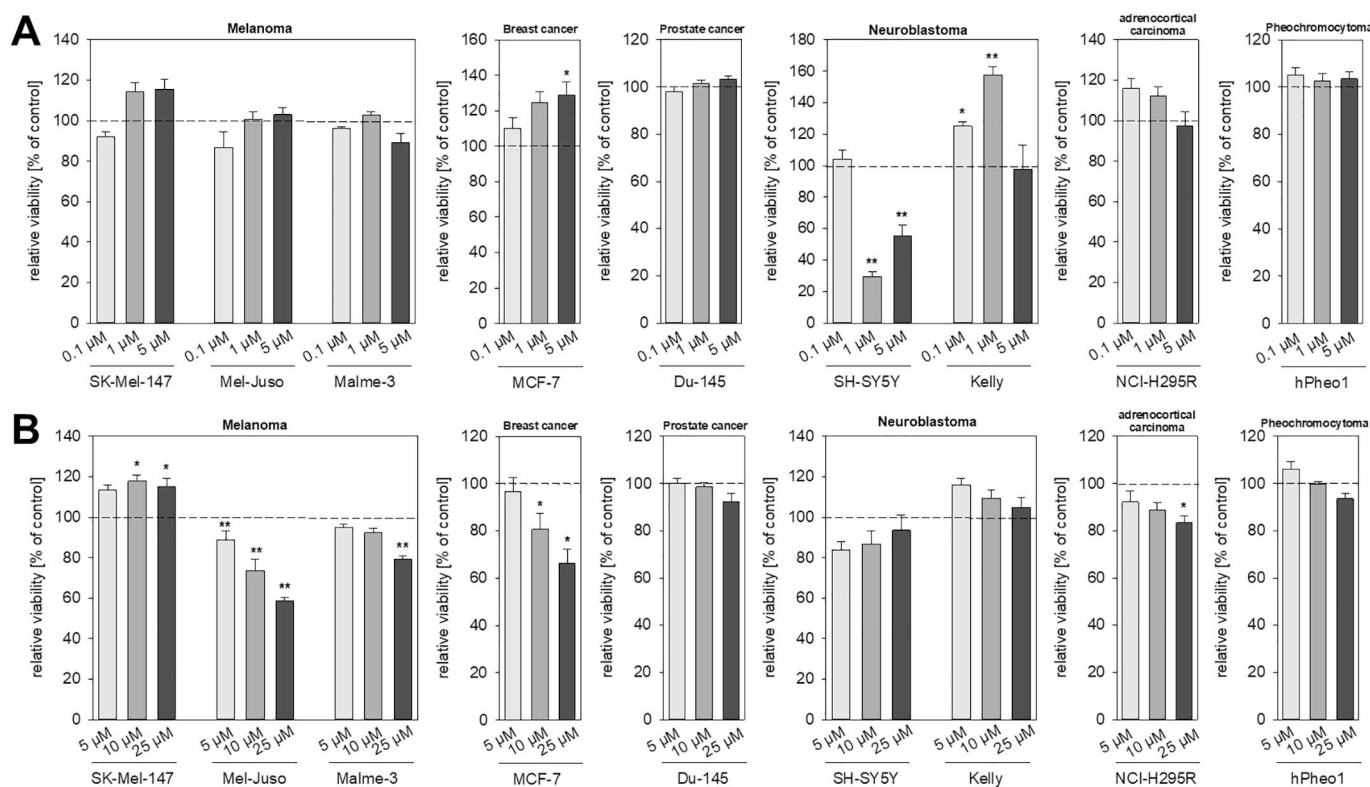


Fig. 4. Cytotoxicity of aeropylsinin-1 (A) and isofistularin-3 (B). Cell viability studies verified different sensitivities of several cancer cell lines towards aeropylsinin-1 and isofistularin-3 in a concentration dependent manner. Four independent experiments ($n = 12$). Data are presented as mean $\pm$ SEM; ANOVA and Bonferroni post hoc test comparison vs. DMSO control * $p < 0.05$, ** $p < 0.01$.

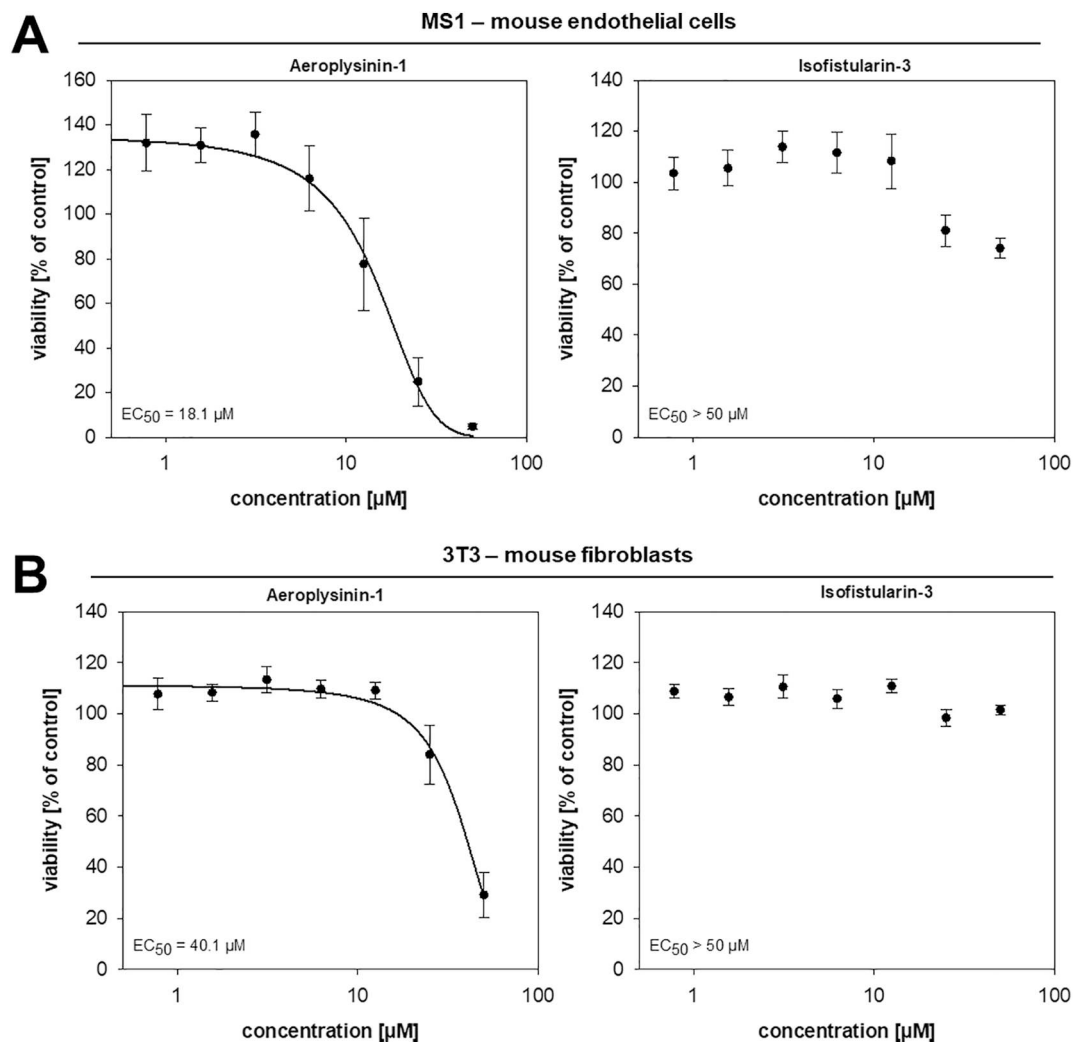


Fig. 5. Normal tissue cell toxicity of aeropylsinin-1 and isofistularin-3 investigated on mouse endothelial cells (MS1 cells) and the mouse fibroblast cell line 3T3. Four independent experiments ($n = 12$). Data are presented as mean $\pm$ SEM. The half maximal effective concentration (EC_{50}) was calculated from dose-response curve by using dose-response fit model.

as well as by the induction of apoptosis [56]. On the one hand, inhibiting of blood vessel formation within the tumor could diminish tumor growth and metastasis, but on the other hand, this effect could also lead to undesirable side effects on the normal vascular tissue. García-Vilas et al. [57] identified new targets for aeropylsinin-1 in endothelial cells, which are involved in the regulation of the redox balance. In contrast to aeropylsinin-1, treatment with isofistularin-3 showed no cytotoxic effect on both normal tissue cell lines. Taken together, these data underline the importance to investigate the underlying mechanism of verongioid sponge-derived secondary metabolites more in detail to identify suitable patients for such a therapeutic approach.

Another important aspect of this study was the evaluation of the potential of the 3D chitin scaffolds isolated from *A. aerophoba* (Fig. 2E) for cell culture and tissue engineering by using iPSC-CMs. A recent study demonstrated the suitability of *A. aerophoba* chitin for the cultivation of human mesenchymal stromal cells (hMSC) [11]. Here, our focus was to examine the potential of this material for iPSC-CMs. The cultivation of beating cardiomyocytes on 3D scaffolds requires a stable cellular adhesion to avoid detachment of the cells caused by the contractile forces and movement. In this study, we compared the cultivation of iPSC-CMs on the pure *A. aerophoba* scaffolds to chitin structures that were pre-coated with Geltrex, which is used to enhance iPSC-CM adhesion [40,42]. Due to the 3D nature and the loose structure of the

sponge scaffold, the seeding of iPSC-CM suspensions leads to short-time interactions between the chitin fibers and cells in the upper region of the scaffold and the accumulation of cells on the bottom of the cell culture plate (transwell plate format, Fig. 6A). In general, iPSC-CMs attached to the Geltrex-coated and uncoated *A. aerophoba* scaffolds. We observed a higher density of iPSC-CMs on the Geltrex-coated samples, which may be caused by a stronger interaction of the cells with adsorbed extracellular matrix proteins, especially in single chitin fibers. However, an exact quantification of iPSC-CM attachment is very challenging due to the variable size and structure of the scaffolds.

The iPSC-CMs on uncoated and pure chitin structures started contracting 24 h after seeding, which is comparable to their behaviour on Geltrex-coated cell culture plates and confirms the biocompatibility of the material with this cell type (Suppl. Video 1). During the culture period, iPSC-CMs in the upper part of the scaffold formed thin layers on the fibers, contracting clusters in the branched regions of the scaffold, as well as connections between different chitin fibers (Fig. 6B, Suppl. Video 2). Furthermore, iPSC-CM layers with integrated chitin fibers formed at the bottom of the cell culture cavity (Fig. 6C). The robust integration of the scaffold segments is revealed by the strong movement of the chitin fibers in accordance with the contractions of the iPSC-CMs (Suppl. Video 2). The attachment of the iPSC-CM layers with the chitin fibers was stable for the complete culture period even in the presence of continuously acting forces.

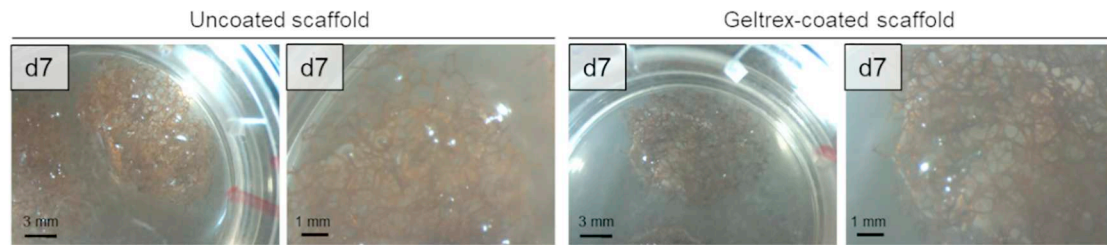
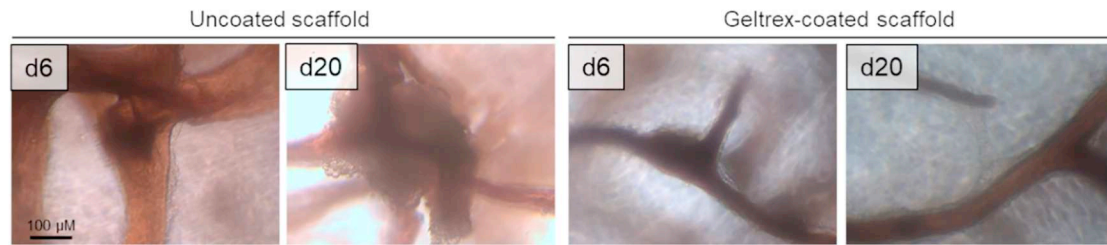
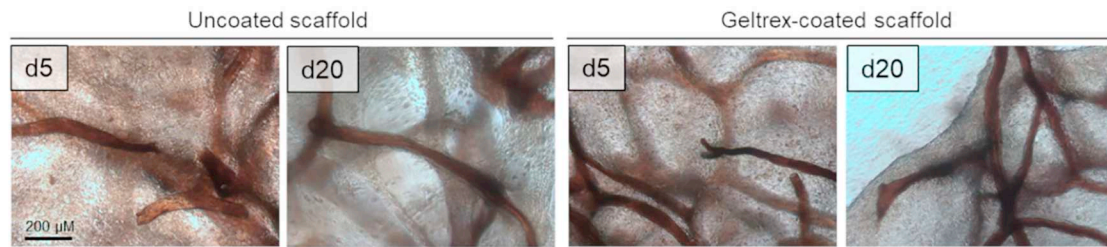
A Overview images**B** Attachment of iPSC-CMs on chitin fibers**C** Integration of chitin fibers in iPSC-CMs layer

Fig. 6. Documentation of attachment and contractile activity of iPSC-CMs on chitin scaffolds of *A. aerophoba*. (A) Overview images of chitin scaffolds and iPSC-CM cell layers (white regions in the scaffolds) in transwell-culture format. (B) iPSC-CMs on the scaffold, which form cell clusters and connections between different chitin fibers. (C) Interaction between iPSC-CM layers and chitin fibers at the bottom of the culture plate.

To examine the attachment and structural organization of the iPSC-CMs in more detail, α -actinin staining was performed to detect the sarcomeres of the cardiomyocytes and thus examine the structural alignment of the cells. These experiments confirmed the robust attachment of iPSC-CMs on the chitin scaffolds and illustrated the nicely ordered sarcomere structures along the chitin fibers (Fig. 7A). Furthermore, the interaction sites between iPSC-CM layers with chitin fibers can be visualized (Fig. 7B, C). This strong alignment and interaction of the cells with the chitin scaffolds may be promising with respect to the integration of *A. aerophoba* scaffolds into advanced models of cardiac tissue, as for example engineered heart muscles (EHM) [40,58]. For these approaches, the 3D architecture of *A. aerophoba* may be beneficial for the stabilization of EHM and the creation of bigger 3D muscle tissue in vitro.

To investigate the proliferative activity of iPSC-CMs on chitin scaffolds, the proliferation marker Ki-67 was stained using specific antibodies. Proliferating cells are detected by the colocalization of Ki-67 with the cell nuclei (Fig. 7). To determine the population of proliferatively active cells with high accuracy, Ki-67-positive cells were counted from 3D reconstructions of microscopy images obtained in different z-positions (Suppl. Video 3). This analysis revealed the presence of 3% Ki-67-positive cells in the Geltrex-coated chitin scaffolds, compared to 6% positive cells on the uncoated material. These results are in line with previous studies which demonstrated the decreasing proliferative activity over time, with comparable amounts of Ki-67-positive cells after 1 month of maturation [59,60]. Therefore, the proliferation of iPSC-CMs on the scaffolds has no or only a minor effect on the results of this study. Overall, our experiments using iPSC-CMs

reveal strong interaction of iPSC-CM layers with the chitin fibers for a longer culture period. Thus, naturally pre-designed 3D chitin scaffolds from *A. aerophoba* may be compatible for the application in advanced cell culture models and tissue engineering approaches [61–63].

Taken together, the 3D scaffolds of *A. aerophoba* and the recently published *I. labyrinthus* [64] demonstrate that chitin skeletons from marine sponges are suitable for the cultivation of iPSC-CMs and may serve as initial scaffolds for the creation of EHMs. Previous studies highlight the lack of a vascular network with an oriented porous scaffold as a major limitation of current tissue engineering approaches for the creation of thick myocardial tissues because of the limitation of oxygen diffusion [65,66]. Recently, Fang et al. reported a chitosan/collagen-based scaffold with oriented micro-pores for the culture of cardiomyocytes in which channel network fabricated with carbohydrate template was embedded for the formation of endothelialized network, however, the fabrication of materials to mimic artificial vasculature is very challenging. As an alternative approach, decellularization of animal or human hearts [67] or plant leaves [66] was performed to obtain perusable scaffolds with an intact vascular network. In the latter approach, hMSCs were seeded in leaf veins and iPSC-CMs were successfully cultured on the leaf surface [66]. Based on these findings, it is of great interest for future studies to seed endothelial cells inside chitin fibers and form EHMs from iPSC-CMs around the chitin fiber. The porous nature of the material will be advantageous here and may facilitate the supply with nutrients and exchange of soluble factors between endothelial cells and iPSC-CMs. Additionally, chitin-based materials have been shown to reduce pro-inflammatory cytokines [68], which might be useful for implant acceptance and regeneration after

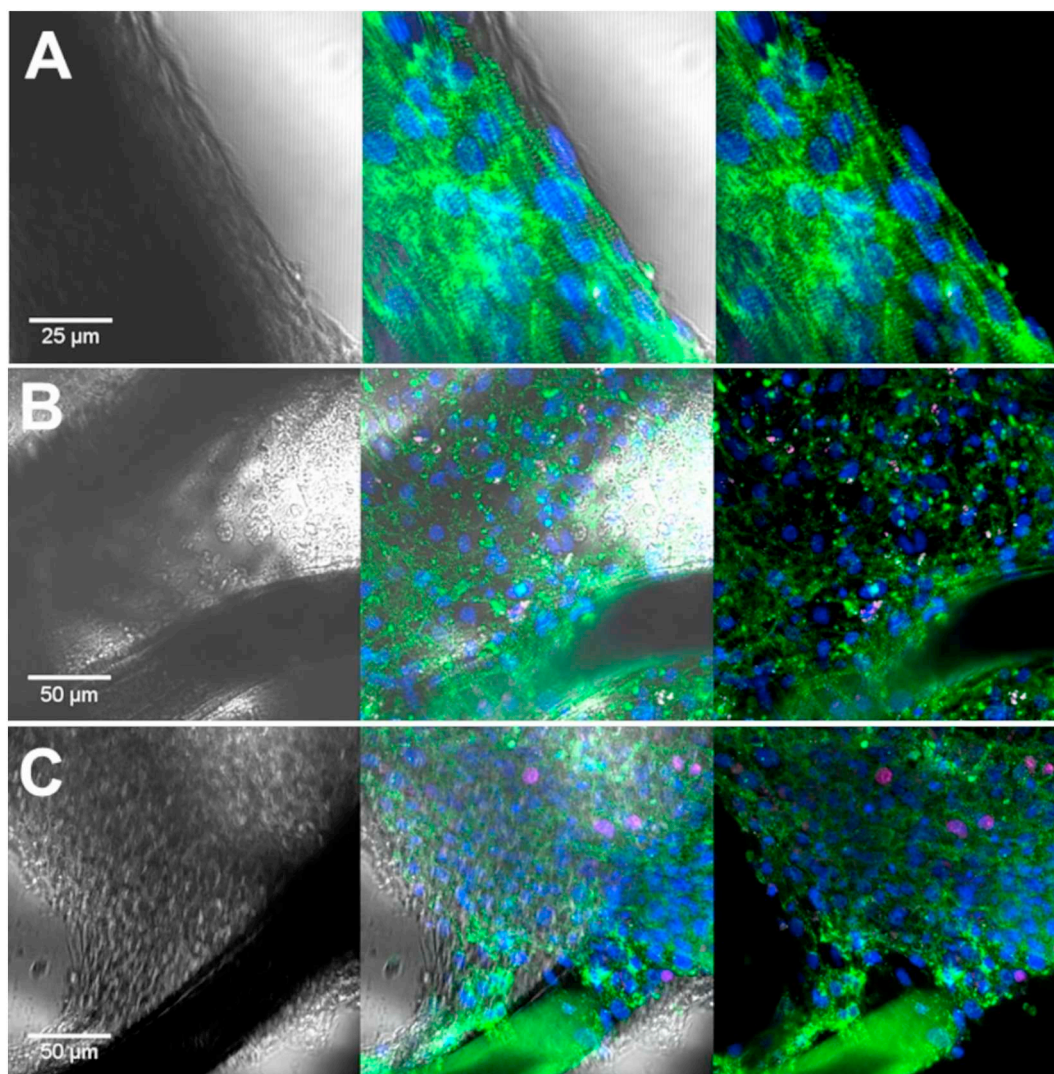


Fig. 7. Fluorescence microscopy studies to characterize iPSC-CM attachment and proliferation on chitin scaffolds of *A. aerophoba*. (A) Representative image of aligned sarcomeric structures of iPSC-CM on chitin fiber (Getrex-coated sample). (B, C) Layers of iPSC-CMs attached to Geltrex-coated (B) and uncoated (C) scaffolds. Specific staining show cell nuclei (blue), α -actinin (green) and Ki-67 (magenta). Images were captured using confocal laser scanning microscopy. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

injury. Furthermore, chitin offers several advantages because it is non-immunogenic, non-toxic and biodegradable with non-toxic products (temporal matrix, tissue supporting materials). Therefore, biological, chitinous 3D scaffolds represent a large source of cost-efficient, simply obtaining biomaterials with various shapes and size, depending on the original species including marine sponges [16,64,69] or spiders [70] for engineering of different tissues, including EHMs, and bone, or as wound dressing.

In contrast to such commercial sponges as bath sponges the economics of verongioid species including *A. aerophoba* is absolutely undeveloped. This situation is not surprising because the use of bath sponges began several thousand years ago [5,71]. Nowadays, countries as Greece and Tunisia with 50 tons of sponges annually are the most important producers of these biomaterials in the Mediterranean sponge industry [5,9]. The prices for commercial sponges range between 10 and 15 U\$ for 1 kg of specimens from the Western Central Atlantic Ocean and 80–100 U\$ for 1 kg of sponges from the Mediterranean sea including the coastal waters of North Africa [5]. Today, bath sponges are commercialized mostly for cosmetic applications. Alternatively, verongioid sponges with their chitinous skeletons and high concentration of bromotyrosines represent an interesting source for tissue engineering and marine pharmacology, but cannot be used as bath tools similar to

spongin-based rigid skeletal constructs typical for keratosan bath sponges.

We carried out an analysis of numerous open access sources to estimate the price situation concerning a few of the commercially available bromotyrosines (aeropylsinin, aerophobin, aerothionin, isofistularin) (for details see Supplementary Material Table S3). The prices range between 100 U\$ and 800 U\$ per milligram depending on the company and the purity of the substances. The chitin-based sponge scaffolds are extremely interesting in materials science since the processing of chitin into sponge-like materials or foams is technologically difficult and expensive. The price for one square centimeter of ready-to-use sterile sponge chitin scaffolds ranges between 25 and 30 U\$.

Recently, researchers from Prof. Bornstein's and Prof. Ehrlich's groups have evaluated the anti-tumorigenic and anti-metastatic potential of bromotyrosines such as aeropylsinin-1 (AP-1) and isofistularin-3 on pheochromocytoma cells [29]. Aeropylsinin-1 diminished the number of proliferating cells and reduced spheroid growth significantly. These in vitro investigations show promise for the application of the sponge-derived marine drug, aeropylsinin-1 as the first bromotyrosine-based anti-metastatic agent. Screening of other bromotyrosines should provide an auspicious strategy to identify novel therapeutic strategies for metastatic disease. However, one major obstacle

that marine pharmacologists and natural product chemists face is the supply problem. The farming of marine sponges can only be an economically relevant alternative against wild collecting if the organisms lend themselves to a cost-effective cultivation [72].

The advantage of *A. aerophoba* is that this source organism does not need to be collected in large quantities to supply the necessary amount for the industry before chemical synthesis of the active compounds is available. First of all, farming of *A. aerophoba* relies on high growth rates (up to 10 cm per year) of colonies which are located at 2–5 m depths in Adriatic sea that provide a sufficient supply. Secondly, we take the liberty to make calculations how much of the selected bromotyrosines can be extracted from this sponge (see Fig. S1) and what are the prices which could be theoretically achieved. For example, using only methanol extraction as described in our study here, it is possible to isolate 1.1 g of isofistularin-3 from 200 g of dry *A. aerophoba* sponge (Fig. 2A). When we calculate 150\$ per 1 mg as a realistic current market price for this compound at 96% purity, the price of 1 g isofistularin-3 is 150,000\$. Thus, we can obtain from 1000 g (1 kg) of dry sponge - 5.5 g of isofistularin-3 with value 825,000 U\$. Consequently, we can suggest that from one *A. aerophoba* colony of 10 m² it will be possible to harvest 18 kg of dried sponge as source for isolation of 99 g of isofistularin-3 as powder with a value of 14,850,000 U\$. Theoretically, one 100 m² marine ranching facility such as we have in Montenegro can bring 180 kg of dried *A. aerophoba* sponge, and 990 g of isofistularin-3 with total value of 148,000,000 U\$. Similar calculations with respect to aeroplysinin-1 lead to the following results: 1 mg of aplysinin-1 - 380\$; from 200 g of dry sponge we obtained 40 mg of this substance; so from 1000 g (1 kg) of dry sponge we could obtain 200 mg of aplysinin-1 with value 76,000 U\$. Consequently, we estimated that from one 10 m² large *A. aerophoba* colony it would be possible obtain 18 kg of dried sponge thus 3600 mg (3.6 g) of aeroplysinin-1 with a value of 1,368,000 U\$. Correspondingly, from 100 m² sponge field it should be possible to obtain 180 kg of dried sponge, 36,000 mg (36 g) of aplysinin-1 with a total value of 13,680,000 U\$. According to personal communication of Dr. Zoran Kljajic [26] who monitored the amounts of verongiids colonies in coastal water of Montenegro in 2007–2011, the whole mass can reach up to 8 tons. Similar quantities of verongiids sponge colonies are to be present in marine environments around Albania and Croatia as well. Thus, if our prognosis from a bioeconomy viewpoint is so optimistic, what are the challenges that must be overcome prior to commercial success?

The main limiting factor is that most sponges are protected species. The second point is the absence of the legal bases on the governmental level to provide legal opportunities for local aquaculture and fishermen communities to cultivate verongiida sponges. Also, there is no one bromotyrosine of poriferan origin which has been approved by FDA and similar agencies.

Farming makes the sponge resource renewable, sustainable, and even more profitable. The high prices for bromotyrosines and the growing requests for 3D chitin scaffolds for regenerative medicine and tissue engineering has stimulated researchers to develop different biotechnological approaches for cultivation of *A. aerophoba* and related sponges under laboratory [73,74] and marine ranching conditions in the Mediterranean Sea [75,76].

Marine biotechnology of sponges is a rapidly growing area that is recognized, by policy makers and the enterprise sector, as having significant potential to develop market opportunities for new biomaterials and pharmaceutical products. Currently, corresponding funding activities should support efforts to create a sustainable bioeconomy is likely to lead a growth in sponges related marine biotechnology research. We plan further to develop processes which enable the utilization of verongiids sponges as unique renewable marine biological resources.

4. Conclusions

Marine-derived products including biomaterials have created a stage for a growing range of new ocean-related economic activities and

constant innovations. Simultaneous isolation of both biomaterials and bioactive compounds from cultivated marine sponges definitely will be an important direction in marine economics for the next years. Scientific discoveries and successful innovations demand bringing a diversity of players (public research institutes, large enterprises, small- and medium-sized enterprises, universities etc.) together into flexibly organized networks, with the end result being in the economic growth of countries like Montenegro, Albania, and Croatia where Verongiida sponges are growing naturally in large amounts. Creating marine farming facilities will contribute also to exports, jobs and value added production and will stimulate growth in other associated industries.

The potential of Verongiida demosponges as renewable source of biomaterials and anti-cancer drugs is strengthened by building and maintaining sustainable technological approaches and practices. Based on the great amount of applied chemicals and the time consumption of the individual extraction steps (up to 7–10 days), the method needs to be improved. Our preliminary results showed that the application of ultrasound decreases the treatment time to 10 h. The application of microwaves may further decrease the treatment time to 7.5 min. Consequently, we plan to optimize new ultrasound- and microwave-based approaches for express simultaneous isolation of both chitinous scaffolds and bromotyrosines from cultivated verongiids demosponges. In the future, we plan to use chitin scaffolds isolated from our cultivated verongiids target sponges for the development of 3D models of the adrenal gland using murine adrenal stem cells/progenitors. Culturing in monolayers, as done in past and present, fails to reflect biologically relevant organ niches. Therefore, the development of 3D models to enable tissue growth is of great importance.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.msec.2019.110566>.

CRediT authorship contribution statement

Björn Binnewerg:Methodology, Investigation, Writing - original draft, Visualization.**Mario Schubert:**Methodology, Investigation, Writing - original draft, Visualization.**Alona Voronkina:**Methodology, Investigation, Writing - original draft.**Liubov Muzychka:**Methodology, Investigation.**Marcin Wysokowski:**Conceptualization, Investigation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition.**Iaroslav Petrenko:**Investigation.**Mirko Djurović:**Resources.**Valentine Kovalchuk:**Investigation.**Mikhail Tsurkan:**Investigation.**Rajko Martinović:**Resources.**Nicole Bechmann:**Investigation, Writing - original draft, Visualization.**Andriy Fursov:**Investigation.**Viatcheslav N. Ivanenko:**Resources, Writing - review & editing.**Konstantin R. Tabachnik:**Resources.**Oleg B. Smolii:**Investigation.**Yvonne Joseph:**Resources.**Marco Giovine:**Resources, Writing - review & editing, Funding acquisition.**Stefan R. Bornstein:**Resources, Supervision.**Allison L. Stelling:**Writing - review & editing.**Antje Tunger:**Investigation, Writing - original draft.**Marc Schmitz:**Methodology, Writing - original draft.**Olga S. Taniya:**Investigation.**Igor S. Kovalev:**Investigation.**Grigory V. Zyryanov:**Investigation.**Kaomei Guan:**Conceptualization, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.**Hermann Ehrlich:**Conceptualization, Resources, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was partially supported by DFG Project HE 394/3, SMWK Project no. 02010311 (Germany), as well as DAAD-Italy Project

“Marine Sponges as Sources for Bioinspired Materials Science”(No. 57397326). M.W is financially supported by Polish National Agency for Academic Exchange (PPN/BEK/2018/1/00071). We thank K. Fischer, J. Müller and J. Pöche for excellent technical assistance and S. Tulok (CFCI TU Dresden) for assistance in microscopy.

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