Biomedicine & Pharmacotherapy 128 (2020) 110275

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

Sphaerococcus coronopifolius bromoterpenes as potential cancer stem cell-targeting agents

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ARTICLE INFO

Keywords:
Marine natural products
Red seaweed
Lung cancer
Cancer stem cells
Microenvironment
Interleukin-6

ABSTRACT

Cancer is one of the major threats to human health and, due to distinct factors, it is expected that its incidence will increase in the next decades leading to an urgent need of new anticancer drugs development. Ongoing experimental and clinical observations propose that cancer cells with stem-like properties (CSCs) are involved on the development of lung cancer chemoresistance. As tumour growth and metastasis can be controlled by tumour-associated stromal cells, the main goal of this study was to access the antitumor potential of five bromoterpenes isolated from *Sphaerococcus coronopifolius* red alga to target CSCs originated in a co-culture system of fibroblast and lung malignant cells. Cytotoxicity of compounds (10–500 μM; 72 h) was evaluated on monocultures of several malignant and non-malignant cells lines (HBF, BEAS-2B, RenG2, SC-DRenG2) and the effects estimated by MTT assay. Co-cultures of non-malignant human bronchial fibroblasts (HBF) and malignant human bronchial epithelial cells (RenG2) were implemented and the compounds ability to selectively kill CSCs was evaluated by sphere forming assay. The interleucine-6 (IL-6) levels were also determined as cytokine is crucial for CSCs.

Regarding the monocultures results bromosphaerol selectively eliminated the malignant cells. Both 12*S*-hydroxy-bromosphaerol and 12*R*-hydroxy-bromosphaerol steroisomers were cytotoxic towards non-malignant bronchial BEAS-2B cell line, IC_{50} of 4.29 and 4.30 μM respectively. However, none of the steroisomers induced damage in the HBFs. As to the co-cultures, 12*R*-hydroxy-bromosphaerol revealed the highest cytotoxicity and ability to abrogate the malignant stem cells; however its effects were IL-6 independent.

The results presented here are the first evidence of the potential of these bromoterpenes to abrogate CSCs opening new research opportunities. The 12*R*-hydroxy-bromosphaerol revealed to be the most promising compound to be test in more complex living models.

1. Introduction

Despite the advances on biology and therapeutics achieved during the last decades, cancer remains one of themajor cause of death across the world, mostly due to aging, lifestyle changes, widespread of smoking habits and the increasing accumulation of atmospheric pollutants. In fact, the most recent statistics show 18.1 millions of new cases and 9.6 millions of deaths in 2018 [1]. Moreover, in 2018, lung cancer remains as one of the lowest 5-year relative survival rate pathologies (18 %) [2].

One of the main factors contributing for the high cancer mortality is therapy failure and consequent tumour relapse [3,4]. Mechanistically, resistance relies either on an inappropriate pharmacological design of the therapeutic approach, or more frequently, on the development of drug resistance [5–7]. Several mechanisms are known to be drug resistance, including drug efflux, detoxification, inactivation, changes in the drug targets, highly efficient DNA repair mechanisms, apoptosis blockage and formation of highly resistant cancer stem cells (CSCs) as result of therapy [8–10]. Moreover, tumour microenvironment and tumour cellular content were also identified as important players in the therapeutic outcome of tumours [11,12]. In fact, studies from the last decades revealed that a cellular population residing inside the tumours and designated CSCs are fundamental in tumorigenesis, tumour maintenance, metastatic widespread and resistance to conventional

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https://doi.org/10.1016/j.biopha.2020.110275
Received 19 March 2020; Received in revised form 9 May 2020; Accepted 15 May 2020

therapeutics [13–15]. CSCs are tumour-associated stem cells that are responsible for tumours' heterogeneity and boosted aggressiveness. In agreement, it has been showed that current therapeutic strategies employed to combat cancer are extremely effective in targeting the rapidly dividing bulk of tumour cells, but spare the slow-dividing quiescent CSCs, that subsequently repopulate a new tumour mass with more resistant tumour cells [16–19]. The aetiologies of this cellular population remain controversial, but recent work from different laboratories definitely confirmed that they can arise from dedifferentiation of terminally differentiated tumour cells, through the action of microenvironment-released paracrine cytokines, particularly IL-6, and Activin-A and G-CSF [20–24]. These observations had tremendous impact in the scientific community, as new therapeutics should not only address the tumour cell mass, but also CSCs and the tumour microenvironment [15,23,25]. Corroborating this idea are some recent *in silico* studies demonstrating that the presence of CSCs biomarkers was associated with a poorer patient prognosis [26–28].

Due to the success of natural products (NPs) and their derivatives in cancer treatments, as well as their ability to mediate several signalling pathways and cause fewer side effects, there is a growing interest to understand their potential as anti-CSCs agents [29]. Numerous studies have reported the great potential of NPs to interfere with CSCs, including extracts or marine natural products [30–33]. For instance, marine extract of *Crambe crambe* sponge inhibited the resistance to apoptosis, self-renewal ability, and proliferation of pancreatic cancer cells with CSCs phenotype [34]. Moreover, its co-administration with gemcitabine drove total tumour abolishment on an *in vivo* cancer model [34]. Additionally, compounds extracted from the red alga *Plocamium cornutum* showed selective activity to inhibit the development of MCF-7 sphere structures, without cytotoxicity on either adherent MCF-7 cells or MCF-12A non-transformed cells [35]; brown alga *Saccharina japonica*, extracts suppressed sphere-forming ability of glioblastoma stem cells [36]. Finally, fucoxanthinol derived from fucoxanthin induced apoptosis and suppressed CSCs, as well as *in vivo* tumorigenesis in human colorectal CSCs [37,38]. Therefore, the main goal of this study was to understand the potential of *S. coronopifolius* bromoterpenes to suppress CSCs on an *in vitro* co-culture cellular model of human lung cancer, as well as to assess the role of IL-6 in the overall suppression process. According with our best knowledge, it is the first study to access the antitumor potential of sphaerococcenol A, 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol, bromosphaerol and sphaerodactylomelol to target CSCs and the tumour microenvironment.

2. Material and methods

2.1. Extraction and isolation of Sphaerococcus coronopifolius compounds

Five bromoterpenes (Fig. 1) were extracted from the red alga *S. coronopifolius* collected in Berlenga Nature Reserve, Peniche, Portugal, according to the procedures described by Rodrigues and co-workers [56]. Subsequent purification was accomplished by chromatographic methods, and structure characterization was performed by NMR and MS techniques. Compounds were dissolved in DMSO, whose concentration in all the performed experiments was lower than 0.2% to avoid toxicity. Controls were always treated with higher concentrations of DMSO.

2.2. Cellular systems

Four cellular systems were used in this study namely, BEAS-2B (immortalized human bronchial epithelial cells), RenG2 (malignant human bronchial epithelial cells), SC-DRenG2 (derivative RenG2 cells) and HBF cells (non-malignant human lung fibroblasts). BEAS-2B cells were acquired from the European Collection of Cell Cultures (ECCAC, Salisbury, UK; ECCAC no. 95,102,433). RenG2 and SC-DRenG2 cells were produced by the group of Alpoim, as previously described by Rodrigues and co-workers [24]. BEAS-2B cells were cultured in LHC-9 medium (Gibco, USA). SC-DRenG2 cells were cultivated in cancer stem cell propagation media (DMEM:F12) supplemented with the same concentration of bFGF and EGF [24]. HBF cell line was obtained from non-malignant human lung tissue from a patient at the Centro Hospitalar e Universitário de Coimbra (CHUC) following protocols established in the laboratory. Appropriate informed consents were signed according to the ethical procedures approved by the Ethical Committee of the Faculty of Medicine of the University of Coimbra. HBF cells were maintained in DMEM medium supplement with 10 % FBS (Biochrom, Germany), 20 U/mL penicillin, 20 μg/mL streptomycin and 50 ng/mL amphotericin B (Biochrom, Germany). Cells were kept at 37 °C in a 95 % air, 5% CO_2 incubator. Culture flasks were coated with a 2% gelatin solution 2 h before use and, cells were seeded at recommended initial density . Subculture was performed using a 0.25 % trypsin-1 mM EDTA solution (Biochrom, Cambridge, UK) whenever cultures reached 80% confluence.

2.3. Cytotoxic assays using the isolated compounds

To assess the compounds cytotoxic capacity in monocultures all the cell lines were seeded at a density of 4×10^3 cells/ well in 96-well plates (SPL-Biosciences®), in the corresponding culture medium, and incubated overnight. Cells were then treated with the compounds at 10, 50 and 500 μM for 72 h. After treatment, the medium was removed and the cells were washed twice with PBS buffer and incubated at 37 °C for 1 h with a PBS-dissolved MTT solution (1.2 mM). After washing off the excess of MTT, cells were disaggregated with DMSO and the absorbance of the crystals of formazan was measured at 570 nm using a spectrophotometer plate reader (Bio-Tek Synergy plate reader, Bedfordshire, UK). Control experiments were performed in parallel in the absence of the algae compounds. At least three independent experiments were performed each in triplicate and results expressed as percentage of control.

2.4. Experimental assays using co-culture systems

To better mimic lung cancer anatomy transwell co-culture systems were implemented as previously described by Rodrigues and collaborators [24]. HBF cells were seeded as feeder layers in 6-well plates (SPL-Biosciences®) at a density of 1.4×10^4 cells/ well. After 3 days, RenG2 cells were seeded in Transwell® inserts (SPL Life Sciences, Korea) at a density of 8×10^3 cells/ well. Co-cultures were kept in the incubator at 37 °C in a 95 % air, 5% CO_2 for two months, and mediums were changed every 15 days. After two months, both cell lines were treated with the algae compounds for 72 h, at the previously defined concentrations. The sphere-forming assay was subsequently employed to evaluate the ability of the bromoterpenes to supress CSCs. Control experiments were performed in parallel in the absence of the algae compounds. At least three independent experiments were carried out in triplicate and results expressed as percentage of control.

2.5. Sphere-forming assay as a screen tool for CSCs

CSCs were isolated using a protocol previously described by Rodrigues and collaborators [24]. 6-well plates were coated with a 2% poli-(2-hydroxyethyl methacrylate) (Sigma, USA) solution to ensure low adherence conditions, and CSCs' isolation medium with appropriate supplements was prepared containing a 2% methylcellulose (Sigma, USA) solution (1:1). CSCs isolation was accomplished after compounds treatment for 72 h. The cells housed in the upper compartment were washed twice with PBS buffer, detached using a 0.25 % trypsin-1 mM EDTA solution (Biochrome, Cambridge, UK) and collected by centrifugation at 380 *g* for 5 min at room temperature. Cells were then resuspended in the isolation medium at a concentration of 3×10^4 cells/ mL, and 2 mL of this suspension were added to each well

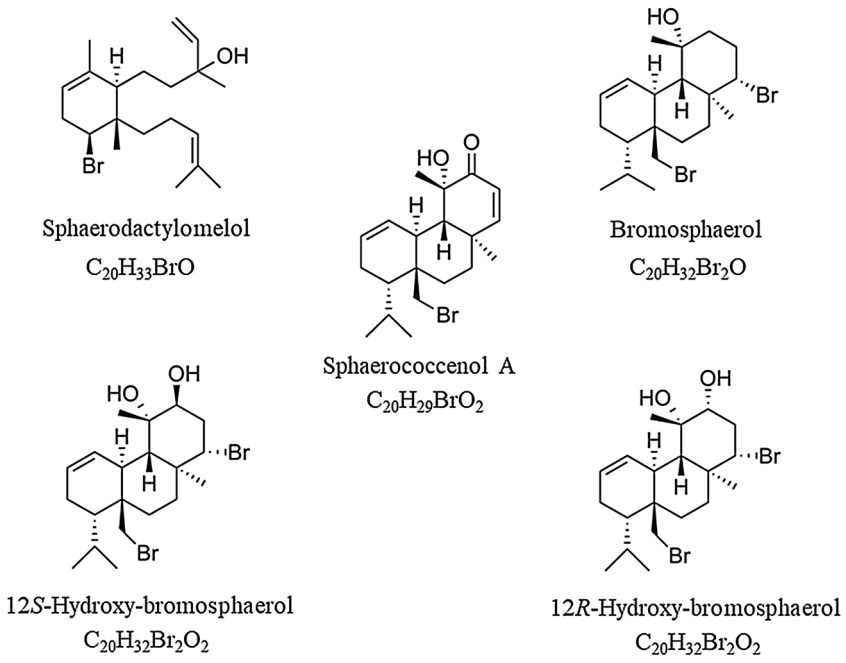

Fig. 1. Chemical structures of bromoterpenes isolated from *Sphaerococcus coronopifolius* collected in the Berlenga Nature Reserve, Peniche, Portugal (Atlantic coast).

of the plate. The isolation medium was supplemented with 10 ng/mL of both human EGF (E9644, Sigma-Aldrich) and bFGF (100−18B, PeproTech, London) and cells were maintained at 37 °C in a 95 % air, 5% CO_2 incubator. Supplements were replaced every two days. Whenever sphere formation was observed and spheres reached a satisfactory volume (which normally happens around 15 days after platting), they were collected and analysed using an optical microscope (Axio observer z1 Carl Zeiss; Camera AxioCam HR R3; Fiji ImageJ software, Wayne Rasband, National Institutes of Health, USA). The spheres' size was registered and photos were taken (Fiji ImageJ software, Wayne Rasband, National Institutes of Health, USA). At least three independent experiments were carried out in triplicate.

2.6. IL-6 levels assessment

The IL-6 levels present in the supernatant of both the upper and bottom compartments were evaluated by enzyme-linked immunosorbent assay (ELISA), using the Human IL-6 Quantikine ELISA Kit (#D6050, R&D Systems) accordingly to the manufacturers' instructions. At least three independent experiments were carried out in triplicate.

2.7. Statistical and data analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison of group means to determine significant differences relatively to control treatment. The Tukey's test was applied for the remaining multiple comparisons. All data were checked for normality and homoscedasticity. Results are presented as mean ± standard error of the mean (SEM). Differences were considered statistically significant at level of 0.05 ($p < 0.05$). Calculations were performed using IBM SPSS Statistics 24 (IBM Corporation, Armonk, NY, USA) and GraphPad v5.1 (GraphPad Software, La Jolla, CA, USA) software.

3. Results

3.1. Evaluation of the cytotoxic activity of the algae-extracted compounds

To ascertain the ideal concentration of the bromoterpenes isolated from *S. coronopifolius* to be used in the co-culture experiments, HBF and RenG2 cells were monocultured for 72 h in the presence either 10, 50 or 500 μM of the isolated compounds (Fig. 2).

The results revealed that only 10 μM sphaerococcenol A induced a significant reduction (55 %) in HBF cells' viability. Moreover, all compounds mediated a marked cytotoxic effect on HBF cells at concentrations above 10 μM (Fig. 2A). Regarding RenG2 malignant cells (Fig. 2B), their viability decreased as the compounds' concentration increased, and the strongest cytotoxic effect was observed with 12*S*-hydroxy-bromopshaerol and bromosphaerol, both at a concentration of 10 μM. The treatment with 50 and 500 μM concentrations decreased cells' viability in more than of 95 %.

Together, these results revealed that drugs concentrations higher than 10 μM mediated marked non-selective cytotoxic effects on both cell lines. Therefore, only the 10 μM concentration proceed to tests in SC-DRenG2 and BEAS-2B cells (Fig. 3).

Unexpectedly, the non-malignant BEAS-2B cells turned out to be resistant to both bromosphaerol and sphaerodactylomelol, but 12*R*-hydroxy-bromosphaerol (1.01 ± 0.28 % of viable cells), 12*S*-hydroxy-bromosphaerol (5.70 ± 2.07 % of viable cells) and sphaerococcenol A (0.82 ± 0.29 % of viable cells) decreased their viability in more than of 90 % (Fig. 3A). However, at a 10 μM concentration, all the compounds decreased significantly SC-DRenG2 cells' viability, with bromosphaerol (3.75 ± 0.54 % of viable cells) and sphaerococcenol A (6.16 ± 1.15 % of viable cells) depicting the highest effect and sphaerodactylomelol (67.25 ± 7.34 % of viable cells) exhibited the lowest effect (Fig. 3B).

Altogether, the results attained so far show that 10 μM of 12*R*-hydroxy-bromosphaerol and 12*S*-hydroxy-bromosphaerol reduce the viability of both BEAS-2B and RenG2 cells but are non-cytotoxic towards HBF. Therefore, it was decided to perform dose-response assays on BEAS-2B cells with these two steroisomers compounds. Drug concentrations tested ranged from 2 to 10 μM, and the results are depicted in Fig. 4.

Treatment of BEAS-2B cells with different concentrations (2−10

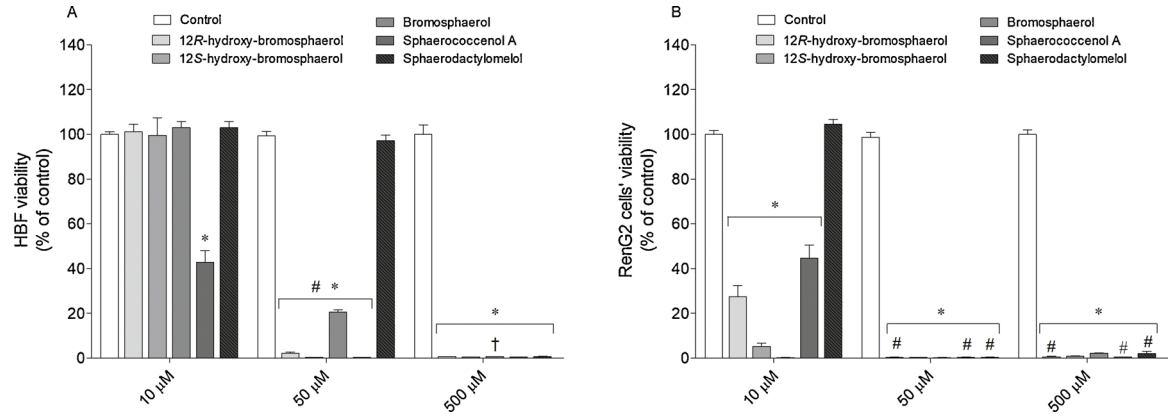

Fig. 2. HBF (A) and RenG2 (B) cells' viability following 72 h of exposure to the *Sphaerococcus coronopifolius* compounds (10, 50 and 500 µM) expressed as % of the control. The values correspond to mean ± SEM of at least three independent experiments were carried out in triplicate. Symbols represent statistically significant differences ($p < 0.05$) when compared to: * control of respective concentration; # 10 µM treatment; † 10 and 50 µM treatment.

µM) of 12*R*-hydroxy-bromosphaerol and 12*S*-hydroxy-bromosphaerol for 72 h revealed that the cytotoxic effects were concentration-dependent, with an IC_{50} of 4.30 µM (Fig. 4A) and 4.29 µM (Fig. 4B), respectively. As the 2 µM 12*S*-hydroxy-bromosphaerol did not affect BEAS-2B cells' viability, it was decided to study the effects of 4µM 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol and bromosphaerol on SC-DRenG2 cells' viability (Fig. 5)

All the aforementioned compounds induced a decrease in SC-DRenG2 cells' viability superior to 80 %, with 12*R*-hydroxy-bromosphaerol and bromosphaerol showing the strongest cytotoxic effect (> 90 %) (Fig. 5). It was then decided to proceed for the co-culture assays with only these two compounds.

3.2. Effects of 12R-hydroxy-bromosphaerol and bromosphaerol on CSCs' formation and IL-6 levels in co-culture systems

According to the results obtained in monocultures, 12*R*-hydroxy-bromosphaerol and bromosphaerol were selected to be tested in transwell® co-cultures of RenG2 and HBF cells. Cells in the co-culture system were treated with 4 µM of the aforementioned compounds for 72 h, and the presence of CSCs, as well as the IL-6 levels on the cell culture media were screened through the sphere-forming assay and ELISA, respectively (Figs. 6 and 7).

The exposure to 12*R*-hydroxy-bromosphaerol totally abolished CSCs, while bromosphaerol only slightly decreased the number of CSCs. The co-administration of both compounds, on the other hand, reverted the effects of 12*R*-hydroxy-bromosphaerol, as no CSCs' inhibition was observed (Fig. 6).

Previously published results evidenced that high levels of IL-6 are mandatory for CSCs formation [24]. In light of such results, it was decided to investigate whether the effects of bromosphaerol and 12*R*-hydroxy-bromosphaerol relied on alterations of the IL-6 levels in the co-culture systems (Fig. 7).

IL-6 levels in the bottom and upper compartments of co-culture did not show significant change following the treatment with 12*R*-hydroxy-bromosphaerol. The treatment with bromosphaerol, however, exhibited a significant increase in IL-6 levels on the upper transwell® compartment. Again, the concomitant treatment with both 12*R*-hydroxy-bromosphaerol and bromosphaerol did not exhibit significant differences compared to control (Fig. 7).

4. Discussion

One of the major challenges in cancer diseases is associated with resistance to conventional therapeutics, being of utmost importance to improve the current strategies and to develop new approaches to fight this burden [39]. Advances in cancer biology showed that this disease is much more complex than the simple continuous uncontrolled proliferation of cancer cells, which is sustained by several factors that contribute for cancer resistance and relapse [40]. Amongst them, tumour heterogeneity, which relies on the presence of CSCs and on the tumour microenvironment, has been shown key players in cancer development and resistance [41,42].

The results attained in the present work revealed that despite the ability of 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol, and bromosphaerol target CSCs (SC-DRenG2) in monoculture, only

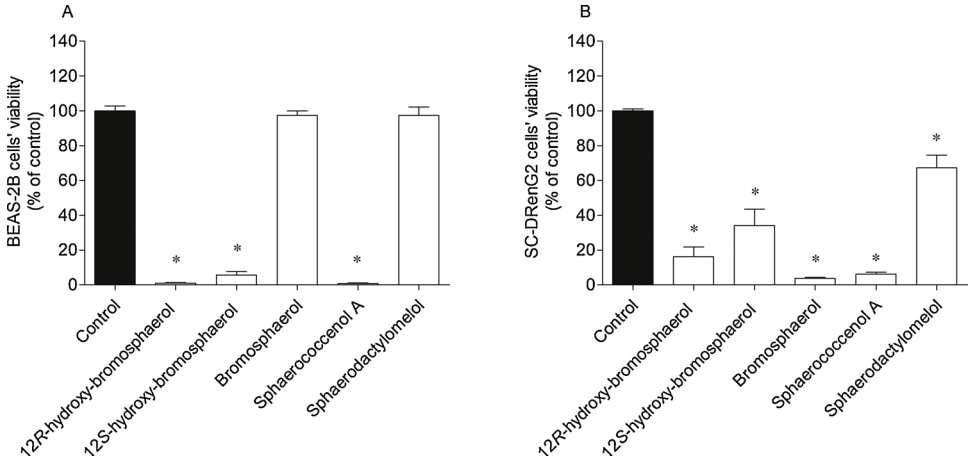

Fig. 3. BEAS-2B (A) and SC-DRenG2 (B) cells' viability following 72 h of exposure to the *Sphaerococcus coronopifolius* compounds (10 µM) expressed as % of the control. The values correspond to mean ± SEM of at least three independent experiments were carried out in triplicate. Symbols (*) represent statistically significant differences ($p < 0.05$) when compared to control.

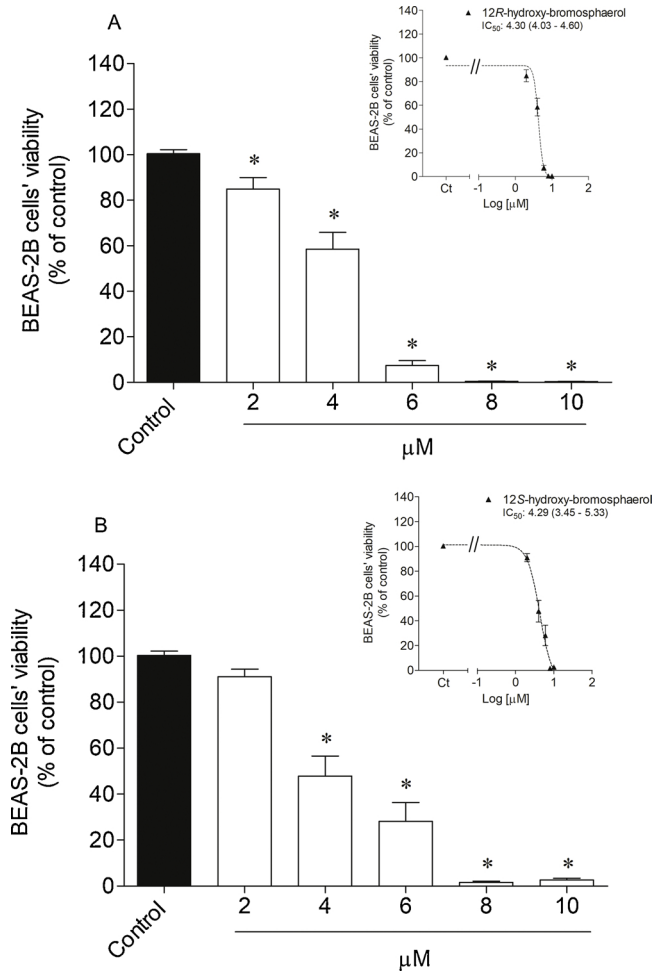

Fig. 4. BEAS-2B dose-response curve following 72h exposure to 12*R*-hydroxy-bromosphaerol (A) and 12*S*-hydroxy-bromosphaerol (B) at concentrations between 2 and 10 μM. Values correspond to mean ± SEM of at least three independent experiments were carried out in triplicate. Symbols (*) represent statistically significant differences ($p < 0.05$) when compared to control.

12*R*-hydroxy-bromosphaerol retained that capacity in co-cultures of malignant human bronchial epithelial cells with normal human bronchial fibroblasts. These results, once again, highlight the importance of the tumour microenvironment in modulating the therapeutic response, since the presence of a normal stroma inhibited the action of 12*R*-hydroxy-bromosphaerol and bromosphaerol over CSCs. They are in line with current literature as, for instance, the doxorubicin antitumour activities were attenuated in prostate cancer cells when co-cultivated with cancer-associated fibroblasts (CAFs). According to the authors, CAFs blocked doxorubicin accumulation in the prostate cancer cells, avoiding ROS production and consequently DNA damage and apoptosis activation [43]. Similar results were observed in co-cultures of breast cancer cells with breast-cancer-tissue-derived mesenchymal stem cells (BC-MSC) treated with cisplatin [44]. According to the authors the resistance of breast cancer cells in co-culture seemed to be associated to IL-6 released by BC-MSC. To progress from bench to bedside, potential therapeutic agents must fulfil a complex and long list of criteria that is updated along the process [45]. Even though the microenvironment plays a pivotal role in the early steps of the carcinogenic process, most of the *in vitro* studies to test new drugs neglect that. In fact most of the preclinical studies of anticancer drugs employ 2D monoculture of cancer cells, where no intercellular communications were considered [46]. Elegant co-culture systems as the one developed by Rodrigues and collaborators [24] and others, allow the addition of complexity to preclinical drug studies, perhaps leading to lower number of drugs that fail to perform in the clinic.

Considering previous observations indicating IL-6 as a mediator of CSCs formation, we hypothesized that drugs targeting this cellular population may decrease IL-6 levels in the tumour microenvironment. Our results did not corroborate this hypothesis, as no significant variation was observed on IL-6 levels, in the co-culture systems, following the 12*R*-hydroxy-bromosphaerol treatment. Nonetheless, they are in line in previous observations revealing that this cytokine, present in the tumour microenvironment, is involved in tumorigenesis by regulating various cancer hallmarks and multiple signalling pathways, being also involved in chemoresistance [47,48]. Of particular interest is the failure of the treatment with bromosphaerol in co-cultures, as compared to the results achieved in monocultures. Apparently, the high levels of IL-6 in the co-culture experiments triggered some pro-survival pathways and circumvented the cytotoxic effect of some CSCs-targeting agents. Finally, our findings are also in agreement with other reports in the literature supporting the potential of extracts or compounds from marine origin to mediate anti-inflammatory and anti-CSC effects[35,37,49–52]. For instance, diterpene glycosides isolated from the soft coral *Antillogorgia elisabethae* were shown to block NF-κB signalling pathway in triple-negative breast cancer and monocytic leukaemia cells [53]. Moreover, two polyhalogenated monoterpene stereoisomers (RU017 and RU018) and one sesquiterpene (smenospongine) isolated from the red alga *Plocamium cornutum* and the sponge *Spongia pertusa esper*, respectively, prevented tumour sphere formation in *in vitro* breast cancer models [35,54]. In the specific case of smenospongine, it promoted cell cycle arrest and intrinsic apoptosis, as well as mediated the down-regulation of specific stem cell markers, namely Nanog, Sox2, and Bmi1 [54].

Our results suggest that 12*R*-hydroxy-bromosphaerol is a potential CSCs-targeting therapeutic agent, whose cytotoxic action seems to be independent of the IL-6 levels in the tumour microenvironment. Although, the cytotoxic effects of some of these bromoterpenes were assessed in monocultures of human lung cancer cells [55], to the best of our knowledge, this is the first study that evaluate the capacity of *S. coronopifolius* compounds to impact CSCs dynamics in a human lung cancer *in vitro* cellular model. Subsequent studies will be needed to confirm the reproducibility of the attained results in more complex living models, and to assess the potential suitability of 12*R*-hydroxy-bromosphaerol to undergo clinical studies. The present study opens new research lines to evaluate the therapeutic potential of these compounds, namely 12*R*-hydroxy-bromosphaerol. In order to understand the mechanism of action underlying the activities observed, the expression of stemness factors (Nanog, oct4, sox2, STAT3), expression of anti-apoptotic proteins (Survivin, XIAP), analysis of cell cycle and hallmarks linked to apoptosis (*e.g.* mitochondrial depolarization, caspases activity, DNA fragmentation) should be studied. On the other hand, the combination of these compounds with anticancer drugs (*e.g.* salinomycin) to improve the therapeutic regimens efficacy as well as their ability to sensitize cancer cells and CSCs to radiotherapy should be addressed in future studies to understand the potential of these compounds to inspire/ create new therapeutic options that contribute to increase the anticancer treatments efficiency.

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.

All authors have approved the manuscript for submission and declare that this manuscript is original and unpublished, and has not been nor will be submitted to another journal for consideration unless it is rejected.

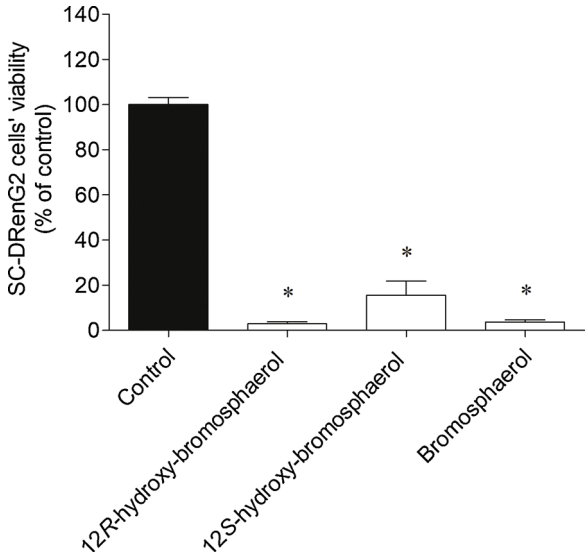

Fig. 5. SC-DRenG2 cells' viability following 72 h of exposure to 4 μM 12*R*-hydroxy-bromosphaerol, 12*S*-hydroxy-bromosphaerol and bromosphaerol. Results are expressed as % of the control. The values correspond to mean ± SEM of at least three independent experiments were carried out in triplicate. Symbols (*) represent statistically significant differences ($p < 0.05$) when compared to control.

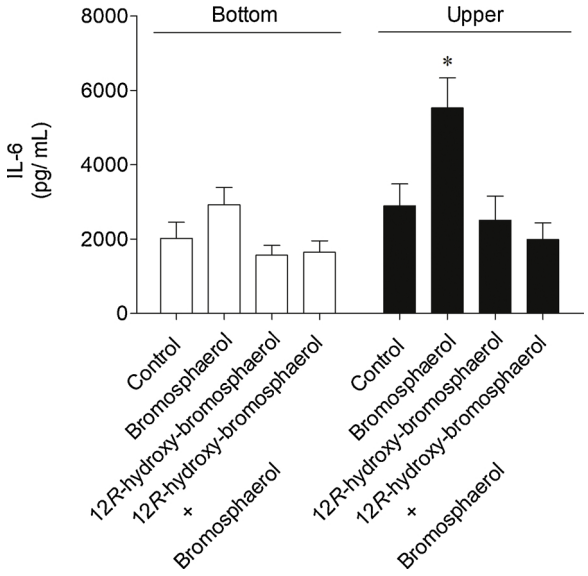

Fig. 7. IL-6 levels assessed by ELISA on the bottom (HBF cells) and upper (RenG2 cells) compartments of the co-culture system, following treatment with 4 μM 12*R*-hydroxy-bromosphaerol, bromosphaerol or both for 72 h. The values correspond to mean ± SEM of at least three independent experiments carried out in triplicate. Symbols (*) represent statistically significant differences ($p < 0.05$) when compared to respective control.

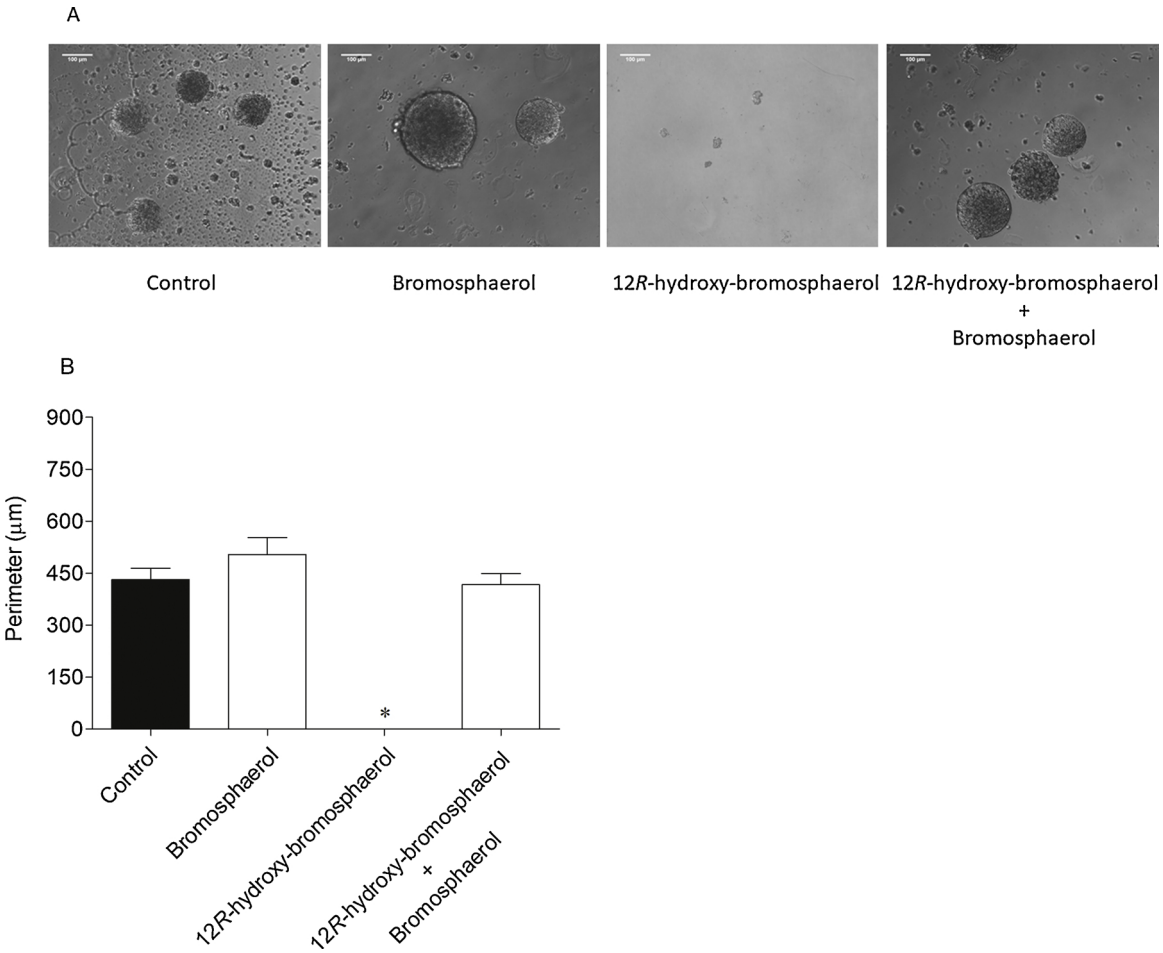

Fig. 6. Spheres attained following the co-culture treatment with 4 μM 12*R*-hydroxy-bromosphaerol, bromosphaerol or both for 72 h. The images are representative of each treatment accomplished (A). Perimeter analysis (μm) of the attained spheres after 2 weeks in culture under low-adherence conditions (B). Twenty spheres were measured per treatment. The results were revealed by the sphere-forming assay. Values correspond to mean ± SEM of at least three independent experiments were carried out in triplicate. Symbols (*) represent statistically significant differences ($p < 0.05$) when compared to control.

Acknowledgments

This work was supported by the Portuguese Foundation for Science and Technology (FCT) through strategic project UID/MAR/04292/2020 and UID/Multi/04046/2020 granted to MARE—Marine and Environmental Sciences Centre and BioISI—BioSystems and Integrative Sciences Institute, respectively, through Red2Discovery project (PTDC/MAR-BIO/6149/2014), co-financed by COMPETE (POCI-01-0145-FEDER-016791), through Oncologia de Precisão: Terapias e Tecnologias Inovadoras project (POINT4PAC) (SAICTPAC/0019/2015 - LISBOA-01-0145-FEDER-016405) and through CROSS-ATLANTIC project (PTDC/BIA-OUT/29250/2017), co-financed by COMPETE (POCI-01-0145-FEDER-029250). This work was also funded by the Integrated Programme of SR&TD "Smart Valorization of Endogenous Marine Biological Resources Under a Changing Climate" (reference Centro-01-0145-FEDER-000018), co-funded by Centro 2020 Programme, Portugal 2020, European Union, through the European Regional Development Fund. FCT is also acknowledged for the grant attributed to J.S. (SFRH/BD/103255/2014).

References

[1] J. Ferlay, M. Colombet, I. Soerjomataram, C. Mathers, D.M. Parkin, M. Piñeros, A. Znaor, F. Bray, Estimating the global cancer incidence and mortality in 2018: GLOBOCAN sources and methods, Int. J. Cancer 144 (8) (2019) 1941–1953.
[2] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2018, CA Cancer J. Clin. 68 (1) (2018) 7–30.
[3] P. Godwin, A.M. Baird, S. Heavey, M.P. Barr, K.J. O'Byrne, K. Gately, Targeting nuclear factor-kappa B to overcome resistance to chemotherapy, Front. Oncol. 3 (2013) 120.
[4] M. Prieto-Vila, R.-u. Takahashi, W. Usuba, I. Kohama, T. Ochiya, Drug resistance driven by Cancer stem cells and their niche, Int. J. Mol. Sci. 18 (12) (2017) 2574.
[5] L. Xie, P. Bourne, Developing multi-target therapeutics to fine-tune the evolutionary dynamics of the cancer ecosystem, Front. Pharmacol. 6 (209) (2015).
[6] I.A. Cree, P. Charlton, Molecular chess? Hallmarks of anti-cancer drug resistance, BMC Cancer 17 (1) (2017) 10.
[7] H. Maeda, M. Khatami, Analyses of repeated failures in cancer therapy for solid tumors: poor tumor-selective drug delivery, low therapeutic efficacy and unsustainable costs, Clin. Transl. Med. 7 (1) (2018) 11.
[8] D.A. Senthebane, A. Rowe, N.E. Thomford, H. Shipanga, D. Munro, M.A.M.A. Mazeedi, H.A.M. Almazyadi, K. Kallmeyer, C. Dandara, M.S. Pepper, M.I. Parker, K. Dzobo, The role of tumor microenvironment in Chemoresistance: to survive, keep your enemies closer, Int. J. Mol. Sci. 18 (7) (2017) 1586.
[9] Y.A. Luqmani, Mechanisms of drug resistance in Cancer chemotherapy, Med. Princ. Pract. 14 (suppl 1) (2005) 35–48.
[10] B. Mansoori, A. Mohammadi, S. Davudian, S. Shirjang, B. Baradaran, The different mechanisms of Cancer drug resistance: a brief review, Adv. Pharm. Bull. 7 (3) (2017) 339–348.
[11] C. Roma-Rodrigues, R. Mendes, P.V. Baptista, A.R. Fernandes, Targeting tumor microenvironment for Cancer therapy, Int. J. Mol. Sci. 20 (4) (2019) 840.
[12] S. Zhong, J.-H. Jeong, Z. Chen, Z. Chen, J.-L. Luo, Targeting tumor microenvironment by small-molecule inhibitors, Transl. Oncol. 13 (1) (2020) 57–69.
[13] J. Zhao, Cancer stem cells and chemoresistance: the smartest survives the raid, Pharmacol. Ther. 160 (2016) 145–158.
[14] P.M. Aponte, A. Caicedo, Stemness in Cancer: stem cells, Cancer stem cells, and their microenvironment, Stem Cells Int. 2017 (2017) 17.
[15] E.Y.-T. Lau, N.P.-Y. Ho, T.K.-W. Lee, Cancer stem cells and their microenvironment: biology and therapeutic implications, Stem Cells Int. 2017 (2017) 11.
[16] N. Nguyen, K.L. Couts, Y. Luo, M. Fujita, Understanding melanoma stem cells, Melanoma Manag. 2 (2) (2015) 179–188.
[17] A.Z. Ayob, T.S. Ramasamy, Cancer stem cells as key drivers of tumour progression, J. Biomed. Sci. 25 (1) (2018) 20.
[18] M.E. Lleonart, E. Abad, D. Graifer, A. Lyakhovich, Reactive oxygen species-mediated autophagy defines the fate of cancer stem cells, Antioxid. Redox Signal. 28 (11) (2018) 1066–1079.
[19] A. Pietras, Cancer Stem Cells in Tumor Heterogeneity, Advances in Cancer Research, Elsevier, 2011, pp. 255–281.
[20] C.L. Chaffer, I. Brueckmann, C. Scheel, A.J. Kaestli, P.A. Wiggins, L.O. Rodrigues, M. Brooks, F. Reinhardt, Y. Su, K. Polyak, L.M. Arendt, C. Kuperwasser, B. Bierie, R.A. Weinberg, Normal and neoplastic nonstem cells can spontaneously convert to a stem-like state, Proc. Natl. Acad. Sci. 108 (19) (2011) 7950–7955.
[21] Piyush B. Gupta, Christine M. Fillmore, G. Jiang, Sagi D. Shapira, K. Tao, C. Kuperwasser, Eric S. Lander, Stochastic state transitions give rise to phenotypic equilibrium in populations of Cancer cells, Cell 146 (4) (2011) 633–644.
[22] Christine L. Chaffer, Nemanja D. Marjanovic, T. Lee, G. Bell, Celina G. Kleer, F. Reinhardt, Ana C. D'Alessio, Richard A. Young, Robert A. Weinberg, Poised chromatin at the ZEB1 promoter enables breast Cancer cell plasticity and enhances tumorigenicity, Cell 154 (1) (2013) 61–74.
[23] E. Kiyoung, H. Seok Won, K. Hyunggee, Cancer stem cell heterogeneity: origin and new perspectives on CSC targeting, BMB Rep. 50 (3) (2017) 117–125.
[24] C.F.D. Rodrigues, E. Serrano, M.I. Patrício, M.M. Val, P. Albuquerque, J. Fonseca, C.M.F. Gomes, A.J. Abrunhosa, A. Paiva, L. Carvalho, M.F. Botelho, L. Almeida, I.M. Carreira, M.C. Alpoim, Stroma-derived IL-6, G-CSF and Activin-A mediated dedifferentiation of lung carcinoma cells into cancer stem cells, Sci. Rep. 8 (1) (2018) 11573.
[25] J.N. Rich, Cancer stem cells: understanding tumor hierarchy and heterogeneity, Medicine 95 (1S) (2016) S2–S7.
[26] D. Horst, L. Kriegl, J. Engel, T. Kirchner, A. Jung, CD133 expression is an independent prognostic marker for low survival in colorectal cancer, Br. J. Cancer 99 (2008) 1285.
[27] K. Eppert, K. Takenaka, E.R. Lechman, L. Waldron, B. Nilsson, P. van Galen, K.H. Metzeler, A. Poeppl, V. Ling, J. Beyene, A.J. Canty, J.S. Danska, S.K. Bohlander, C. Buske, M.D. Minden, T.R. Golub, I. Jurisica, B.L. Ebert, J.E. Dick, Stem cell gene expression programs influence clinical outcome in human leukemia, Nat. Med. 17 (2011) 1086.
[28] B. Cheng, G. Yang, R. Jiang, Y. Cheng, H. Yang, L. Pei, X. Qiu, Cancer stem cell markers predict a poor prognosis in renal cell carcinoma: a meta-analysis, Oncotarget 7 (40) (2016) 65862–65875.
[29] W.F. Taylor, E. Jabbarzadeh, The use of natural products to target cancer stem cells, Am. J. Cancer Res. 7 (7) (2017) 1588–1605.
[30] N. Bhummaphan, P. Chanvorachote, Gigantol suppresses Cancer stem cell-like phenotypes in lung Cancer cells, Evid. Based Complement. Altern. Med. 2015 (2015) 10.
[31] P. Chanvorachote, S. Chamni, C. Ninsontia, P.P. Phiboonchaiyanan, Potential anti-metastasis natural compounds for lung Cancer, Anticancer Res. 36 (11) (2016) 5707–5717.
[32] G. Sun, L. Wei, J. Feng, J. Lin, J. Peng, Inhibitory effects of Hedyotis diffusa Willd. On colorectal cancer stem cells, Oncol. Lett. 11 (6) (2016) 3875–3881.
[33] H.-W. Leung, C.-H. Ko, G.G.-L. Yue, I. Herr, C.B.-S. Lau, The natural agent 4-vinylphenol targets metastasis and stemness features in breast cancer stem-like cells, Cancer Chemother. Pharmacol. (2018).
[34] O. Sabine, K. Anne, R. Vanessa, L. Li, K. Georgios, G. Wolfgang, G. Martha-Maria, B. Franz, H. Ingrid, Targeting of pancreatic and prostate cancer stem cell characteristics by Crambe crambe marine sponge extract, Int. J. Cancer 130 (7) (2012) 1671–1681.
[35] J.-A. De la Mare, J.N. Sterrenberg, M.G. Sukhthankar, M.T. Chiwakata, D.R. Beukes, G.L. Blatch, A.L. Edkins, Assessment of potential anti-cancer stem cell activity of marine algal compounds using an in vitro mammosphere assay, Cancer Cell Int. 13 (2013) 39.
[36] K.S. Yeon, K. Jeong-Yub, S. Woon-Seob, L.S. Joon, C. Sung-Gil, L. Ji-Yun, P. Myung-Jin, Saccharina japonica extract suppresses stemness of glioma stem cells by degrading epidermal growth factor Receptor/Epidermal growth factor receptor variant III, J. Med. Food 21 (5) (2018) 496–505.
[37] M. Terasaki, H. Maeda, K. Miyashita, T. Tanaka, S. Miyamoto, M. Mutoh, A marine bio-functional lipid, fucoxanthinol, attenuates human colorectal cancer stem-like cell tumorigenicity and sphere formation, J. Clin. Biochem. Nutr. 61 (1) (2017) 25–32.
[38] M. Terasaki, M. Mima, S. Kudoh, T. Endo, H. Maeda, J. Hamada, K. Osada, K. Miyashita, M. Mutoh, Glycine and succinic acid are effective indicators of the suppression of epithelial-mesenchymal transition by fucoxanthinol in colorectal cancer stem-like cells, Oncol. Rep. 40 (2018) 414–424.
[39] G. Housman, S. Byler, S. Heerboth, K. Lapinska, M. Longacre, N. Snyder, S. Sarkar, Drug resistance in Cancer: an overview, Cancers 6 (3) (2014) 1769.
[40] H. Zahreddine, K. Borden, Mechanisms and insights into drug resistance in cancer, Front. Pharmacol. 4 (28) (2013).
[41] A. Albini, A. Bruno, C. Gallo, G. Pajardi, D.M. Noonan, K. Dallaglio, Cancer stem cells and the tumor microenvironment: interplay in tumor heterogeneity, Connect. Tissue Res. 56 (5) (2015) 414–425.
[42] X.-x. Sun, Q. Yu, Intra-tumor heterogeneity of cancer cells and its implications for cancer treatment, Acta Pharmacol. Sin. 36 (10) (2015) 1219.
[43] E.H. Cheteh, M. Augsten, H. Rundqvist, J. Bianchi, V. Sarne, L. Egevad, V.J.N. Bykov, A. Östman, K.G. Wiman, Human cancer-associated fibroblasts enhance glutathione levels and antagonize drug-induced prostate cancer cell death, Cell Death Dis. 8 (6) (2017) e2848.
[44] H. Xu, Y. Zhou, W. Li, B. Zhang, H. Zhang, S. Zhao, P. Zheng, H. Wu, J. Yang, Tumor-derived mesenchymal-stem-cell-secreted IL-6 enhances resistance to cisplatin via the STAT3 pathway in breast cancer, Oncol. Lett. 15 (6) (2018) 9142–9150.
[45] R. Cagan, P. Meyer, Rethinking cancer: current challenges and opportunities in cancer research, Dis. Model. Mech. 10 (4) (2017) 349–352.
[46] Y. Miki, K. Ono, S. Hata, T. Suzuki, H. Kumamoto, H. Sasano, The advantages of co-culture over mono cell culture in simulating in vivo environment, J. Steroid Biochem. Mol. Biol. 131 (3) (2012) 68–75.
[47] Z. Culig, M. Puhr, Interleukin-6: A multifunctional targetable cytokine in human prostate cancer, Mol. Cell. Endocrinol. 360 (1–2) (2012) 52–58.
[48] N. Kumari, B.S. Dwarakanath, A. Das, A.N. Bhatt, Role of interleukin-6 in cancer progression and therapeutic resistance, Tumor Biol. 37 (9) (2016) 11553–11572.
[49] T. Reya, S.J. Morrison, M.F. Clarke, I.L. Weissman, Stem cells, cancer, and cancer stem cells, Nature 414 (2001) 105.
[50] M. Al-Hajj, M.S. Wicha, A. Benito-Hernandez, S.J. Morrison, M.F. Clarke, Prospective identification of tumorigenic breast cancer cells, Proc. Natl. Acad. Sci. 100 (7) (2003) 3983–3988.
[51] S. Ottinger, A. Klöppel, V. Rausch, L. Liu, G. Kallifatidis, W. Gross, M.-M. Gebhard, F. Brümmer, I. Herr, Targeting of pancreatic and prostate cancer stem cell characteristics by Crambe crambe marine sponge extract, Int. J. Cancer 130 (7) (2012)

1671–1681.
[52] S.Y. Kim, J.-Y. Kim, W.-S. Shin, S.J. Lee, S.-G. Chi, J. Lee, M. Park, Saccharina japonica Extract Suppresses Stemness of Glioma Stem Cells by Degrading Epidermal Growth Factor Receptor/Epidermal Growth Factor Receptor Variant III, J. Med. Food 21 (5) (2018) 496–505.
[53] J. Sperlich, R. Kerr, N. Teusch, The marine natural product pseudopterosin blocks cytokine release of triple-negative breast Cancer and monocytic leukemia cells by inhibiting NF-κB signaling, Mar. Drugs 15 (9) (2017) 262.
[54] J. Tang, W. Wu, F. Yang, L. Liu, Z. Yang, L. Liu, W. Tang, F. Sun, H. Lin, Marine sponge-derived smenospongine preferentially eliminates breast cancer stem-like cells via p38/AMPKα pathways, Cancer Med. 7 (8) (2018) 3965–3976.
[55] V. Smyrniotopoulos, C. Vagias, C. Bruyère, D. Lamoral-Theys, R. Kiss, V. Roussis, Structure and in vitro antitumor activity evaluation of brominated diterpenes from the red alga Sphaerococcus coronopifolius, Bioorg. Med. Chem. 18 (3) (2010) 1321–1330.
[56] D. Rodrigues, C. Alves, A. Horta, S. Pinteus, J. Silva, G. Culioli, R. Pedrosa, Antitumor and antimicrobial potential of bromoditerpenes isolated from the red alga, Sphaerococcus coronopifolius, Marine Drugs 13 (2) (2015) 713–726, https://doi.org/10.3390/md13020713.