Universidade de Aveiro Departamento de Biologia
2018

MAFALDA SUTIL TABUADA

Potencial de bactérias marinhas para produção de compostos com aplicação médica

Potential of marine bacteria to synthesize bioactive compounds for medical applications

DECLARAÇÃO

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Potential of marine bacteria to synthesize bioactive compounds for medical applications

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Catarina Ribeiro Marques, Cientista Convidada do Departamento de Biologia da Universidade de Aveiro e membro integrado do Centro de estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro.

Dedico especialmente ao meu pai por ter tornado tudo possível.
Dedico aos de casa pela paciência a ter comigo.
Dedico aos meus avós e à minha família em geral.
Dedico ao meu namorado pelo apoio dado.

o júri

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palavras-chave

Cancro do pâncreas, terapia, sintetases peptídicas não ribossomais, sintases de policetídeos, compostos naturais bioativos, bactérias marinhas simbiontes, cnidários, extratos brutos, ensaio MTT.

resumo

O cancro do pâncreas é uma das doenças mais letais do mundo. A melhor esperança para reduzir a mortalidade provocada por este cancro reside no diagnóstico e tratamento precoces, mormente na deteção de lesões que podem conduzir ao seu desenvolvimento. Por outro lado, as terapias tradicionalmente aplicadas para combater o cancro do pâncreas têm vários inconvenientes, relacionados nomeadamente com uma eficácia limitada, toxicidade para os pacientes e aquisição de resistência aos medicamentos. Para contrariar esta tendência, tem sido dedicado um maior esforço na descoberta de novos fármacos anti-cancro pancreático, a partir de recursos naturais biológicos. Neste âmbito, organismos provenientes de ambientes marinhos (*e.g.*, microorganismos simbiontes, algas, invertebrados) possuem um amplo potencial genético para a biossíntese de compostos naturais. No entanto, os ecossistemas marinhos estão ainda consideravelmente sub-explorados para este propósito. Os simbiontes bacterianos marinhos, em particular, podem produzir vários metabolitos secundários com alto interesse biotecnológico, especialmente para aplicações biomédicas como as direcionadas para o tratamento anticancerígeno. Portanto, os principais objetivos deste trabalho foram: (1) desenvolver uma breve revisão sobre o cancro do pâncreas e suas atuais terapias, bem como sobre os produtos naturais bioativos e vias biossintéticas existentes em microorganismos marinhos; (2) desvendar o potencial de simbiontes bacterianos isolados de cnidários marinhos para sintetizar compostos bioativos contra o cancro do pâncreas. Algumas bactérias têm o potencial de sintetizar novos produtos através da atividade das enzimas policetídeo sintases (PKS), sintetases peptídicas não ribossomais (NRPS), ou uma combinação de ambas. Estas enzimas multifuncionais produtoras de policetídeos (PKs), péptidos não ribossomais (NRPs) ou híbridos PKs-NRPs podem ter várias estruturas químicas e bioatividades *(e.g.,* antimicrobiana, antioxidante, anti-inflamatória, anticancerígena). O rastreio molecular de fragmentos do gene NRPS em simbiontes bacterianos marinhos de cnidários evidenciou a sua presença em 22,3% dos isolados, os quais pertencem aos filos Proteobacteria e Actinobacteria. A maioria das sequências de aminoácidos obtidas para o grupo de bactérias selecionadas por serem potenciais produtores de NRPs, apresentou uma homologia relativamente elevada em relação a sequências de aminoácidos depositadas para NRPS. Além disso, os extratos preparados a partir das frações celulares e sobrenadante de culturas das bactérias selecionadas inibiram significativamente a atividade de uma linha celular humana de adenocarcinoma do ducto pancreático, mediante o ensaio MTT. Assim, o potencial anticancerígeno dos simbiontes bacterianos de cnidários demonstrou ser promissor, pelo que o seu estudo deve ser mais aprofundado no futuro.

keywords

Pancreatic cancer, therapeutics, nonribosomal peptide synthetases, polyketide synthases, bioactive natural compounds, marine bacteria symbionts, cnidarians, crude extracts, MTT assay.

abstract

Pancreatic cancer is one of the deadliest diseases in the world. The best hope for reducing mortality by cancer lies in its early diagnosis and treatment, namely in the detection of lesions that can evolve into cancer stages. On the other hand, traditionally applied therapeutics to fight pancreatic cancer have several drawbacks, namely related with limited efficacy, toxicity to patients, and acquisition of drug resistance. As to counteract this trend, a greater effort has been devoted to the discovery of new anti-pancreatic cancer drugs from biological natural resources. In this context, organisms from marine environments (*e.g.*, microbial symbionts, algae, invertebrates) possess a wide genetic potential for the biosynthesis of natural compounds. However, marine ecosystems are yet severely overlooked for that purpose. Marine bacterial symbionts, in particular, can produce several secondary metabolites with high biotechnological interest, especially for biomedical applications like those directed to anticancer treatment. Therefore, the major goals of this work were to: (1) develop a brief review on pancreatic cancer and current therapeutics, as well as on the pool of natural products and associated biosynthetic pathways in marine microbes; (2) unravel the potential of bacterial symbionts isolated from marine cnidarians to synthesize bioactive compounds against pancreatic cancer. Some bacteria have the potential to synthesize new products through the activity of the modular enzymes polyketide synthases (PKS), nonribosomal peptide synthetases (NRPS), or a combination of both. These multifunctional enzymes are involved in the production of polyketides (PKs), nonribosomal peptides (NRPs) or PKs-NRPs hybrid compounds that can have various chemical structures and bioactivities (*e.g.*, antimicrobial, antioxidant, anti-inflammatory, anticancer). The molecular screening of NRPS gene fragments in marine bacterial symbionts of cnidarians showed their presence in 22.3% of the isolates, which belong to Proteobacteria and Actinobacteria phyla. Most aminoacid sequences obtained for a selected group of these potential NRPs-bacterial producers presented a relatively high homology with deposited aminoacid sequences of NRPS clusters. Furthermore, crude extracts prepared from the cell and cell-free fractions of cultures from the selected bacteria could generally significantly inhibit the activity of a human pancreatic ductal adenocarcinoma cell line, according to the MTT assay. Thereby, the anticancer potential of cnidarian bacterial symbionts was promising, hence reinforcing the need for deepening its study in the future.

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Abbreviations list

A- adenylation domain

ACP- acyl carrier protein domain

AT- acetyltransferase domain

BRCA1- breast cancer 1 DNA repair-associated gene (*BRCA1*) or protein (BRCA1)

BRCA2- breast cancer 2 DNA repair-associated gene (*BRCA2*) or protein (BRCA2)

C- condensation domain

CDKN2A- cyclin-dependent kinase inhibitor 2A

EMT- epithelial mesenchymal transition

gDNA- genomic DNA

KCs- Kupffer cells

KRAS- Kristen ras oncogene

KS- ketosynthase domain

MAPK- mitogen-activated protein kinase

MDR- multidrug resistance

NRPS- nonribosomal peptide synthetases

NRPs- nonribosomal peptides

OS- oxidative stress

PanIN- Pancreatic Intraepithelial Neoplasia

PCP- peptidyl carrier protein domain

PDAC- pancreatic ductal adenocarcinoma

PCR- polymerase chain reaction

PKS- polyketide synthases

PKs- polyketides

ROS- reactive oxygen species

TE- thioesterase domain

TNF-α - tumour necrosis factor alfa

TP53-.tumour protein p53 gene

Chapter I

1-Pancreatic cancer: what is it and how it evolves?

Pancreatic cancer is estimated to be the 7th deadliest cancer in the world in 2018[1], due to its fast progress, high malignancy and early metastasis, making therapeutics efficacy practically impossible for most patients[2,3]. Early detection gives hope for a cure, but its lack of symptoms transforms pancreatic cancer diagnosis quite difficult and unsuccessful[3].

1.1-Epidemiology & etiology

1.1.1- *Incidence and mortality rates*

Pancreatic ductal adenocarcinoma (PDAC) and its variants is responsible for 85-90% of all pancreatic neoplasms, being thereby most commonly called as pancreatic cancer[4]. Globally, there is a prediction for the year of 2018 of about 458,918 new cases of pancreatic cancer, representing 2.5% of all cancers, and 432,242 deaths (4.5% of all cancers deaths)[1]. According to GLOBOCAN statistics for the year 2012, 338,000 new cases (2.4% of all cancers) and 331,000 deaths (4% of all cancers deaths) were recorded[5], what clearly suggests an increased impact of this disease. Pancreatic cancer incidence rate for men and women is around 5.5 individuals per 100,000 and 4.0 individuals per each 100,000, respectively[1]. For both sexes, however, it has been observed an increasing incidence of pancreatic cancer with age, and most diagnosed cases occur after the 55 years of age[6,7]. In what concerns its worldwide incidence, this disease has been mostly diagnosed in developed countries, where 55.5% of new cases occurred in the year of 2012[5]. For 2018, is estimated higher incidence rates for both genders in Eastern Europe (9.9 per 100,000 men, and 5.8 per 100,000 women) and Western Europe (9.5 per 100,000 men, and 7.2 per 100,000 women), comparatively to those previewed for Northern America (8.7 per 100,000 men, and 6.5 per 100,000 women) and Australia/New Zealand (7.4 per 100,000 men, and 6.4 per 100,000 women)[1].

1.1.3- Risk factors

Among the risk factors for PDAC development, they can be roughly divided into intrinsic and extrinsic factors. As intrinsic factors, genetic mutations at different genes like *KRAS* (encode for proteins that normally promote the abnormal proliferation of cancer cells[8,9]), *Tp53* (tumour suppressor gene that when is mutated enhances tumour growth[9] through the control of DNA

damage responses), *BRCA1*/*BRCA2* (associated with the regulation of DNA repair[10]), *PALB2* (chromatin adaptor for *BRCA2* and promotes the connection between *BRCA1* and *BRCA2*[11]) and *CDKN2A*[12] (tumour suppressor gene that when altered causes chromosomic mutations leading to tumor growth[13]), can trigger PDAC carcinogenesis[9]. Similarly, several pancreatic diseases may promote PDAC pathogenesis. The most concerning one is diabetes, as far as it arises from the dysregulation of pancreatic function. On the other hand, diabetes can be a manifestation of PDAC, especially in the case of type I (chronic) diabetic patients. However, type II diabetes can indeed be a major pancreatic cancer triggering factor, because it increases the risk of hyperglycaemia due to the impairment of glucose levels in blood[14,15], which increase cancer risk by guaranteeing the prevalence and evolution of *KRAS* mutations[15,16]. Chronic pancreatitis also provides an increased risk of advancing into PDAC as a result of a large number of cell divisions due to the disruption of DNA repair systems[17]. Obesity may as well promote the appearance of PDAC since adipose tissues may boost inflammatory and hormonal responses, besides the potential disruption of the energy balance related with an increase of food consumption[18] and induction of chronic hyperinsulinemia[19,20]. Inherited genetic susceptibility is also a PDAC risk factor, existing the possibility of familial pancreatic cancer associated with susceptibility *loci*[20]. Age, gender, blood group and ethnicity (related to the incidence among countries/continents) constitute as well intrinsic factors affecting PDAC development and incidence[4].

Additional external or behavioural factors associated with PDAC are dietary habits, alcohol consumption and smoking[7,21,22], being the last one a great contribution to the occurrence of DNA damages/mutations in pancreatic cells that further promote initiation and proliferation of PDAC[23,24].

1.1.4- Types of pancreatic cancer by their location

Malignant pancreatic tumours can be classified according to their location, *i.e.*, in the exocrine (ductal or acinar cancers) or endocrine pancreas[25]. As aforementioned, the exocrine ductal epithelial adenocarcinoma is responsible for about 90% of the cases[4], while the endocrine cancer is more rare[25]. The two types are heterogeneous and have different histological and clinical features, as well as different epidemiologic expression[26]. It is also possible to find a combined tumour with exocrine and endocrine location, although this is not very frequent. This pancreatic cancer type is usually characterized by an exocrine-ductal component, in which the endocrine tumour may represent *ca.* one third to one half of the total tumour[25].

1.2- Diagnosis

Current diagnostic techniques of PDAC rely on imaging and/or blood analyses. Among the available imaging tests there are computerized tomography scanning, magnetic resonance imaging, endoscopic ultrasonography and endoscopic retrograde cholangiopancreatography. However, these are expensive, may be invasive, require more time for achieving the results and cannot accurately detect tiny lesions at a premalignant stage[27]. In turn, the most common blood (serum) biomarkers known for pancreatic cancer diagnosis and monitoring of response to therapeutics are proteins/epitopes like CA 19.9, CA50, CA125, Laminin gamma C and CEA[28,29]. Nevertheless, these markers may present drawbacks related with low sensitivity and uncertainty to accurately detect PDAC. For instance, CA 19.9 is not expressed in Lewis-negative phenotype (Lewis antigen system based on chromosome 19/Lewis gene) and may even give false positives in the presence of obstructive jaundice[30].

So, the currently available diagnostic tools and markers fail to provide the occurrence of PDAC within a safe period for its effective treatment, what combined with its strong metastatic behaviour and resistance to current therapies, makes it a life-threatening disease. Therefore, there is an urgent need for an ideal biomarker that facilitates pancreatic cancer detection. In this context, there are three main steps that need to be performed for an early detection and treatment of pancreatic cancer. The first is to distinguish the lesions that leads to advanced pancreatic cancers. Second, there should be an opportunity to detect those curable lesions through the creation of a sensitive screening test capable of responding along the disease progression time. Third, the disease prevalence in the population must be high enough to be accurately screened by the tests[3].

The search for new biomarkers towards the early diagnosis of PDAC may rely on crucial pathways or mechanisms responsible for its proliferation, namely the formation of pre-metastic niches, which precedes distant metastasis from primary tumour site. Exosomes are microvesicles derived from pancreatic lesions that have a role in the formation of pre-metastatic niches in liver[31] and other body locations like breast and lungs[32]. The Kupffer cells (KCs) in the liver phagocytize exosomes from the blood stream, hence activating fibrotic pathways and pro-inflammatory responses (*e.g.*, synthesis of fibronectin and transforming growth factor β) leading to a metastatic microenvironment[31]. The circulating exosomes can therefore be explored as biomarkers for pancreatic cancer, though the cellular and molecular mechanisms by which exosomes alter metastasis are yet to be established. Some experiments in mice carrying PDAC had focused on how the upregulation of migration inhibitory factor is a main event during cancer

progression, which can be detected in exosomes isolated from the plasma of mice and patients with PDAC[31].

1.3 -Biology and carcinogenesis

1.3.1- Carcinogenesis

Pancreatic cancer possess a multifaceted biology and its current knowledge assumes that it is constituted by several components, such as pancreatic cancer somatic cells and stem cells, and the tumour stroma[33]. In particular, the pancreatic cancer stellate cells (*i.e.*, myofibroblasts), are the main components of the tumour stroma[33]. In what concerns cancer stem cells in the primary tumour, they have been pointed out as the responsible for shorter patients' survival, resistance to chemo- and radiotherapies, as well as for the increasing of PDAC metastatic potential[34,35].

Pancreatic cancer may begin with a with gene mutation(s) and/or pancreatic lesions. In what regards genetic mutations in normal pancreatic cells, they may target genes involved in several signalling pathways like RAS-ERK signalling (extracellular signal-regulated kinase signalling pathway**)** , G1/S phase transition (checkpoint in cell cycle), DNA damage response, TGFβ (transforming growth factor beta) signalling, SWI/SNF (SWItch/Sucrose Non-Fermentable) pathway, KMT2 (family of methyltransferases), cell stress response, axonal guidance, RNA splicing, homophilic cell adhesion[36,37]. Regarding the inherited genetic susceptibility, the disabling of DNA repair system-encoding genes (*e.g.*, *BRCA1*, *BRCA2*, Fanconi anaemia *FANCC* and *FANCG*, ataxia telangiectasia mutated genes) have been associated with an increased risk of PDAC[38,39]. Also mutation on genes involved in cell division cycle [*e.g.*, the genes cyclin-dependent kinase inhibitor 2A (*CDKN2A*)[15,38,40], nuclear receptor subfamily 5 group A member 2 (*NR5A2*), telomerase reverse transcriptase (*TERT*), zinc ring finger 3 (*ZNFR3*) and *TP63*][41–43] have been studied. Overall, a single mutation alone is not enough for tumour development[15] due to the poor proliferation capacity of the pancreatic tissue[44]. As such, cells divide and increase their number, ending up in cellular populations with the same driver gene mutation, leading to clonal expansion. Hence, the tumour propagates upon increasing cell divisions, consequently enabling the accumulation of somatic alterations[15,45] that are responsible for pancreatic cancer heterogeneity. The (population of) cells carrying mutations can afterwards cross the surrounding membrane and disperse to other near or distant locations/organs, hence forming metastases. For that, pre-metastatic niches

are developed through several events that help establishing the tumour microenvironment in distant metastatic sites, thereby leading the flux of cancer cells from the primary tumour to those sites, while simultaneously guaranteeing the survival of the newly arrived metastatic cells[46,47]. As briefly referred in the previous section, metastatic cells may rely on tumour-derived exosomes[48,49], which are microvesicles (*ca.* 30-150 nm diameter) of endocytic origin constituted by extracellular matrix components[50–54], with a key role on intercellular communication between cancer cells and their respective microenvironment. Exosomes abundance has been normally correlated with tumour malignancy[55], since they contribute for the successful adhesion and colonization of circulating cells into other tissues[56], hence mediating the process of metastases dissemination and tumour development/proliferation[15].

There are evidences that premalignant lesions in normal ductal epithelium, such as Pancreatic Intraepithelial Neoplasia (PanIN), have also a strong involvement in cancer development[57], though 16 to 45% of them are not associated with invasive carcinomas[58,59]. These lesions occur and start in the small ducts of the exocrine pancreas[33]. According to their neoplastic progression and stage, they may be classified as PanIN-1 (low grade), PanIN-2 (medium grade) or PanIN-3 (high grade)[60,61]. The PanIN-1 lesions are observed in normal pancreatic tissues, whilst PanIN-2 occur in the tissue of neoplastic pancreata, and PanIN3 lesions are usually associated with an established pancreatic cancer. The mutations responsible for PanIN progression into a carcinoma frequently rely on the activation of *KRAS2* oncogene (most frequent and it occurs in 95% of pancreatic tumors), and inactivation of tumour suppressor genes such as *CDKN2A*, *INK4A*, *TP53* and *DPC4/SMAD4*[15,34,62,63]. Although PanIN are major cancer-inducing lesions, other pancreatic neoplasic lesions may be indeed precursors of invasive carcinomas, namely the intraductal papillary mucinous neoplasm (IPMN) and mucinous cystic neoplasms (MCN)[3].

The biochemical and metabolic traits associated with PDAC development highlight the capacity of changes in cell functions that are needed for tumour initiation and progression[64]. As such, besides the high energetic (*i.e.*, ATP) and biochemical (several macromolecules) requirements to support PDAC proliferation, tumour microenvironment partly sustained by desmoplastic stromal reactions is also an extremely relevant influencing factor. The stroma is the connective and structural part of a tissue, but in PDAC the characteristic desmoplastic stroma is enhanced comparatively to the epithelial counterpart of the tumour, being responsible for the hypoxic and low nutrient loads determined in PDAC metabolic features[65–67]. Moreover, PDAC is indeed typically characterized by a fibroinflammatory microenvironment, which induce T cells that possess a mechanism of p38 protein kinase activation (MAPK)[68], which lead to an enhanced

production of cytokines[69,70]. The microenvironment of PDAC is the major source of tumour-promoting cytokines that cause angiogenesis, metastasis, and resistance to chemotherapy and disruption of the host defences[71–73]. Angiogenesis, in particular, promotes the formation of a dense blood vessels net what is very important towards the growth, invasion and metastasization of the tumours, as far as it provides nutrients and oxygen to the tumour cells[74,75]. Furthermore, angiogenesis entails the proliferation and migration of endothelial cells. Some growth factors are part of tumour angiogenesis such as the fibroblast growth factor, epidermal growth factor, vascular endothelial growth factor, placental growth factor, tumour necrosis factor-alfa (TNF-α), and angiogenin [76–78].

1.3.2- Apoptosis pathway

Together with the cancer pathways mentioned above, other pathways like Hedgehog pathway, integrin signalling, JNK, Wnt-β-caterin, Small GTPases and mainly apoptosis[67], have very important roles in PDAC development. Herein is described the apoptosis pathway in particular since it is one of the first mechanisms that act against cell dysregulation in our system, and hence give some information on how the disease can be treated. Besides, apoptosis functions in combination with the other signalling pathways, therefore maximizing its potential to eliminate cancer cells[79]. Plus, it is a natural mechanism to prevent cancer dissemination by killing the dysregulated cells, but it can also influence cell mutations when cancer cells can escape from programmed cell death[80]. So, apoptosis is not always a safe pathway, and can actually favour disease proliferation under certain circumstances[79].

Apoptosis is a natural programmed cell death that emerges from two specific pathways in cells. The first is extrinsic to the cell and depends on its surface receptors. The second is an intrinsic pathway lead by mitochondria-produced molecules[81]. A strong therapeutic strategy is to block the apoptosis pathways in cancer cells[78,82]. Some peptides are responsible to induce apoptosis in such cells, which makes them potential targets of treatment. Necrosis is another relevant natural cell death pathway, though not programmed. Necrosis involves the degeneration of cell cytoplasm, causing the release of cytoplasmic contents[78,83,84]. Apoptosis and necrosis can be originated by physical traumas to the cells or pathological cell damages[85,86].

In the human body are generated radicals called reactive oxygen species (ROS), such as the superoxide anion (O^-_2), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) which are derived from oxygen metabolism and help to balance the oxidative homeostasis in normal cells[87,88]. Therefore, depending on its levels, ROS can either be beneficial for cell functioning or harmful[81,89].

Free radicals and ROS produced in normal cell metabolism are beneficial (at reasonable concentrations) because of the regulation of intracellular redox signalling[90–92]. Some ROS can even induce the production of anticancer molecules like phenethylisothiocyanate, which promote pathways committed to the selective killing of cancer cells[81]. However, if the antioxidant systems are inhibited and/or an excess of ROS is produced in response to oxidative stress scenarios, then a loss of cellular functions and even apoptosis may take place[93]. Pancreatic cancer cells create medium levels of ROS to operate in their proliferation, migration and metastasis. Nevertheless, if ROS levels are too high it can promote PDAC cell death, because they create an adverse environment[81,94–96].

Some natural compounds influence apoptosis by interacting with different signalling pathways targets, like transcription factors, protein kinases, angiogenesis, and inflammatory cytokines that are often unbalanced in cancer stages[81]. Available studies confirm that using Nimbolide (a plant secondary metabolite) to induce overexpressed levels of ROS could inhibit pancreatic cancer proliferation and migratory capacity through the regulation of apoptosis and autophagy[15]. Besides natural compounds, we possess our own proteins that function like tumour suppressor p53 that is a known example of protein responsible for protecting genome stability and have a very important role in the control of cell cycle, apoptosis, maintenance of genomic integrity and DNA repair in response to cell stress[97,98]. When p53 is activated it attaches to regulatory molecules that enhances the expression of specific genes leading cells response against tumour formation[9].

1.4- Pancreatic cancer therapeutics

Surgery remains the most used method for controlling pancreatic cancer in an attempt of therapeutic cure, because there is still no effective treatment and the available disease screening/diagnostic methods are not robust enough to detect pancreatic cancer at a more curative stage[78,99]. Notwithstanding, in some cases PDAC surgery may be supported by adjuvant therapies that usually include chemotherapeutic treatments relying on the administration of *e.g.*, gemcitabine or 5-fluorouracil (5-FU). However, some studies were explored the effect of combining these drugs with other chemotherapeutics like capecitabine, pemetrexed, erlotinib, irinotecan, exatecan, and/or targeted therapies[78,100–102], though they revealed not to be effective yet.

One of the major problems tailoring therapeutic effectiveness is the different cancer phenotypes, which may develop resistance to anticancer compounds or even to pro-apoptotic effects. Therefore, it is important to have this into consideration, and gather a robust knowledge on PDAC phenotypes of each tumour for applying adjusted and more precise therapeutics[103].

In this context, targeted therapies (*e.g.*, hormone therapy, immunotherapy) have been the target of many studies for that purpose as already referred, though it is yet on its infancy. A variety of bioactive peptides capable of modulating immune responses can be explored as new anticancer drugs and used towards cancer immunotherapy[103]. Those peptides can provide many advantages relatively to conventional chemotherapeutics, such as high selectivity, favourable pharmacokinetic action, easy to access tumor sites, generally low toxicity and immunogenicity. although they have a weakness due to their limited stability and difficulties in penetrating cell membranes[103].

Bacterially-based therapies have also been raising interest among the biomedical scientific community. Some bacteria like *Salmonella typhimurium* A1-R have been explored to help suppress tumour growth. The action of bacteria does not involve exerting a direct cytotoxic effect on tumour cells, but instead inducing the expression of anticancer agents through chemotaxis[104]. Notwithstanding, *Salmonella* may induce negative side-effects due to infection development[105]. Thereby, major challenges should be addressed regarding toxicity, ability to specifically reach the tumour site and attain regions not easily reachable by current treatments[105], bacteria proliferation in tumour microenvironment and capacity to be genetically engineered[105]. Anyway, bacteria are offering and opening new therapeutic possibilities, which have been also associated with their enormous potential of biosynthesis of bioactive secondary metabolites or natural products. The combination of these bacterially-based therapies with chemotherapeutic agents can indeed contributes to a new era of alternative PDAC treatments in the future.

2- Search for new anti-PDAC natural products

Nowadays, novel compounds have been urgently claimed by clinics in order to overcome the limitations of current PDAC therapeutics, which range from toxicological side-effects, low specificity and efficacy, to multidrug resistance[105]. As such, focusing the discovery of new drugs on natural resources has been encouraged again, particularly by the pharmaceutical industry, given the need to find novel chemical structures with enhanced anticancer activity, more easily absorbed, stable, less toxic and highly tumour-specific. In a chemical perspective, natural products

are broadly considered as molecules with varied structures and molecular weights that belong to different chemical classes (*e.g.*, peptides, fatty acids, nucleosides) and may present, besides anticancer activity, antifungal, antiviral, antibiotic, anti-inflammatory and/or antioxidant activities[106]. Given their variety and range of potential applications, the number of studies devoted to the search of bioactive natural products synthesized by living organisms from different environmental sources has been increasingly rising in the last decades[80]. The aquatic[107] or terrestrial environments[108] are two major compartments targeted for searching natural compounds. In particular, ecosystems characterized by extreme conditions of temperature, pH, pressure, oxygen availability, nutrient loads and contamination profiles[109], or even under-explored environments (*e.g.*, the marine environment[108]) may offer great pharmaceutical opportunities. This is because the organisms inhabiting such extreme environments developed genetic, metabolic and physiological adaptations to withstand harsh conditions. Thereby, different organisms have been explored for their potential to synthesize bioactive molecules through a secondary metabolism that often results from that evolutionary selection or adaptation[110]. Although the ecological role of the secondary metabolites may not be thoroughly disclosed or known for many organisms, it has been referred their role on organisms' responses as to endure and proliferate under stressful conditions (*e.g.*, acidic pH, chemical contamination)[111] and species interrelations (*e.g.*, interspecies competition, quorum sensing[111], microbial pathogens)[112,113].

Among the organisms producing bioactive compounds with anti-PDAC activity, plants have been demonstrating a great potential. Curcumin, which was isolated from a turmeric plant, has antitumor, antioxidant and anti-inflammatory properties[112]. The anticancer properties of curcumin are derived from its capacity to block the transcriptional nuclear factor kappa beta (NFkb), which is a cancer cell regulator in inflammatory, proliferation, apoptosis and multidrug resistance[114]. In fact, a curcumin analogue, GO-Y030, has been found to be even more active against STAT3[115], a signal transducer and activator of transcription 3 that is associated with promotion of metastasis in PDAC[115]. Capsaicin, which is the main compound of chilli pepper plants, has been used to fight multidrug resistance. Capsaicin targets Trx-ASK1 (apoptosis signal-regulating kinase) to induce apoptosis in BxPC-3 cells, a line of pancreatic adenocarcinoma cells[106]. Mogroside V was another compound isolated from a Chinese plant that inhibits the survival of PANC-1 cells, leading to apoptosis by blocking cell division at the G0/G1 phase[116]. It also upregulates genes like *CDKN1A* (that is controlled by p53) and *CDKN1B* functioning as cyclin kinase inhibitors and tumour suppressors. It is suggested that Mogroside V can indeed control PDAC growth by angiogenesis inhibition through the VEGF (vascular endothelial growth factor)-

dependent mechanism[116]. In fact, since Mogroside V can be daily consumed as a food sweetener, it may be used as a preventive natural treatment against PDAC[116].

Great part of the work developed recently has also been directed to get further knowledge on the secondary metabolites produced by bacteria, mainly from Actinobacteria phylum[117]. The biosynthesis of natural products by bacteria is sustained by different enzymes, being the most known, the polyketide synthases (PKS)[118] and nonribosomal peptide synthetases (NRPS)[119]. These multifunctional and modular enzymes mediate the production of polyketides (PKs) and nonribossomal peptides (NRPs) compounds[120]. For more details on these biosynthetic pathways and genetic machinery, please see Chapter II.

2.1- *Marine sources of natural compounds*

The marine ecosystem corresponds to 95% of the Earth biosphere and has been pointed out as a great provider of multiple sources of bioactive natural compounds[121,122] Among the marine organisms, bacteria, cyanobacteria, archaea, fungi, micro- and macroalgae, and even invertebrates have been explored for the extraction and purification of natural compounds that exhibit several properties with significant biological activities, such as anticancer, anti-inflammatory, antiviral, antibacterial and anticoagulant[112,123]. Overall, despite some of the marine natural products discovered so far present anti-PDAC activity, the potential of marine ecosystems to provide additional drug options is far from being deeply known or covered.

2.1.1- Bacteria and Archaea

Among the marine microbes, representatives of Archaea and Eubacteria kingdoms have been investigated for new and diverse metabolites with bioactivity[112]. Although these microbial groups are geographically ubiquitous, Archaea are often identified in extreme environmental conditions like in deep sea and thermal vents[112]. But bacteria can also withstand a wide range of environments and hostile conditions[110]. Given their adaptations, marine bacteria evolved abilities associated with the production of many secondary metabolites. Different peptides synthesized by bacteria revealed to be promising compounds for medical applications, for example, lasso peptides[124], bacteriocins like microcins, colicins and lantibiotics[124], and nonribosomal peptides like cyanobacterial peptides[112]. Cyanobacteria were found to produce anabaena peptides (from *Anabaena sp.*), cyanopeptolins (a class of oligopeptides produced by *Microcystis*), aeruginosins

(protease inhibitors produced by *Microcystis aeruginosa*) and microviridins (serine protease inhibitors produced by various genera of cyanobacteria)[112]**.** Other type of bacteria, Myxobacteria, have a complex life cycle and are proved to be a useful source of natural compounds with huge potential for new drugs[125]. In marine Actinobacteria, the genus *Streptomyces* produces almost 80% of the natural compounds of Actinomycete[126]. *Streptomyces* species are producers of compounds with some properties like antifungal (*e.g.*, natamycin), antibacterial (*e.g.*, streptomycin), and antiparasitic (*e.g.*, ivermectin) activities[194-196]. Against PDAC, was particularly found capsimycins derived from *Streptomyces xiamenensis* that induced cytotoxicity against several pancreatic cancer cell lines[129].

2.1.2- Fungi

Marine fungi as *Acremonium*, *Aspergillus*, *Fusarium*, *Epicoccum* and others genera[130] often live as symbionts of marine algae or invertebrates[112,123], evidencing the ability to synthesize several compound like PKs, alkaloids[112,131], sesquiterpenes[112], and aromatic compounds[23,132]. These natural products were discovered to induce cytotoxic, neuroactive, antibacterial, antiviral and antifungal activities[131,133]. *Penicillium chrysogenum* is a penicillin producer, whilst *Penincillium griseofulvum* is a producer of griseofulvin (for antifungal treatment) and are the most studied biosynthetic fungi[130]. Another marine fungi representative of the genus *Trichoderma* are also producers of several different bioactive metabolites, like the antimycobacterial aminolipoptide trichoderins[134] and the antifungal trichodermaketone A[135]. Varioxepine is a recently discovered alkaloid isolated from the endophytic fungus *Paecilomyces variotii* (lives in association with an algae) that has activity against the phyto-pathogenic fungus *Fusarium graminearum*[136].

2.1.3- Algae

Algae are photosynthetic organisms, and major producers of biomass and organic products in the marine environment[112]. Marine microalgae include five groups, Chlorophyta, Chrysophyta, Pyrrhophyta, Euglenophyta and Cyanophyta[112]. Microalgae are considered a great source of natural bioactive compounds, namely carotenoids, which constitute several food products[23,137]. Carotenoids produced by microalgae can act on oxidative stress by diminishing free radicals and ROS, which have been pointed out as drivers of many human diseases[88], such as Alzheimer, Parkinson and other neurological diseases, as well as cancer, among others[88]. β-

carotene from *Dunaliella salina* is a well-known carotenoid produced by this marine microalgae, with antioxidant properties and hence used to remove free radicals[88].

Marine macroalgae can normally be utilized as vegetables, medicines, fertilizers, and raw materials in different industries, given their anticancer, anticoagulant, and nutritional properties[112]. Natural products from these macro organisms include polysaccharides (*e.g.*, from Rhodophyta), which can present anticoagulant properties by interfering with fibrin polymerization, what is usually essential for blood coagulation[112]. Included in the polysaccharides studied from macroalgae, fucoidans (from brown algae), carrageenan (from red algae) and ulvan (from green algae) can have significant roles in cancer control and have been investigated for that purpose[138].

2.1.4- Invertebrates and their symbionts

Among the marine invertebrates, tunicates (phylum Chordata), cnidarians (phylum Cnidaria), and especially sponges (phylum Porifera)[113] may produce a diverse classes of compounds, like terpenes, sterols, fatty acids, cyclic peptides, amino acid derivatives and alkaloids[23]. Some of the invertebrate-derived marine drugs have already been approved or are in tests phase. For example, aplidine (approved in March 2018) is a depsipeptide originally isolated from de tunicate *Aplidium albicans*[121] that possess anticancer activity but it was removed from market in December 14th 2018 for its lack of activity range. Jorumycin is an alkaloid from the mollusc *Jorunna funebris* and has been applied in schizophrenia cases[107]. The marine sponge *Oceanapia* sp. produces the antibacterial C14 acetylenic acid that is a fatty acid and is effective against *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtillis and Staphyloccocus aureus*[132]. From a sponge of the genus *Leiodermatium* was extracted leiodermatolide, a polyketide with antibiotic capacity that exhibits cytotoxicity against several pancreatic cancer cell lines, like AsPC-1, PANC-1, BxPC-3 and MIA PaCa-2[139].

When it was realized that marine invertebrates harbour many bacterial symbionts, the source of bioactive compounds was thought to be also of bacterial origin. Tetrodotoxin is capable to treat neuropathic pain and is in phase III trials[140]. Nojirimycin, originally isolated from the sponge symbionts *Streptomyces roseochromogenes* and *Streptomyces lavendulae*, is a natural product abundantly used to fight type II diabetes[141]. *Bacillus tequilensis* isolated from the sponge *Sarcotragus fasciculatus* has β-glucosidase inhibitors also with anti-diabetic potential[142]. *Pseudomonas piscicida* from the sponge *Hymeniacidon perleve* has Norharman (from β-carbolins compound class) with anti-microbial activity[143]. Some compounds with anti-tumorigenic potential

like Yondelis® (drug approved in 2007 in Europe by the European Medicines Agency) can be used in the treatment of ovarian and breast cancers, melanomas, soft tissue sarcomas, being extracted from bacterial symbionts of marine invertebrates[144].

Similarly to sponges, the cnidarians are also quite distributed in seas and oceans worldwide[145,146]. They possess specialized cells (cnidocytes) that are used to kill preys for feeding purposes, as well as to guarantee their defence and movement[147]. The phylum Cnidaria is divided into Anthozoa that includes sea anemones and corals, and Medusozoa, which includes classes like Staurozoa, Hydrozoa, Schyphozoa and Cubozoa[112]. Hydrozoan species can host bacteria as part of their tissues[148]. The symbiotic bacterial community is specific, for example, of each *Hydra* species (a well-known hydrozoan) and their endoderm is structured as to fight bacterial pathogens through the production of antimicrobial peptides, thereby constituting an alternative for the absence of a physical barrier[149,150]. In extracts of *Hydra*, Hydramacin-1 is a peptide obtained with high antimicrobial activity[145]. This peptide seems to be enhanced by other microbial products, like lipopolysaccharides (LPS), which have antibacterial activity against *Bacillus megaterium, Escherichia coli*, *Klebsiella oxytoca* and *Klebsiella pneumoniae* strains, but barely shows activity against *Pseudomonas aeruginosa*[145]. Regarding medusozoans, jellyfish like *Pelagia notiluca* have also been approached in some studies focusing on the isolation of compounds[151]. Moreover, a symbiont isolated from the jellyfish *Cyanea capillata* belonging to *Pseudomonas* sp. genus, could synthesize the antimicrobial protein CAP-1 that is highly inhibitory of the growth of marine pathogenic microorganisms like *Vibrio vulnificus*[152]. CAP-1, however, is not cytotoxic to human tumoural and normal cells of the skin[152]. Notwithstanding, this bioactive potential should be further analysed against other targets and applications.

3- Objectives and structure

This work had two major objectives. One of them intended to perform the state-of-the-art regarding pancreatic cancer, as well as its main therapeutic approaches and gaps that can be potentially fulfilled by the discovery of new bioactive natural compounds produced through specific biosynthetic pathways, especially occurring in living resources of the marine environment. The other aim is to unravel the potential of bacterial symbionts isolated from marine cnidarians to synthesize bioactive compounds against PDAC. In order to accomplish these goals, the following specific objectives were established:

● to make a literature review on pancreatic cancer etiology, epidemiology, diagnosis, biology, carcinogenesis and current treatments, as well as on the discovery of novel natural products, especially from the marine environment;

● to summarize the biosynthetic pathways sustained by NRPS and PKS, and the bacterially-synthesized NRPs and PKs natural compounds;

● to screen the potential bioactivity of bacteria isolated from cnidarians sampled in the Atlantic Ocean, through the application of a molecular approach and testing of crude extracts against a pancreatic ductal adenocarcinoma cell line.

This document is thereby structured in chapters that are lined with these objectives, being some of the chapters organized in a paper-directed format:

● Chapter I: General Introduction

● Chapter II: NRPS, PKS and hybrid genes – structure, derived marine natural compounds, and genetic engineering

● Chapter III: Screening of marine bacteria for the potential biosynthesis of NRPs bioactive compounds against pancreatic cancer

● Chapter IV: Final Considerations

4- Scientific outputs during the MSc research

Poster - Scientific poster entitled as "Potential of marine bacteria to synthesize bioactive compounds for medical application". Authors: Mafalda Tabuada, Carlos Moura, Amadeu M.V.M. Soares, Catarina R. Marques. Poster presented at the IV Post-Grad Symposium in Biomedicine, ibiMed, UA. p. 59.

Review/ research articles:

Chapter I and II- A review paper will be prepared from the state-of-the art and literature search performed for the topics focused in chapters I and II.

Chapter III- An original research paper will be submitted to the international peer-reviewed scientific journal *Research in Microbiology*, being the title: "Screening of marine bacteria for the potential biosynthesis of NRPS bioactive compounds".

Chapter II

NRPS, PKS and hybrid genes – structure, derived marine natural compounds, and genetic engineering

Abstract

Nonribosomal peptides (NRPS), polyketide synthases (PKS) and NRPS/PKS hybrids are genes that became known for encoding huge and complex modular enzymes involved in the microbial synthesis of secondary metabolites. These secondary metabolites are natural products with important ecological and biological activities that may serve a broad use for medical and industrial purposes. According to their bioactivity or properties they may be antibiotics, siderophores, surfactants, anticancer substances, immunosuppressive and anti-inflammatory compounds. Given the relevance of these genes, the present chapter focus on PKS, NRPS and hybrids structure and biosynthesis, their occurrence in marine microbes, mediation of the biosynthesis of marine natural products, and on the main advancements with regard to the genetic engineering of those modular enzymes as producers of natural compounds derived from marine bacteria.

Key-words: enzymes, nonribossomal peptides, polyketides, bioactive compounds, marine bacteria

Structure and biosynthesis of NRPS and PKS enzymes

The nonribosomal peptide synthetases (NRPS) and polyketides synthases (PKS) are multimodular enzymes with remarkable structural diversity that sustain the biosynthesis of many secondary metabolites or natural products, broadly referred as nonribosomal peptides (NRPs) and polyketides (PKs), respectively. The NRPs- and PKs-encoding genes and genetic machinery are hence frequently found in the three domains of life represented by Bacteria, Archaea and Eukarya[153].

Overall, their biosynthetic pathways rely on sequential metabolic steps mediated by each module of NRPS or PKS enzymes, which resulting organization of the monomers in the compounds produced depend on the organization of the modules composing the enzymes[154]. The chemical structural unit (or monomers) incorporated by NRPS and PKS modules is an aminoacid (for peptide synthesis) or a carboxylic acid (*e.g.*, for fatty acid synthesis where monomers are acyl-CoA), respectively[155]. The great variability and arrangement of the enzymes modules and, consequently, of the monomers and their combinations, definitely contribute to the wide diversity of NRPs and PKs compounds, in terms of size and chemical structure[120]. Indeed, such diverse chemical structures of NRPS- and PKS-based products, coupled to the possibility of interacting with different (bio)molecular targets/receptors, provides a wide range of bioactivities relevant for different applications[156]. A major relevance of these compounds is that they may present a variety of biological activities with high pharmaceutical interest[157], besides other properties relevant for other applications[158].

The number of NRPS modules (or catalytic units) depend on the number of aminoacids to be synthesized, but each one can be divided into three major domains: (i) the adenylation (A) domain with properties that allow the identification and activation of certain amino acid residues which are then transported to the second domain; (ii) the peptidyl carrier protein (PCP) (or thiolation domain); (iii) the condensation domain (C) that catalyzes the formation of C-N bonds for the binding of the aminoacid to the elongated oligopeptide, and transport this oligopeptide to the next module[158–160] (Fig. 1a). The termination module contains a thioesterase (TE) domain responsible for the release of the peptide/protein via hydrolysis or cyclization[118,119,154,161,162]. Additional domains can be present in each module of NRPS enzymes that catalyse aminoacids epimerization, reduction, oxidation, methylation (Fig. 1a). As such, each module add one monomer to the elongated oligopeptide chain, until being synthesized the final peptide/protein (Fig. 1a).

Although NRPS is normally referred as modular enzymes, 10% of bacteria gene clusters were found to codify nonmodular enzymes, what was associated with an evolutionary trend mediated by horizontal gene transfer[153]. Nonmodular enzymes are not organized in modules, but in catalytic domains which codify separate proteins. Nonmodular NRPS occur in biosynthetic pathways of siderophores, such as those peptides synthesized from adenylation (EntE, VibE) or condensation (VibH) domains respectively in enterobactin and vibriobactin biosynthetic pathways[163,164]. Natural products synthesized by NRPS can be arranged according to their biological activities. Different NRPS-derived products have antibiotic and antifungal activities, like tyrocidine, bacitracin, surfactin, vancomycin, fengycin, telomycin, griselimycin, nannocystin A[165] (Table 1). Lipopeptides are amphiphilic compounds, such as the surfactin produced by *Bacillus subtilis*, which can easily penetrate the microbial cell membrane and cause its disruption, thereby facilitating its use as an antimicrobial[166]. Cyclic peptides like nannocystin A have a huge antiproliferative and antitumor activity, targeting the translation factor 1 of eukaryotic cells[167]. The cyclic depsipeptide griselimycin was proved to possess strong activity against *Mycobacterium tuberculosis*[168–170].

PKSs are subdivided into three groups, type I, type II and type III, according to the structure of the modular enzyme. Type I PKS modular enzymes, however, are the most known and represented in the microbial secondary metabolism[171]. They consist of multi-enzyme complexes derived from various functional catalytic units or domains in each module (as explained above)[158]. Type I-PKS can yet be divided into interactive (one sole organized module that is iteratively synthesized until the final peptide; *e.g.*, erythromycin[172]) or modular (different modules, *i.e.*, each module corresponding to different set of domains are subsequently synthesized up to the final peptide; *e.g.*, lovastatin[172]) enzymes (Fig. 1b). Similarly to NRPS, the type I PKS modules contain three domains: (i) acetyltransferase (AT); (ii) ketosynthase (KS); and (iii) acyl carrier protein (ACP). These domains are essential for PKS elongation. The AT domain adds the monomers malonyl or methylmalonyl-CoA. The KS unit is responsible for the C-C binding and linkage of the building blocks to the oligopeptide chain. The ACP domain is similar to the PCP domain in NRPS, and works as a support for the synthesis of PKS by promoting a covalent binding of the elongated chain of monomers[158] (Fig. 1b). Alternative domains suchlike ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), and methyltransferase (MT) may also be organized into PKS modules[173] (Fig. 1b). Type I PKSs are responsible for the biosynthesis of complex and short polyketides like macrolides, polyether and polyenes[169,170,174] (Table 1). The Type II PKS are mono- or bifunctional enzymes composing a stable complex[175]. They have a unique characteristic that is

their high amino acid sequence homology and highly conserved gene sequences[176]. This type of PKSs in bacteria produce aromatic polyketides, like oxytetracycline and pradimicin[159]. Type III PKS are small homodimer enzymes that have been more associated with the secondary metabolism of plants, though also identified in some bacteria. Among the compounds they produce there are chalcones, pyrones, acridones, phloroglucinols, stilbenes, and resorcinolic lipids[157].

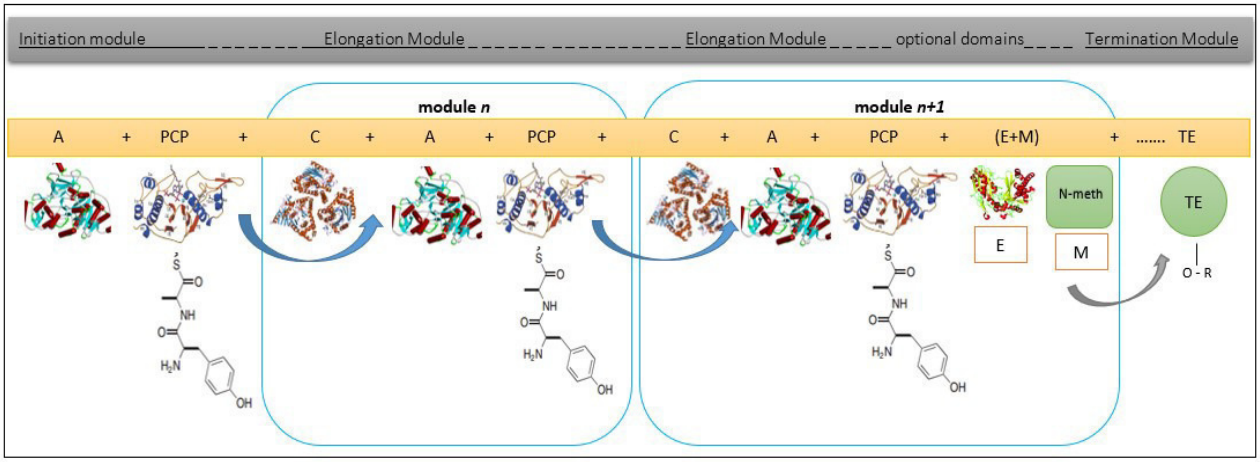

Fig.1a: Example of the modular organization in NRPS, with adenylation (A), peptidyl carrier protein (PCP), epimerization (E), N-methylation (M) and termination (TE) domains[118,119,154,161,162]

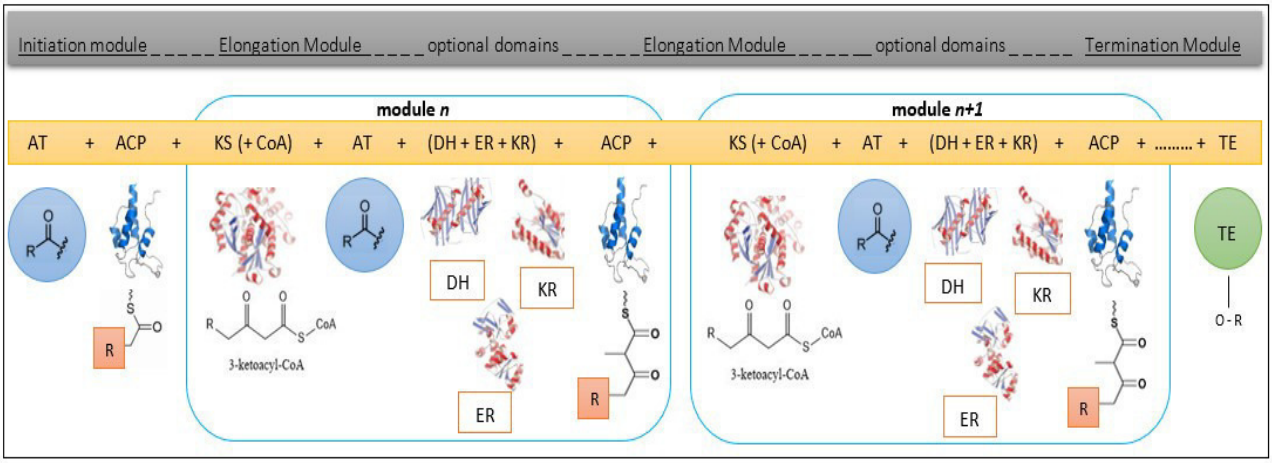

Figure 1b: Example of the modular organization of type I PKS modules, with adenyl-transferase (AT), ayl carrier protein (ACP), ketosynthase (KS), dehydratase (DH), enoylreductase (ER), ketoreductase (KR) and termination (TE) domains[173].

The combination of PKS and NRPS in a biosynthetic cluster, leads to the synthesis of PK/NRP hybrids. This type of compounds can be broadly divided into two classes according to their synthesis. One includes the hybrids that are separately synthesized by NRPS and PKS enzymes, and afterwards combined into a final hybrid compound. The other class involves the

peptide-polyketide products that are directly synthesized from the functionally combined NRPS and PKS enzymes[177] (Table 1).

Table 1: Examples of bioactive compounds associated with NRPS, PKS and NRPS/PKS hybrids.

Enzymes		Examples of compounds	Chemical class/structure	Activities	References
NRPS		Surfactin	Cyclic lipopeptide	Antibiotic, antifungal, antiviral, hemolytic	178,179
		Tyrocidine	Cyclic decapeptides	Anticancer	120,179
		Vancomycin	Glycosylated peptide	Antibiotic	180
		Fengycin	Lipopetide	Antibiotic	160
		Bacitracin	Cyclic peptide	Antibiotic, antifungal	181
		Telomycin	Lipopetide	Antibiotic	182
		Nannocystin A	Macrolactone peptide	Anticancer	183,184
		Griselimycin	Cyclic peptide	Antibiotic	151
PKS	type I	Erythromycin	Macrolides	Antibiotic, antifungal, anticancer	172,185,186
		Nanchangmycin, monensin, nigericin, tetronomycin	Polyethers	Antifungal, phytotoxic	160,187
		Rapamycin	Polyenes	Antifungal, anticancer, immunosuppressive	172
	type II	Lipopetide biosurfactants	Biosurfactants	Antimicrobial, antibiofilm	163
		Glicopeptide biosurfactants		Antimicrobial	165
		Various polyketides	Aromatic polyketides	Anti-inflammatory, antioxidant	166,188
	type III	C_{21} heptaketide	Chalcones	Antibiotic	157,189
		Salinipyrone A	Pyrones	Anticancer	108
		Triazoleacridone	Acridones	Anticancer	157,190
		C_{18} heptaketide	Phloroglucinol	Antithrombotic, profibrinolytic, anticoagulant	157,191
NRPS/PKS hybrids		Equisetin, Pseurotin A	Tetramic acids	Antifungal, neurogenic	175,176,192

Marine environment as a pool of PKS/NRPS genes

Marine natural products have been isolated and identified from different marine organisms like macroalgae, sponges, tunicates[143]. However, several studies have been focusing on the potential of microbial symbionts that live in association with marine invertebrates towards the production of bioactive secondary metabolites[193]. One of the strategies followed for the discovery of new natural products is based on the culture of microorganisms and screening of their bioactive potential. But with the onset of next generation (sequencing) tools together with the revolution of bioinformatics tools, other frameworks have been conducted, which reveal a diversity of silent gene clusters in marine microbes that potentially encode a tremendous range of bioactive metabolites[159] These molecular- and genetic-based technologies help to overcome

culture difficulties, while simultaneously help to uncover the hidden biosynthetic potential in marine (microbial) resources. Notwithstanding the natural synthesis and isolation of the compounds are limited or compromised if the microorganisms are uncultured. As such, other studies performed the bioprospection of microbes biosynthetic abilities through a preliminary molecular screening, before going into the application of compounds extraction procedures[153]. For instance, sequencing and genome mining techniques has been used to identify biosynthetic gene clusters encoding NRPS, PKS and NRPS-PKS hybrids in the genomes of marine bacteria, such as Gram-negative representatives of the Alpha- and Gammaproteobacteria clades[194]. *Vibrio, Roseobacter* and *Pseudoalteromonas* evidenced several bioactive metabolites capable of inducing chitinolytic, iron quenching (siderophore production) and/or quorum sensing properties[111,195] Brito et al.[155], started by doing a molecular screening of the genes encoding NRPS and PKS enzymes in marine cyanobacteria isolated from the Portuguese coastline (Atlantic Ocean). The authors found a greater presence of PKS genes than NRPS. Moreover, some cyanobacteria could synthesize natural compounds (analogues of known and unknown compounds; *e.g.*, antanapeptin C, dolastatin 16, malyngamides cluster, and cyanotoxins as nodularin and microcystin) under laboratorial conditions, as determined by LC-MS and molecular networking. The metabolite barbamide was also isolated from a marine cyanobacteria (*Lyngbya majuscula*), which biosynthetic gene cluster (*bar*) encodes a hybrid PKS-NRPS enzymatic system[184,198,199]. Furthermore, it encompasses unusual aspects, specifically, the A and PCP domains of NRPS for leucine/trichloro leucine are encoded in separated open reading frames[198], and the PKS module of this cluster is split into two proteins (BarE/BarF)[200]. Although there is a co-linear arrangement of PKS-NRPS systems, the produced natural compounds are constrained by complex protein-protein interactions[185]. NRPS and PKS genes in marine microbes have been frequently detected in bacterial representatives of Proteobacteria, Actinobacteria, Firmicutes and Cyanobacteria, and in Ascomycota fungi[153]. Marine Actinobacteria, in particular, enclose a vast pool of unexplored bioactive products. For instance, *Salinispora tropica* evidenced a complex metabolome of secondary metabolites[201]. Later on, it was concluded that it can produce natural compounds (salinosporamide A and B) through a hybrid PKS-NRPS pathway using the building blocks acetate, β-hydroxy-2'-cyclohexenylalanine, and either butyrate or a tetrose-derived chlorinated molecule. These compounds present various activities, namely anti-cancer activity[196,197,202,203].

Biotechnological applications and genetic engineering of PKS/NRPS

Although the culture of bacteria for natural products bioprospection is a valuable biotechnological avenue, it may bring some limitations if their production at large scale is intended. One major limitation concerns bacteria domestication, which may gradually lead to the reduction of NRPs and PKs gene expression and synthesis, as far as laboratorial culture conditions fail to reproduce environmental conditions that promote the expression of an adaptive secondary metabolism[109]. Furthermore, the chemical structures of naturally-obtained compounds may not be biocompatible or provide a stable activity, especially if a pharmacological application is required. However, the determination of NRPS, PKS and hybrids genetic diversity and machinery, the chemical structure of the assembled compounds, mechanistic/enzymatic pathways, and networks, can provide information for the generation of new chemicals, through the application of genetic engineering tools[118,204].

The engineering of PKS-NRPS hybrids may take advantage of the natural diverse chemical structures/compositions of the compounds, in order to produce new customized chemicals that may be difficult to obtain through artificial chemical synthesis[205–207]. NRPS can be indeed engineered to achieve optimized natural products with enhanced bioactivity, and help with pharmaceutical needs to fight different diseases[204]. As such, NRPS gene clusters were already re-designed. A first attempt was made on surfactin synthetase, being the natural A-PCP domains (responsible for leucine addition) replaced by others of bacterial and fungal origin[208]. The haemolytic capacity of the altered surfactin was kept, though peptide production was not greatly increased. The discovery of gene sequences encoding a link (composed by aminoacids with no particular function) between NRPS domains allowed their exploitation for engineering enzymatic fusions without losing the integrity/activity of enzymes[209]. Also, the engineering of NRPS A domain-specificity by the introduction of site-mutations of substrate amino acids, provides an option for the manipulation of NRPS biosynthetic clusters towards the creation of new antibiotics[126].

Another strategy that has been exploited for the controlled biosynthesis of new compounds relies on the heterologous expression of NRPS, PKS or NRPS-PKS hybrid gene clusters, either identified via metagenome analysis or previously engineered[209], by bacterial vectors like *Streptomyces*, which genetic machinery may be tailored to overexpress and produce the compounds[210]. *Streptomyces toyocaensis* NRRL 15009, which is the producer of the non-glycosylated "glycopeptide" A47934, is an expression vector capable of synthesizing hybrid glycopeptide antibiotics[211,212]. Genome mining of this species revealed its reduced size, and the

presence of only four NRPS, one type II PKS and none type I PKS gene clusters. These genetic features highlight the value of *S. toyocaensis* NRRL 15009 for the heterologous expression of cryptic NRPS gene clusters[213]. Awakawa et al.[185] also re-designed the biosynthesis of modular NRPS/PKS hybrids producing the antimycins JBIR-06 (tri-lactone) and neoantimycin (tetra-lactone) through heterologous expression, hence resulting in novel antimycin analogues. These approaches mostly consider the modular organization and structure of the chemicals synthesized by particular Actinomycetes, but it requires their validation in other bacterial systems (*e.g.*, marine-derived species) and their respective NRPS/PKS assemblies[193]. Other example was the engineering of *Streptomyces avermitilis* (synthesizes avermectin) and *Saccharopolyspora erythraea* (synthesizes erythromycin) PKSs to produce spinosyn derivatives. The specificities of erythromycin and avermectin loading modules allowed the generation of hybrid PKs, which once replaced by the spinosyn loading module lead to the production of novel spinosyn analogues with new and/or enhanced activity for insect control[202,214]. Hence the modular organization of erythromycin and avermectin PKS provided the means to employ genetic engineering and custom the chemical structures of their products in a targeted way.

Thus, the success of biosynthetic engineering approaches in the production of a wide range of novel compounds has been accomplished. Therefore, the creation of optimized novel compounds, not known in nature, is likely to soon assume a breakthrough in biotechnology and a major relevance in clinics, as well as other sectors (*e.g.*, industry).

Conclusions

The complex modular enzymes PKS, NRPS and PKS-NRPS hybrids are ubiquitously and diversely catalysing the synthesis of natural polyketides, peptides or polyketide-peptide compounds, respectively, by microbes from different environments. The marine environment, however, is believed to harbour a large pool of yet unknown biosynthetic microorganisms. As such, a great effort has been lately devoted to the search of novel marine compounds that could help with the lack of therapeutics for different diseases (*e.g.*, bacterial infections, cancer) or drugs to control multi-resistant bacteria, as well as with the need of compounds to serve different industrial, agricultural or environmental processes/services. As the laboratorial culture of microbes may present several limitations to mimic the environmental traits stimulating the production of PKs, NRPs and hybrid compounds, new approaches have been searching towards their large and controlled production for different (bio)technological applications. In this context,

genetic engineering together with next-generation sequencing and bioinformatics tools is contributing to the re-design of the naturally-occurring enzymes/compounds and biosynthetic pathways. Consequently, new compounds or analogues have been generated with optimized structures, stability and enhanced activities, tailoring new opportunities for supplying environmental, technological and social requirements.

Chapter III

Screening of marine bacteria for the potential biosynthesis of NRPs bioactive compounds against pancreatic cancer

Abstract

The high malignancy of pancreatic cancer together with the severe limitations of the currently available treatments, namely involving drug resistance and inefficacy, the search for new anticancer drugs from natural sources has been claimed. Natural products that can modulate multidrug resistance have characteristics such as lower toxicity due to potentially high tumour selectivity, which may offer attractive opportunities. Therefore, the aim of this work is to apply a molecular approach to screen for the biosynthetic genetic potential, as well as, the bioactivity of crude extracts of marine bacterial symbionts isolated from cnidarians. Among the strains tested, in 39 was amplified a conserved region of the nonribosomal peptide synthetase (NRPS) A domaingenes. These bacteria were mainly assigned to the Proteobacteria and Actinobacteria phyla. Fourteen were selected given their ease of culture and the success on NRPS gene fragments amplification. The greatest part of them proved to harbour genes of NRPS biosynthetic cluster with high homology, and the crude extracts (cell and cell-free fractions) were generally cytotoxic to Capan-1 human pancreatic ductal adenocarcinoma cell line, as indicated by the MTT assay. The results were promising and gave the confirmation of association between the presence of NRPS in marine bacteria and potential production of bioactive compounds with anti-cancer activity.

Key-words: molecular screening, biosynthetic gene, crude extracts, bacterial symbionts, cnidarians, pancreatic ductal adenocarcinoma, MTT assay

Introduction

Although pancreatic ductal adenocarcinoma (PDAC) is ranked in the 7th position among the deadly cancers in the world[1], it is a quite threatening cancer given its silent and quick progression and metastization, low responsiveness to current therapeutics, high probability of recurrence after surgery, and a great ability to develop multidrug resistance. In order to cope with these clinical hurdles, some effort has been put towards the discovery of new drugs to fight PDAC.

Natural products synthesized by living organisms can present anticancer bioactivity and simultaneously be less toxic than conventional chemotherapeutic drugs (*e.g.*, gemcitabine), if

highly specific for targeting the tumour site. The testing of natural compounds against PDAC has been increasing, and several reports have been highlighting specific compounds capable of being used as potential adjuvant therapeutic drugs (*e.g.*, curcumin and capsaicin)[105,115] resents a thorough review on the natural compounds that have been tested in combination with current chemotherapeutic pharmaceuticals in preclinical and clinical trials, with the aim of controlling PDAC progression.

Microbes, and bacteria in particular, can be promising producers of anticancer natural compounds, once many of them evolved a secondary metabolism to sustain different biological and eco-physiological adaptations, which lead to the formation of metabolites with peculiar bioactivities with interest for medical applications, like cancer treatment[215]. Under this context, marine bacteria and bacterial symbionts living in association with invertebrates are major sources of natural compounds, evidencing a widely unknown resistome[205] and metabolome[217]. Hence, the sequenced environmental microbial genomes so far had revealed that a potential diversity of novel bioactive compounds exists[207,218,219], what broadens the horizons regarding new compounds discovery. among the chemical classes of compounds lately explored, nonribsomal peptides (NRPs) synthesized through the action of modular and multifunctional enzymes broadly named as nonribsomal peptides synthetases (NRPS), have been a great opportunity for the development of novel anticancer medicines, like bleomycin in preclinical trials and primarily isolated from *Streptomyces verticillus*[220]. The multiple modules and domains composing the modular NRPS enzymes, as well as the diversity of the genetic machinery and biosynthetic gene clusters encoding NRPS[194], renders an enhanced relevance given the diverse chemical structures and activities that can consequently arise[156,209,221]. Deep-sea marine bacterial strains, symbionts of sponges, tunicates or cnidarians and been evidencing a genome enriched in NRPS clusters[126,222]. Moreover, bacteria from different phyla well known to produce secondary metabolites, especially Actinobacteria, have been screened for NRPS gene clusters[117], and the possible products associated to NRPS sequences have also been predicted[145]. According to a study, marine actinomycetes that potentially synthesize anti-cancer compounds possess a certain diversity of NRPS gene clusters[210]. Notwithstanding, many bioactive compounds produced by marine bacteria are related to a combined activity of both NRPS and polyketides, as the hybrid compounds can offer enhanced and more specific activities[223]. Despite the great discoveries that have been reached and efforts to unravel new anticancer natural drugs, the marine environments are still greatly overlooked and underrated[122]. Multiple marine ecological niches yet to be screened enclose promising genetic and biosynthetic diversities with potential relevance to fight PDAC.

Thereby, the aim of this work is to screen for the biosynthetic potential of marine bacteria isolated from cnidarian species through the use of molecular techniques directed to the amplification of NRPS gene fragments. Furthermore, in a way to confirm the production and activity of compounds produced by the NRPS-harbouring marine bacteria, their crude extracts were tested against a human PDAC cell line.

Materials and Methods

Bacterial symbionts, culture and storage conditions

The bacterial strains used in this study were previously isolated from nine cnidarian individuals, which were sampled in the Atlantic Ocean. The bacterial isolates were individually grown in marine agar plates at room temperature (20 ± 2ºC), being stored every time needed in 15% glycerol at -80ºC.

Molecular screening of NRPS gene fragments

In order to identify the potential of bacterial isolates to synthesize bioactive secondary metabolites, the presence of genes encoding NRPS were screened through polymerase chain reaction (PCR). For that, a set of degenerate primers targeting conserved genetic regions of the adenylation domain (A) of NRPS was used. A total of 175 bacterial strains isolated from all the cnidarians hosts were subjected to this molecular screening approach. Before PCR, bacterial genomic DNA (gDNA) was extracted by heat lysis at 105ºC during 5 minutes in a dry bath, followed by a cooling step at 4ºC for at least 15 minutes. The NRPS genes were PCR-amplified from bacterial gDNA using the degenerate primers A2F (5'-AAGGCNGGCGSBGCSTAYSTGCC-3') and A3R (5'TTGGGBIKBCCGGTSGINCCSGAGGTG-3'). Reactions for NRPS were performed in a final volume of 25 µL containing *ca.* 20 ng of gDNA, 400 pmol of each primer and 12.5 µL of Dream Taq Master Mix. The PCR run was conducted in a C-1000 Touch™ Thermal cycler from Bio-Rad, according to the next profile for NRPS: 4 min at 95°C, and 30 cycles 1 min at 95°C, 1 min at 70°C and 1 min at 72°C, followed by 10 min at 72°C. It was applied an agarose gel electrophoresis (CS-300V, Frilabo) for the separation of the PCR products in a 1% agarose gel. The amplicons were then observed in an UV Transilluminator Chemidoc XRS+ (Bio-Rad). The PCR amplicons were purified with a PCR-product purification kit and sequenced. The nucleotidic sequences were converted into aminoacid sequences and compared with available protein databases in BlastP®. The annotation was performed based in NCBI non-redundant protein database. Bacterial strains

that did not present culture constraints and for which was possible to get high amplification of the A domain-encoding amplicons, were selected for the preparation of crude extracts.

Bacterial identification through 16S rRNA gene sequencing

The 16S rDNA gene was amplified from gDNA (extracted as previously described) through the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1522R (5'-AAGGAGGTGATCCAGCC-3') [224]. Reactions for 16S were performed with the Dream Taq Master Mix for a final volume of 25 µL containing *ca.* 20 ng of gDNA and 0.3 µM of each primer. The PCR run was conducted for 9 min at 94°C, followed by 29 cycles of 30s at 94°C, 30s at 56°C and 90s at 72°C, and a final elongation step of 10 min at 72°C. The 16S amplicons were separated in an electrophoresis (see above), being the corresponding band excised from the gel, purified with a DNA purification kit, and subjected to Sanger sequencing. The nucleotide sequences were compared with NCBI database entries using BlastN® program.

Preparation of bacteria crude extracts

The bacteria previously selected (*i.e.*, presenting an intense band of A-domain amplicons) were grown in Marine broth at 23ºC up to the stationary phase. The obtained bacterial suspension was centrifuged to separate cells from the supernatant, thereby obtaining cell and cell-free fractions, which were extracted with acetone (2:1) and ethyl acetate (1:1), respectively. After incubation with agitation, the solvent layer of cell and cell-free fractions were separated and evaporated in a BÜCHI rotary evaporator at 40ºC. The dried crude extracts were weighted, dissolved in DMSO and stored in aliquots at -20ºC.

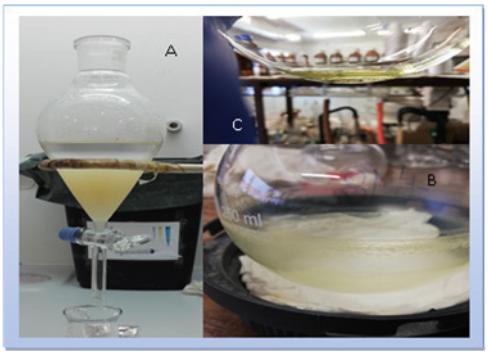

Fig.2: Representation of the metabolite extraction process with A) decantation, B) evaporation and C) obtained extract.

Cancer cell line culture and MTT assay

The human PDAC cell line Capan-1 was cultivated in RPMI medium supplemented with FBS 10% and maintained at 37ºC and 5% CO_2, being used for the assays at *ca.* 70% confluence. The prepared crude extracts from both bacterial fractions were tested for their cytotoxicity against Capan-1 cells through the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] colorimetric assay. This functional assay allows determining the impact of the extracts on the activity of mitochondrial enzymes, which catalyse the reduction of MTT to formazan, hence forming violet crystals that can be spectrophotometrically measured[225] at 550-590 nm. Before performing the assay with the bacterial extracts, the conditions for running it were optimized in regard to: (i) initial cell density ($1x10^3$, $5x10^3$, $1x10^4$ and $5x10^4$ cell/mL); (ii) DMSO concentration [0%, 0.5%, 0.8%, 1% and 2% (v/v)] since extracts were dissolved in DMSO, (iii) measuring times after dissolution of formazan crystals (5 min, 10 min and 15 min after adding the solvent to dissolve formazan crystals), (iv) measured wavelengths (500, 540, 570, 590, 650 nm). From a 70% confluent cell culture was prepared a cell suspension, which cell density was counted in a Neubauer chamber. The different cell densities were prepared in RPMI medium added with FBS and dispensed in the respective 96-well cell culture microplates, for cells attachment during approximately 24h (see culture conditions above). Then the culture medium was removed and replaced my DMSO-containing medium (200 µL) at the appropriate concentrations. Four experimental replicates were considered per cell density and DMSO concentration, being empty wells used as a blank. After 24h exposure under the same culture conditions, the MTT assay was initiated for about 2-3h. At the end of this exposure time, the MTT solution was discarded and the formazan crystals were dissolved with concentrated DMSO. After 5, 10 and 15 min was measured the optical density at 500, 540, 570, 590 and 650 nm, being the latter used to correct any potential effect associated with turbidity that should not be accounted for the measurement of the biological parameter.

At the light of the optimization outcome, the cytotoxicity assay performed with the crude extracts (cell and cell-free fractions) was performed with 1% of extract per well, and an increased initial cell density of $7x10^4$ cell/mL due to the low absorbances obtained with $5x10^4$ cell/mL. In this assay were also included: a blank, a negative control (cells + culture medium) and a solvent control (cells + culture medium with 1% DMSO). For each treatment (*i.e.*, controls and extracts) were tested four technical replicates (*i.e.*, 4 wells) and two experimental replicates (*i.e.,* 2 microplates). Cell seeding, attachment time, duration and conditions of exposure to bacterial

extracts was performed as for the optimization assays. The spectrophotometric measurements were made after 5 min dissolution at 570 nm and 650 nm.

To the spectrophotometric readings was removed the absorbances obtained in the blank and at 650 nm. After this treatment, the average and standard deviation of the four technical replicates and the two experimental replicates were computed and depicted in charts. A one-way analysis of variances (ANOVA) followed by the post-hoc multicomparison Dunnett's test were applied to detect statistically significant differences on cells viability exposed to DMSO concentrations or bacterial extracts in relation to that of the control ($\alpha = 0.05$). Statistical analyses were performed in SigmaPlot® v14 software.

Results and discussion

On the search for novel biomedical drugs from marine bacteria, besides getting knowledge on their potential biosynthetic abilities (*e.g.*, by genome mining) it is also relevant to realize if they are actually expressed. For that, the isolation and culture of the bacteria can be a crucial step to explore the production of natural compounds and get more information on the biosynthetic bacteria (*e.g.*, taxonomic identification). In this context, it was followed a molecular strategy for detecting the presence of NRPS-encoding genes in the gDNA of the marine bacterial strains isolated from cnidarian species[193].

Detection of NRPS A domain

From the screening of 175 bacterial strains for NRPS gene fragments presence, 39 were positive (=22%) (Table 2). This value is approximate to those reported in other studies focusing on bacteria from marine sediments (30% of positives)[158] or from sea sponges (with 13% positives)[219]. These percentages are however a rough and very relative estimation that depend on much factors like the ecological niche and environmental conditions, extraction and isolation methods, primers used and gene sequences targeted. Indeed, some studies got much higher percentage of NRPS-positive bacteria (63%) from marine sediments[117]. However, none of the mentioned studies developed this type of screening in bacteria isolated from cnidarians, what represents a promising and pioneer outcome towards the possibility of prospecting bioactive compounds.

All cnidarians had symbionts harbouring NRPS genes, although the number of NRPS-positive bacteria from each host is not very uniform along the cnidarian individuals, which can be

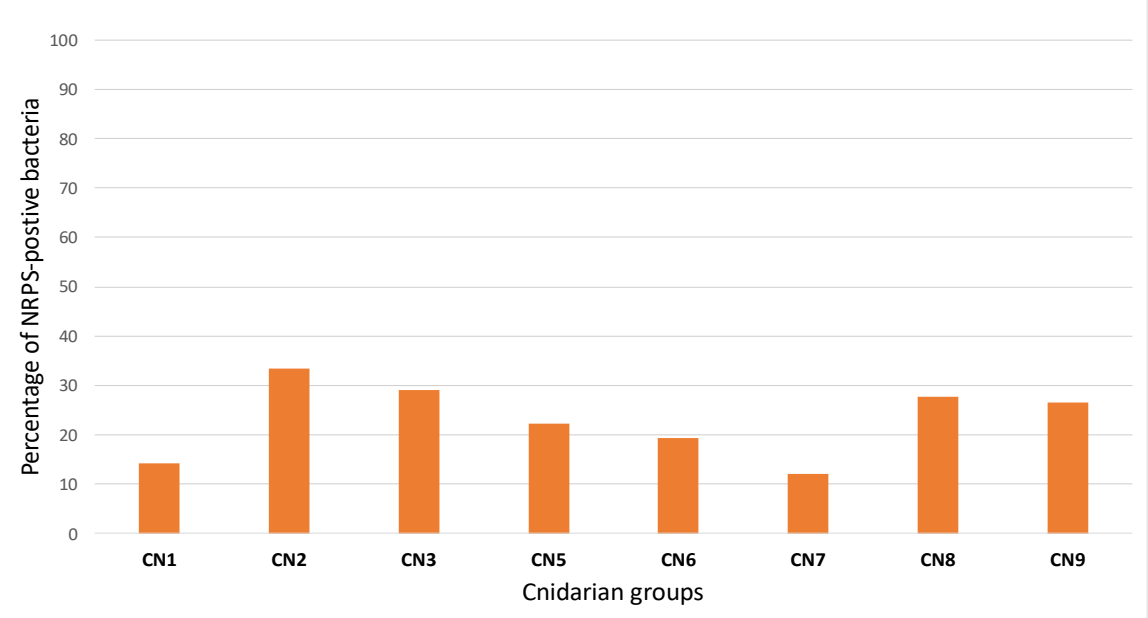

Fig.3: Percentage of bacteria presenting NRPS gene fragments in each group of cnidarians considered.

ranked by decreasing order as CN2 > CN3 > CN9 > CN5/CN8 > CN6 > CN1 > CN7 (Fig. 3). CN4 (Tables 2 and 3; not plotted) presented 100% percentage of bacteria potentially producing bioactive compounds, however this group only possessed one bacterial clone. This outcome is in line with the potential production of NRPs by symbionts of different cnidarians. A jellyfish symbiont, *Pseudomonas* sp., was found to be a producer of CAP-1 protein, an antimicrobial compound that has also the capacity to inhibit several marine microorganisms like pathogenic *Vibrio* species[152]. Soft-corals have also been found to contribute to a new source of antibiotics based on the presence of NRPS genes in bacterial symbionts (*e.g.*, *Pseudomonas* sp.), which are not harmful for the host, but in turn have activity against *Streptococcus equi*[226]. Some beneficial symbiotic microbes have indeed proved to produce antifungal compounds in the tissue surface of hydrozoan species[149]. In cnidarians like *Aglaophenia* sp., *Vibrio* species can have an important ecological role to this type of organisms[227], and in the present study, *Vibrio* spp. were identified in CN3, CN6 and CN9 cnidarians. *Obelia* sp. have the capacity to gather bacteria in their gastric cavity[228]. Bacteria makes part of Obelia "diet", while they filtrate them to their stomach cavity and use them as bacteriophages[228]. In the present study, *Pseudomonas* spp. are well represented in CN2, CN4 and CN7 cnidarian groups with four different species, namely *Pseudomonas alcaliphila* in CN7 group (Table 3) that has the capacity of biodegrading bile acids and use them as a carbon source[229].

Table 2: Bacterial clones isolated and screened for NRPS gene fragments. (+) stands for the positive and (-) for the negative presence of the NRPS A domain gene fragment; CN# - cnidarian individual number (#); ns - not screened.

CN1		CN2		CN3		CN4		CN5				CN6				CN7				CN8		CN9	
Clones	NRPS	Clones	NRPS	Clones	NRPS	Clones	NRPS	Clones	NRPS	Clones	NRPS	Clones	NRPS	Clones	NRPS	Clones	NRPS	Clones	NRPS	Clones	NRPS	Clones	NRPS
M85	(+)	M58	(+)	M1	(-)	M157	(+)	M130	(-)	M154	(-)	M99	(-)	M123	(+)	M25	(-)	M49	(-)	M158	(-)	M70	(-)
M86	(-)	M59	(-)	M2	(-)			M131	(-)	M155	(-)	M100	(-)	M124	(-)	M26	(-)	M50	(-)	M159	(+)	M71	(+)
M87	(-)	M60	(+)	M3	(-)			M132	(-)	M156	(-)	M101	(+)	M125	(+)	M27	(-)	M51	(+)	M160	(-)	M72	(+)
M88	(-)	M61	(+)	M4	(-)			M133	(+)			M102	(-)	M126	(-)	M28	(-)	M52	(-)	M161	(-)	M73	(-)
M89	(-)	M62	(-)	M5	(+)			M134	(-)			M103	(-)	M127	(-)	M29	(-)	M53	(+)	M162	(-)	M74	(-)
M90	(-)	M63	(-)	M6	(+)			M135	(-)			M104	(-)	M128	(-)	M30	(-)	M54	(-)	M163	(-)	M75	(-)
M91	(-)	M64	(-)	M7	(-)			M136	(-)			M105	(-)	M129	(+)	M31	(-)	M55	(-)	M164	(-)	M76	(-)
M92	(-)	M65	(-)	M8	(-)			M137	(-)			M106	(-)			M32	(-)	M56	(-)	M165	(+)	M77	(-)
M93	(-)	M66	(-)	M9	(-)			M138	(+)			M107	(-)			M33	(-)	M57	(-)	M166	(-)	M78	(-)
M94	(-)	M67	(-)	M10	(+)			M139	(+)			M108	(-)			M34	(-)			M167	(+)	M79	(-)
M95	(+)	M68	(+)	M11	(-)			M140	(-)			M109	(-)			M35	(-)			M168	(-)	M80	(+)
M96	(-)	M69	(-)	M12	(-)			M141	(+)			M110	(-)			M36	(-)			M169	(+)	M81	(-)
M97	(-)			M13	(-)			M142	(-)			M111	(-)			M37	(-)			M170	(-)	M82	(+)
M98	(-)			M14	(-)			M143	(-)			M112	(-)			M38	(-)			M171	(-)	M83	(-)
				M15	(+)			M144	(-)			M113	(-)			M39	(-)			M172	(-)	M84	(-)
				M16	(-)			M145	(-)			M114	(-)			M40	(+)			M173	(+)		
				M17	(-)			M146	(+)			M115	(+)			M41	(-)			M174	(-)		
				M18	(-)			M147	(-)			M116	(-)			M42	(+)			M175	(-)		
				M19	(+)			M148	(-)			M117	(-)			M43	(-)						
				M20	(-)			M149	(+)			M118	(-)			M44	(-)						
				M21	(+)			M150	(-)			M119	(-)			M45	(-)						
				M22	(-)			M151	(-)			M120	(-)			M46	(-)						
				M23	(+)			M152	(-)			M121	(+)			M47	(-)						
				M24	(-)			M153	(-)			M122	(-)			M48	(-)						

Identification of NRPS-positive bacteria

For all the 16S rRNA gene sequences of the bacteria evidencing NRPS gene fragments, an homology between 81% and 99% was achieved (Table 3) to the closest relative in BLASTn. It has been defended that 98% is the limit below which two species are considered to be different, whilst below 95% may indicate it is a new species[230]. NRPS genes have been often found in the phyla Proteobacteria, Actinobacteria, Firmicutes and Cyanobacteria[153,159]. In the present study, it was possible to observe a distribution of the marine bacterial strains between the Proteobacteria and Actinobacteria phyla only (Table 3). Besides, Proteobacteria strains were assigned to Alphaproteobacteria or Gammaproteobacteria classes. The four principal genera observed among the NRPS gene fragments-harbouring strains were *Ruegeria, Pseudomonas, Halomonas*, *Pseudoalteromonas* and *Vibrio.* The remaining genera retrieved from the 16S rRNA sequencing analysis were *Dietzia, Brachybacterium, Erythrobacter, Rhodococcus, Psychrobacter, Kribella, Pseudoalteromonas, Paracoccus, Sulfitobacter, Microbacterium, Brevibaterium, Cobetia* and *Alteromonas.* This diversity is in accordance with currently available or consulted literature. The *Vibrio* and *Pseudoalteromonas* genera have been found to present NRPs- and NRP/PK hybrids-encoding genes[194]. In the same study, the genus with most NRPS clusters was *Pseudoalteromonas,* which strains proved to have antibacterial activity[194]. In another study devoted to the screening of NRPS- and PKS-encoding genes in marine bacteria living in symbiosis with invertebrates, the authors verified that *Sulfitobacter, Paracoccus* and *Vibrio* were among the potential biosynthetic strains[221]. It is also common to find *Ruegeria* and *Pseudomonas* representatives as producers of antimicrobial compounds (*e.g.*, cyclic peptides) with proved activity against other bacteria[121]. *Halomonas* species were already reported to possess both PKS and NRPS genes[231,232]. *Alteromonas, Sulfitobacter* and *Halomonas* strains were proved to genetically carry and express at least one of the genes, and in the case of bacteria from *Sulfitobacter* and *Halomonas* genera showed to be able to induce high anticancer activity[231]. In relation to the other genera mentioned, the lack of bibliography suggests that they are poorly studied and highlight an unexplored resource for natural product discovery.

Table 3: Outcome of the 16S rRNA gene sequencing analysis for the identification and homology of NRPS-positive marine bacteria isolated from different cnidarian species. The light-green shaded clones correspond to the bacteria selected for further anticancer activity testing. Homologies ≤97% are represented in blue.

Cnidarian	Clone	Phylotype	Closest relative phylotype in BlastN	Class	Phylum	Homology (%)
CN1	M85	*Rhodococcus yunnanensis*	*Rhodococcus yunnanensis* YIM 70056	Actinobacteria	Actinobacteria	96
	M95	*Psychrobacter celer*	*Psychrobacter celer* SW-238	Gammaproteobacteria	Proteobacteria	93
CN2	M68	*Rhodococcus cercidiphylli*	*Rhodococcus cercidiphylli* YIM 65003	Actinobacteria	Actinobacteria	95
	M60	*Pseudomonas monteili*	*Pseudomonas monteili* CIP 104883	Gammaproteobacteria	Proteobacteria	98
	M61	*Pseudomonas monteili*	*Pseudomonas monteili* CIP 104883	Gammaproteobacteria	Proteobacteria	97
	M58	*Alteromonas addita*	*Alteromonas addita* R10SW13	Gammaproteobacteria	Proteobacteria	98
CN3	M23	*Vibrio gigantis*	*Vibrio gigantis* LGP 13	Gammaproteobacteria	Proteobacteria	98
	M10	*Ruegeria pelagia*	*Ruegeria pelagia* NBRC 102038	Alphaproteobacteria	Proteobacteria	99
	M15	*Ruegeria pelagia*	*Ruegeria pelagia* NBRC 102038	Alphaproteobacteria	Proteobacteria	97
	M5	*Ruegeria pelagia*	*Ruegeria pelagia* NBRC 102038	Alphaproteobacteria	Proteobacteria	96
	M6	*Dietzia maris*	*Dietzia maris* AUCM A-593	Actinobacteria	Actinobacteria	97
	M21	*Brachybacterium paraconglomeratum*	*Brachybacterium paraconglomeratum* LMG 19861	Actinobacteria	Actinobacteria	98
	M19	*Erythrobacter flavus*	*Erythrobacter flavus* SW-46	Alphaproteobacteria	Proteobacteria	96
CN4	M157	*Pseudomonas plecoglossicida*	*Pseudomonas plecoglossicida* FPC951	Gammaproteobacteria	Proteobacteria	95
CN5	M146	*Halomonas meridiana*	*Halomonas meridiana* DSM 5425	Gammaproteobacteria	Proteobacteria	98
	M149	*Halomonas meridiana*	*Halomonas meridiana* DSM 5425	Gammaproteobacteria	Proteobacteria	97
	M141	*Halomonas meridiana*	*Halomonas meridiana* DSM 5425	Gammaproteobacteria	Proteobacteria	95
	M139	*Sulfitobacter faviae*	*Sulfitobacter faviae* S5-53	Alphaproteobacteria	Proteobacteria	81
	M138	*Rhodococcus yunnanensis*	*Rhodococcus yunnanensis* YIM 70056	Actinobacteria	Actinobacteria	97
	M133	*Halomonas denitrificans*	*Halomonas denitrificans* M29	Gammaproteobacteria	Proteobacteria	88
CN6	M115	*Psychrobacter pacificensis*	*Psychrobacter pacificensis* NIBH P2K6	Gammaproteobacteria	Proteobacteria	97
	M101	*Vibrio gigantis*	*Vibrio gigantis* LGP 13	Gammaproteobacteria	Proteobacteria	96
	M123	*Pseudoalteromonas flavipulchra*	*Pseudoalteromonas flavipulchra* NCIMB 2033	Gammaproteobacteria	Proteobacteria	97
	M121	*Pseudoalteromonas flavipulchra*	*Pseudoalteromonas flavipulchra* NCIMB 2033	Gammaproteobacteria	Proteobacteria	98
	M125	*Paracoccus rhizosphaerae*	*Paracoccus rhizosphaerae* CC-CM 15-8	Alphaproteobacteria	Proteobacteria	96
	M129	*Paracoccus haeundaensis*	*Paracoccus haeundaensis* BC74171	Alphaproteobacteria	Proteobacteria	98
CN7	M40	*Pseudomonas chloritidismutans*	*Pseudomonas chloritidismutans* AW-1	Gammaproteobacteria	Proteobacteria	97
	M51	*Pseudomonas alcaliphila*	*Pseudomonas alcaliphila* NBRC 102411	Gammaproteobacteria	Proteobacteria	97
	M42	*Kribbella alba*	*Kribbella alba* YIM 31075	Actinobacteria	Actinobacteria	94
	M53	*Pseudoalteromonas paragorgicola*	*Pseudoalteromonas paragorgicola* KMM 3548	Gammaproteobacteria	Proteobacteria	95
CN8	M165	*Cobetia amphilecti*	*Cobetia amphilecti* 46-2	Gammaproteobacteria	Proteobacteria	97
	M169	*Brevibacterium permense*	*Brevibacterium permense* VKM Ac-2280	Actinobacteria	Actinobacteria	84
	M173	*Microbacterium oxydans*	*Microbacterium oxydans* DSM 20578	Actinobacteria	Actinobacteria	93
	M167	*Sulfitobacter pontiacus*	*Sulfitobacter pontiacus* ChLG-10	Alphaproteobacteria	Proteobacteria	97
	M159	*Microbacterium hydrocarbonoxydans*	*Microbacterium hydrocarbonoxydans* BNP48	Actinobacteria	Actinobacteria	97
CN9	M80	*Vibrio gallaecicus*	*Vibrio gallaecicus* Rd 8.15	Gammaproteobacteria	Proteobacteria	94
	M71	*Vibrio gigantis*	*Vibrio gigantis* LGP 13	Gammaproteobacteria	Proteobacteria	95
	M72	*Ruegeria mobilis*	*Ruegeria mobilis* NBRC 101030	Alphaproteobacteria	Proteobacteria	98
	M82	*Ruegeria mobilis*	*Ruegeria mobilis* NBRC 101030	Alphaproteobacteria	Proteobacteria	97

Analysis of NRPS A domain sequences

Fourteen strains (*cf.* Tables 2, 3) with the best results on NRPS screening (band intensity) and ease of culture were selected for further study: M85, M60, M51, M139, M146, M61, M123, M157, M72, M125, M169, M15, M68 and M138. The primary step was to sequencing the amplicons of the NRPS A domain as to obtain the respective aminoacid sequences (Table 4). From the sequencing and analysis of the NRPS gene fragments only 8 amino acid consensus was obtained, which were related with NRPS, PKS or NRPS/PKS hybrid aminoacid sequences, previously determined in bacteria belonging to *Pseudomonas*, *Pseudoalteromonas*, *Halomonas*, *Rhodococcus* and *Sulfitobacter* genera (Table 4). These genera were coherent to the 16S rRNA identification obtained for the cnidarian bacterial symbionts herein studied (Table 3). The homology between the sequenced and BlastP-deposited aminoacid sequences ranged between 37% and 96%, though it was broadly above 61%. These high similarities to NRPS peptides and/or A domain sequences are in agreement with the principal domain targeted by the degenerate primers used in the screening, hence reinforcing their reliability despite the known genetic diversity of NRPS genes[233]. Additionally, such diversity can be even more increased and become a quite valuable adaptation if NRPS/PKS hybrids occur. This might be the case of clone M157 that shows the genetic potential to synthesize a hybrid compound, which have been frequently suggested to be related with chemical scaffolds presenting enhanced bioactivity, complementarity to biological receptors, and stability[122,126,234]. Notwithstanding, considering that no reliable aminoacid sequence was obtained for 6 strains, either the sequencing of the amplified NRPS fragments should be repeated or other primers must be further tested.

Bacteria crude extracts and MTT assay

Although NRPS gene fragments sequencing demonstrated consensus only for 8 of the selected bacteria, the 14 bacteria selected according to the molecular screening were considered for the preparation of extracts from cell and cell-free fractions of grown cultures. A total of 28 extracts were hence obtained. The concentrations of crude extracts varied between 2 and 22 mg/mL DMSO for the cell fractions and between 3 and 25 mg/mL DMSO for the cell-free fractions (Table 5). These concentrations were considerably high considering that resulted from a 100 mL culture.

Table 4: Prediction of correspondences for aminoacid sequences obtained from A domain of NRPS gene sequences to the selected bacterial strains (only sequences with reliable consensus are presented).

Strain	Name	Closest match in BlastP	Similarity (%)	Accession nr.	Database
M85	*Rhodococcus yunnanensis*	MULTISPECIES: non-ribosomal peptide synthetase [*Rhodococcus*]	88	WP_094621983.1	nr
M60	*Pseudomonas monteili*	Non-ribosomal peptide synthetase [*Pseudomonas putida*]	96	WP_084850711.1	nr
M51	*Pseudomonas alcaliphila*	non-ribosomal peptide synthase domain TIGR01720/amino acid adenylation domain-containing protein, partial [Pseudomonas indica]	66	SDK21005.1	nr
M139	*Sulfitobacter faviae*	amino acid adenylation domain protein [Halomonas sp. KO116]	75	AJY51867.1	nr
M146	*Halomonas meridiana*	RecName: Full=Polyketide synthase PksN	79	O31782.3	sw
		amino acid adenylation domain protein [Halomonas sp. KO116]	82	AJY51867.1	nr
M61	*Pseudomonas monteili*	non-ribosomal peptide synthetase, partial [Pseudomonas hunanensis]	85	PKF22537.1	nr
M123	*Pseudoalteromonas flavipulchra*	RecName: Full=Nonribosomal peptide synthase atnA; AltName: Full=Aspercryptin biosynthesis cluster protein A	37	Q5AUZ6.1	sw
M157	*Pseudomonas plecoglossicida*	non-ribosomal peptide synthase/polyketide synthase [Pseudomonas sp. p21]	61	WP_063912751.1	nr

Table 5: Concentrations obtained of the crude extracts prepared.

Clones	Extracts from cell fractions [ ] (mg/ml DMSO)	Extracts from cell-free fractions [ ] (mg/ml DMSO)
M51	2	20
M157	20	10
M61	13	11
M85	10	3
M139	10	10
M72	20	20
M68	20	20
M146	10	20
M60	20	20
M125	20	20
M169	20	20
M138	9	25
M15	22	20
M123	20	20

For the MTT assays, a preliminary test was made in order to establish the optimal assay conditions (*i.e.*, initial cell density, wavelength, and measuring time) and maximum DMSO concentration that did not affect Capan-1 cells growth. Figure 4 shows the cell viability response ranges of Capan-1 cell line in regard to different experimental variables that can influence the outcome of MTT assay. In general, a similar absorbance trend was obtained in the three reading times and wavelengths tested. As such, it was decided to set 5 minutes for formazan crystal dissolution and perform the absorbance readings at 570 nm. However, the initial cell density that provided better absorbance levels was $5x10^4$ cells/mL, although still out of the recommended range (~0.75 – 1.25). Therefore, the MTT assay of the extracts was performed for an initial cell density of $7x10^4$ cells/mL. The cytotoxic effect of DMSO was verified in order to evaluate which would be the maximum non-inhibiting concentration for the future testing of bacterial extracts, which were solubilized in DMSO. The cytotoxic effect of DMSO at different cell densities is shown in figure 5. In comparison to the control, significant inhibitory effects of DMSO occurred almost in all DMSO concentrations for cell densities equal or lower than $1x10^4$ cells/mL, for all the wavelengths tested except at 500nm. In the $1x10^3$ cells/mL cell density it can be verified that all the DMSO concentrations except 0.5% revealed to be toxic for Capan-1 cells. For the $5x10^4$ cells/mL cell density no significant cell viability inhibition occurred, except under 2% of DMSO at all wavelengths, except for 0.8% DMSO at 590 nm. Therefore, and as broadly stated in the literature, the initial cell density may constrain the cytotoxicity outcomes. Anyway, at the light of these results, the following MTT assays aiming to test the effect of bacterial extracts on Capan-1 cellular viability were run at higher initial cell densities coupled with an optimal dose of 1% of extract (*i.e.,* 1% DMSO) and absorbance readings at 570 nm, because as it could be observed, at higher cell densities ($5x10^4$ cells/mL), 1% of DMSO did not affect them. Although the effect of DMSO concentration was not herein included in this set of optimization experiments for the initial cell density of $7x10^4$ cells/mL, a control with culture medium and 1% (v/v) DMSO was considered in the MTT assay performed for the bacterial extracts.

The cytotoxic effect of the 28 extracts is shown in figure 6. Except for the cell fraction extract of M123 strain, all extracts of cell-free and cell fractions induced a significant inhibition ($p<0.05$) of cellular activity. Overall, the percentage of viable/active Capan-1 cells after exposure to cell-free fraction extracts ranged between 38 and 76%, whilst to cell fraction extracts 40 to 97% of viable cells were calculated, what indicates that the cell-free fraction was generally the most cytotoxic. Thereby, this result suggests that the NRPs or hybrids potentially synthesized by these marine bacteria cross the cellular membranes and are released to the surrounding environment.

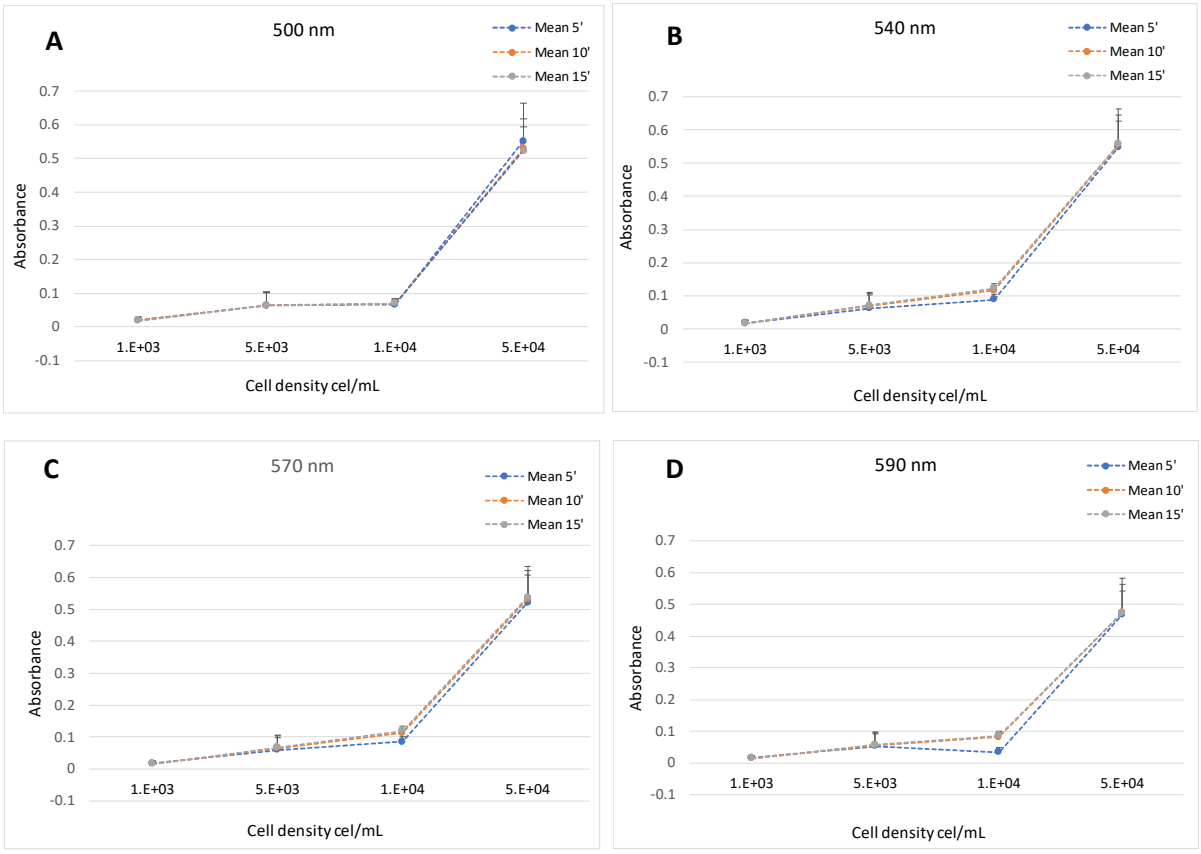

Fig.4: Analysis of the average absorbance values measured at different reading times (5 min, 10 min and 15 min), wavelengths (A - 500 nm, B – 540 nm, C – 570 nm, D – 590 nm) and initial cell densities of Capan-1 human PDAC cell line, resulting from MTT assay.

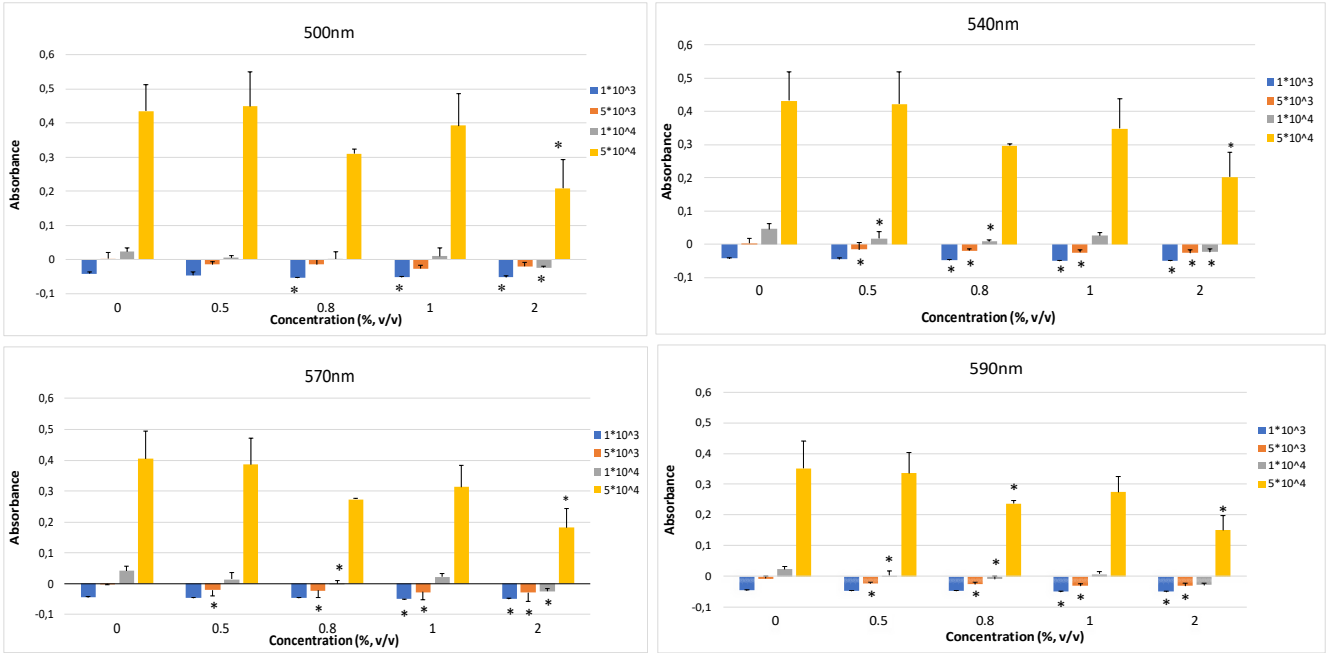

Fig.5: Average absorbance values obtained in MTT assay for the testing of DMSO effect considering different initial cell densities and measuring wavelengths. Error bars indicate standard deviation and *

stands for statistically significant inhibition of cell viability provoked by DMSO relatively to the control, within the respective initial cell density ($p< 0.05$).

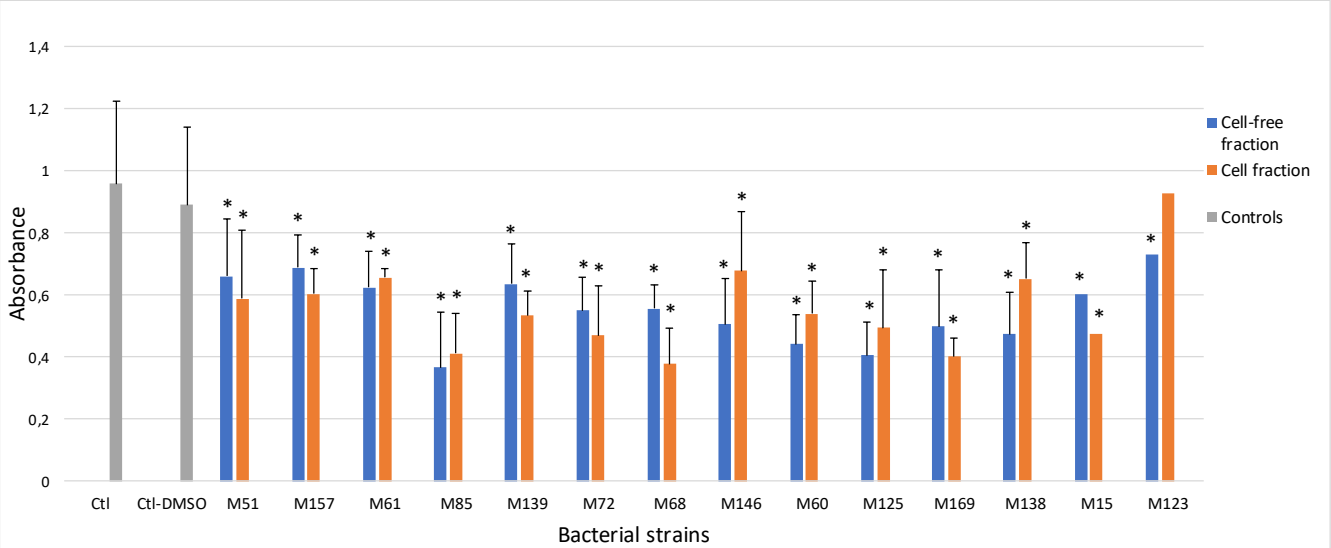

Fig.6: Average absorbance values obtained in MTT assay for testing the anticancer activity of bacterial crude extracts (cell and cell-free fractions) against Capan-1 PDAC cells. The exposure was run with an initial cell density of $7x10^4$ cells/mL and the absorbance readings were performed at 570 nm. Error bars indicate standard deviation and * stands for statistically significant inhibition of cell viability provoked by the extract relatively to the control ($p< 0.05$).

The more pronounced inhibitions occurred under both fractions of M85, the cell fraction of M68 and M169, and the cell-free fraction of M125.

According to the 16S rDNA-based phylogenetic analysis, they corresponded to species belonging to the genera *Rhodococcus* spp. (M85, M68, M138), *Brevibacterium* sp. (M169), *Paracoccus rhizosphaerae* (M125), respectively, but also significant inhibitions were detected for the extracts of *Pseudomonas* spp. (M51, M60-M61, M157), *Ruegeria* spp. (M15, M72), *Halomonas* sp. (M146), *Sulfitobacter* (M139), and *Pseudoalteromonas* sp. (M123) species (Fig. 6, Table 3). The bioactivities of M60, M61, M85, and M146 are likely to rely on NRPs compounds, given the high homology (≥82%) in aminoacid sequences obtained upon comparison with BlastP deposited sequences of NRPS biosynthetic gene clusters (Table 4). Though with a lower identity percentage, the activity of M51, M123, M139, and M157 strains can also result from NRPS biosynthesis, though a more thorough analysis has yet to be undertaken. No studies regarding the *Paracoccus* and *Ruegeria* genera and its potential of anticancer agents biosynthesis could be found. In relation to *Rhodococcus* genera, the current literature does not show many related reports on their cytotoxic effect. However, a study was performed with *Rhodococcus* strains isolated from a

polluted soil, towards the evaluation of the effect of crude extracts obtained from its cell-free fraction on human hepatocellular carcinoma cells (HepG2 cell line) and cervical carcinoma cells (HeLa cells)[235]. The authors verified the cytotoxic effect of the *Rhodococcus* extracts on cell viability, hence indicating their antitumor activity against both cell lines. A study with *Brevibacterium* from several coastal areas of South Arabia[236], showed the anticancer activity of the cell-free fraction of crude extracts against MCF-7 human breast cancer cells, according to the outcome of MTT assay, but no effect was recorded against HTC 116 colorectal cancer cells[236]. Considering *Halomonas* and *Sulfitobacter* genera (to which belongs M146 and M139, respectively), a study was performed to test the bioactivity of strains isolated from deep sea brines on MCF-7 cell line, DU145 prostate carcinoma cells and HeLa cells, using the MTT assay[237]. The authors also used the cell-free fraction of the crude extracts and the results turned out to show their significant inhibitory action to the three addressed cell lines[237]. Bacterial representatives of the *Pseudomonas* genus (as M51, M60, M61 and M157) obtained from coastal waters were indeed reported to produce anticancer products capable of inhibiting hepatocellular carcinoma cells viability, as well as an extract dose-dependent trend could be identified[238]. In this study, the bacteria with anti-hepatocellular-carcinoma abilities presented gene clusters for PKs biosynthesis, although the authors believe that other biosynthetic gene clusters might be present as well, given the extremely high inhibitory effect observed[238]. However, those genes could be silenced or the primers used for their amplification were not the most adequate[238].

Although, M123 was the only bacterial strain whose cell fraction did not significantly affect Capan-1 cells activity, two studies performed with *Pseudoalteromonas* bacterial strains reached opposite results. In the first study, the authors used strains isolated from intertidal zones in China Sea[239]. Among the 29 strains analysed, the cell-free-based crude extracts of two of them were active against HeLa cells and BGC-823 stomach cancer cell line[239]. The specific compound responsible for that cytotoxicity was purified and identified as “norharman”, an already known compound for its high toxicity[239]. Also the cell-free crude extracts of other *Pseudoalteromonas* strains collected from marine cold water could inhibit the human A549 lung cancer cell line, based on the outcome of MTT assay, being the cytotoxic compound the 4-HBA (4-hydroxybenzoic acid)[240].

Conclusions

Marine cnidarian symbionts can enclose a great biosynthetic potential, which can be of major relevance to fight pancreatic cancer. The outcomes herein attained strengthen that a molecular screening of NRPs-producing bacteria as a first step to select potential producers of bioactive secondary metabolites against PDAC is reliable and pin point a solid study direction. As such, from a set of 14 bacterial strains selected through this approach, most derived extracts inhibited the cellular activity of a pancreatic cell line. Such activity was partly associated to NRPS biosynthetic clusters given the presence of gene sequences of NRPS A domain in those bacteria. More tests will be done to better understand the anticancer activity of the extracts of marine bacteria, but the promising results reinforce the search for new anticancer drugs in under explored marine resources.

Chapter IV

Final considerations

As final conclusions of this study it is recognized the diversity of possibilities that the marine environment offers in terms of biological diversity of macro and, especially, of microorganisms. Among these, marine bacteria often living in symbiosis with marine cnidarians, enclose a potential to help reaching alternative treatments for several human diseases or health problems, given their ability to synthesize bioactive natural compounds.

Bacterially-produced secondary metabolites or natural compounds result frequently from the expression of NRPS, PKS or NRPS-PKS hybrids gene clusters, which encode the biosynthesis of nonribosomal peptides, polyketides and nonribosomal-polyketide hybrids, respectively, with varied chemical structures, properties and activities. Getting a deeper knowledge on the genetic composition and machinery sustaining the diversity and evolutionary trend of those modular enzymes as well as their ubiquity, not only has been widen the discovery of new products but also the fundamentals to create novel optimized chemicals through genetic engineering. Such approaches will favour the biosynthesis of active compounds to counteract medical limitations related with drug resistance against resistant microbial infections or with the low efficacy of current cancer therapeutics, among others. Still, more studies directed to the marine environment and the biotechnological potential of bacterial symbionts are needed.

In this context, bacterial symbionts of cnidarian species from the Atlantic Ocean were herein explored for screening the biosynthetic potential of NRPs and test the activity of the respective bacterial extracts. The results and research accomplished, support the hypothesis that a range of bacteria, mainly Proteobacteria and Actinobacteria are responsible for the production of diverse NRPs. These clinically relevant products associated with NRPS genes, may represent a vigorous and large source of clinical options for the treatment of current threatening and invasive diseases, such as pancreatic cancer. Indeed, the corresponding band of NRPS A domain was detected in the genomic DNA of 39 strains (22.3% of the tested isolates), among which 14 were selected and their crude extracts (cell and/or cell-free fractions) presented activity against a human pancreatic cell line (Capan-1), according to the MTT assay outcome. This constituted a valuable first step to proceed for isolation and discovery of the anti-cancer NRPs synthesized by those marine bacteria for the potential anti-pancreatic cancer control.

As such, this study brings novel scientific knowledge regarding NRPs bacterial producers isolated from cnidarians, reinforcing the pharmaceutical potential hidden in marine biological and ecological niches. In the future, these marine bacteria possessing NRPS genes should be further

tested to find out if the produced secondary metabolites exhibit activity against other tumours, bacteria, fungi, inflammatory and immunosuppressive diseases, among others. It is hence encouraging to look forward and open horizons for the best hope, through the application of modern sequencing, chemistry and enzymology tools to uncover bacterial metagenomic/metabolomics, to further manipulate and examine their capacity to produce relevant NRPS as well as PKS and hybrid enzymes enrolled in the synthesis of novel natural products.

References:

1. Bray, F. *et al.* Global Cancer Statistics 2018: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J CLIN* **68,** 394–424 (2018).
2. Erdek A., M., Fishman K., E., Herman M., J., Laheru A., D. & Wolfgang L., C. Recent Progress in Pancreatic Cancer. *Cancer J. Clin.* **63,** 318–348 (2013).
3. Lennon, A. M. *et al.* The Early Detection of Pancreatic Cancer : What Will It Take to Diagnose and Treat Curable Pancreatic Neoplasia ? *Cancer Res.* **74,** 3381–3390 (2014).
4. Midha, S., Chawla, S. & Kumar, P. Modifiable and non-modifiable risk factors for pancreatic cancer : A review. *Cancer Lett.* **381,** 269–277 (2016).
5. Ferlay, J. *et al.* Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. *Int. J. Cancer* **136,** 359–386 (2015).
6. Malvezzi, M. *et al.* European cancer mortality predictions for the year 2016 with focus on leukaemias. *Ann. Oncol.* **27,** 725–731 (2016).
7. Bosetti, C. *et al.* Pancreatic cancer: Overview of descriptive epidemiology. *Mol. Carcinog.* **51,** 3–13 (2012).
8. Bryant, K. L., Mancias, J. D., Kimmelman, A. C. & Der, C. J. KRAS: feeding pancreatic cancer proliferation. *Cell Press* **39,** 91–101 (2014).
9. Morton, J. P. *et al.* Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. *Proc. Natl. Acad. Sci.* **107,** 246–251 (2010).
10. Yoshida, K. & Miki, Y. Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Sci.* **95,** 866–871 (2004).
11. Nepomuceno, T. C. *et al.* The role of PALB2 in the DNA damage response and cancer predisposition. *Int. J. Mol. Sci.* **18,** 1–20 (2017).
12. Hu, C. *et al.* Association between inherited germline mutations in cancer predisposition genes and risk of pancreatic cancer. *JAMA - J. Am. Med. Assoc.* **319,** 2401–2409 (2018).
13. Hélias-Rodzewicz, Z., Lourenco, N., Bakari, M., Capron, C. & Emile, J. F. CDKN2A Depletion Causes Aneuploidy and Enhances Cell Proliferation in Non-Immortalized Normal Human Cells. *Cancer Invest.* **36,** 338–348 (2018).
14. Pannala, R. & et.al. New-onset diabetes : a potential clue to the early diagnosis. *Lancet Oncol.* **10,** 88–95 (2009).
15. Makohon-moore, A. & Iacobuzio-donahue, C. A. Pancreatic cancer biology and genetics from an evolutionary perspective. *Nat. Publ. Gr.* **16,** 553–565 (2016).
16. Micucci, C., Valli, D., Matacchione, G. & Catalano, A. Current perspectives between metabolic syndrome and cancer. *Oncotarget* **7,** 38959–38972 (2016).
17. Weiss, F. U. Pancreatic cancer risk in hereditary pancreatitis. *Front. Physiol.* **5,** 1–5 (2014).
18. Bracci, P. M. Obesity and pancreatic cancer: Overview of epidemiologic evidence and biologic mechanisms. *Mol. Carcinog.* **51,** 53–63 (2012).
19. Gukovsky, I., Li, N., Todoric, J., Gukovskaya, A. & Karin, M. Inflammation, Autophagy, and Obesity: Common Features in the Pathogenesis of Pancreatitis and Pancreatic Cancer. *Gastroenterology* **144,** 1–22 (2014).
20. Stolzenberg-Solomon, R. Z. & Amundadottir, L. T. Epidemiology and Inherited Predisposition for Sporadic Pancreatic Adenocarcinoma. *Hematol. Oncol. Clin. North Am.* **29,** 619–640 (2015).
21. Tramacere, I. *et al.* Alcohol drinking and pancreatic cancer risk: a meta-analysis of the dose-risk relation. *Int. J. cancer J. Int. du cancer* **126,** 1474–1486 (2010).
22. Brandt, A. Van Den, Calle, E. E., English, D. R., Folsom, A. R. & Jo, L. A POOLED ANALYSIS OF 14 COHORT STUDIES OF ANTHROPOMETRIC FACTORS AND PANCREATIC CANCER RISK. *Int J*

Cancer. **129,** 1708–1717 (2012).
23. Blockley, A., Elliott, D. R., Roberts, A. P. & Sweet, M. Symbiotic microbes from marine invertebrates: Driving a new era of natural product drug discovery. *Diversity* **9,** 1–13 (2017).
24. Blackford, A. *et al.* Genetic Mutations Associated With Cigarette Smoking in Pancreatic Cancer. *Cancer Res.* **69,** 3681–3688 (2010).
25. Ballas, K. D. *et al.* Mixed exocrine-endocrine tumor of the pancreas. *J. Pancreas* **6,** 449–454 (2005).
26. Zhou, J. *et al.* Incidence rates of exocrine and endocrine pancreatic cancers in the United States. *Cancer Causes Control* **21,** 853–861 (2010).
27. Chan, A., Diamandis P., E. & Blasutig M., I. Strategies for discovering novel pancreatic cancer biomarkers. *J Proteomics* **81,** 126–134 (2013).
28. Ballehaninna, U. K. & Chamberlain, R. S. Biomarkers for pancreatic cancer: Promising new markers and options beyond CA 19-9. *Tumor Biol.* **34,** 3279–3292 (2013).
29. Chan, A. *et al.* Validation of biomarkers that complement CA19.9 in detecting early pancreatic cancer. *Clin Cancer Res* **20,** 5787–5795 (2014).
30. Ballehaninna, U. K. & Chamberlain, R. S. The clinical utility of serum CA 19-9 in the diagnosis, prognosis and management of pancreatic adenocarcinoma: An evidence based appraisal. *J. Gastrointest. Oncol.* **3,** 105–119 (2012).
31. Costa-silva, B. *et al.* Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat. Cell Biol.* **17,** 816–826 (2015).
32. Massagué, J. & Obenauf, A. C. Metastatic colonization by circulating tumour cells. *Nature* **529,** 298–306 (2016).
33. Hidalgo, M. New insights into pancreatic cancer biology. *Ann. Oncol. 23* **23,** 135–138 (2012).
34. Feldmann, G., Beaty, R., Hruban H., R. & Maitra, A. Molecular genetics of pancreatic intraepithelial neoplasia Georg. *J Hepatobiliary Pancreat Surg.* **14,** 224–232 (2007).
35. Li, C. *et al.* Identification of Pancreatic Cancer Stem Cells. *Am. Assoc. Cancer Res. Journals* **67,** 1030–1038 (2007).
36. Biankin, A. V *et al.* Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature* **491,** 399–405 (2012).
37. Jones, S. *et al.* Core Signaling Pathways in Human Pancreatic Cancers Revealed by Global Genomic Analyses. *Science (80-.).* **321,** 1801–1806 (2008).
38. Petersen, G. M. Familial Pancreatic Adenocarcinoma. *Hematol. Oncol. Clin. North Am.* **29,** 641–653 (2015).
39. Bunting, S. F. & Nussenzweig, A. END-JOINING, TRANSLOCATIONS AND CANCER. *Nat Rev Cancer.* **13,** 2781–2789 (2015).
40. Becker, A. E. *et al.* Pancreatic ductal adenocarcinoma : Risk factors , screening , and early detection. *World J. Gastroenterol.* **20,** 11182–11198 (2014).
41. Grant, R. C. *et al.* Exome-Wide Association Study of Pancreatic Cancer Risk. *Gastroenterology* **154,** 719–722.e3 (2018).
42. Huaman, M. A. *et al.* Genome-wide association study identifies multiple susceptibility loci for pancreatic cancer. *Nat Genet.* **46,** 994–1000 (2014).
43. Petersen, G. M. *et al.* A genome-wide association study identifies pancreatic cancer susceptibility loci on chromosomes 13q22.1, 1q32.1 and 5p15.33. *Nat Genet.* **42,** 224–228 (2010).
44. Klein, W. M. & *et.al*. Direct correlation between proliferative activity and dysplasia in pancreatic intraepithelial neoplasia (panIN): Additional evidence for a recently proposed model of progression. *Mod. Pathol.* **15,** 441–447 (2002).
45. Iacobuzio-donahue, C. A. Genetic evolution of pancreatic cancer: lessons learnt from the

pancreatic cancer genome sequencing project. *Gut.* **61,** 1085–1094 (2012).
46. Peinado, H., Lavotshkin, S. & Lyden, D. The secreted factors responsible for pre-metastatic niche formation : Old sayings and new thoughts. *Semin. Cancer Biol.* **21,** 139–146 (2011).
47. Sceneay, J., Smyth, M. J. & Möller, A. The pre-metastatic niche : finding common ground. *Cancer Metastasis Rev* **32,** 464–449 (2013).
48. Hood, J. L. *et al.* Exosomes Released by Melanoma Cells Prepare Sentinel Lymph Nodes for Tumor Metastasis. *Cancer Res.* **71,** 3792–3801 (2011).
49. Peinado, H. *et al.* Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat. Med* **18,** 883–891 (2013).
50. M.Stromnes, I., E.DelGiorno, K., D.Greenberg, P. & R.Hingorani, S. Stromal reengineering to treat pancreas cancer ingunn. *Carcinogenesis* **35,** 1451–1460 (2014).
51. Neesse, A., Algül, H., Tuveson, D. A. & Gress, T. M. Stromal biology and therapy in pancreatic cancer: A changing paradigm. *Gut* **64,** 1476–1484 (2015).
52. Cohen, R. *et al.* Targeting cancer cell metabolism in pancreatic adenocarcinoma. *Oncotarget* **6,** 16832–16847 (2015).
53. Sousa, C. M. & Kimmelman, A. C. The complex landscape of pancreatic cancer metabolism. *Carcinogenesis* **35,** 1441–1450 (2014).
54. Vonderheide, R. H. & Bayne, L. J. Inflammatory networks and immune surveillance of pancreatic carcinoma. *Curr Opin Immunol.* **25,** 200–205 (2014).
55. Ratajczak, J., Wysoczynski, M., Hayek, F., Janowska-Wieczorek, A. & Ratajczak, M. Z. Membrane-derived microvesicles: Important and underappreciated mediators of cell-to-cell communication. *Leukemia* **20,** 1487–1495 (2006).
56. Weidle, U. H., Birzele, F., Kollmorgen, G. & Rüger, R. The multiple roles of exosomes in metastasis. *Cancer Genomics and Proteomics* **14,** 1–16 (2017).
57. Murphy, S. J. *et al.* Genetic Alterations Associated With Progression From Pancreatic Intraepithelial Neoplasia to Invasive Pancreatic Tumor. *Gastroenterology* **145,** 1098–1109 (2013).
58. Lennon, A. M. *et al.* The early detection of pancreatic cancer: What will it take to diagnose and treat curable pancreatic neoplasia? *Cancer Res.* **74,** 3381–3389 (2014).
59. Andea, A., Sarkar, F. & Adsay, V. N. Clinicopathological Correlates of Pancreatic Intraepithelial Neoplasia: A Comparative Analysis of 82 Cases With and 152 Cases Without Pancreatic Ductal Adenocarcinoma. *Mod. Pathol.* **16,** 996–1006 (2003).
60. Saavedra, J. A. *et al.* Pancreatic Intraepithelial Neoplasia. *Am. J. Surg. Pathol.* **25,** 579–586 (2001).
61. Hanada, K. *et al.* Effective screening for early diagnosis of pancreatic cancer. *Best Pract. Res. Clin. Gastroenterol.* **29,** 929–939 (2015).
62. Yachida, S. & et.al. Clinical Significance of the Genetic Landscape of Pancreatic Cancer and Implications for Identification of Potential Long- term Survivors. *Clin. Cancer Res.* **18,** 6339–6348 (2012).
63. Biankin, A. V. & et.al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature* **491,** 399–405 (2012).
64. Cohen, R., Neuzillet, C., Tijeras-raballand, A. & Faivre, S. Targeting cancer cell metabolism in pancreatic adenocarcinoma. **6,** (2015).
65. Mahadevan, D. & Hoff, D. D. Von. Review Tumor-stroma interactions in pancreatic ductal adenocarcinoma. *Mol. Cancer Ther.* **6,** 1186–1198 (2007).
66. Chu, G. C., Kimmelman, A. C., Hezel, A. F. & Depinho, R. A. Stromal Biology of Pancreatic Cancer. *J. Cell. Biochem.* **101,** 887–907 (2007).
67. Progress, M. & Hidalgo, M. Pancreatic Cancer. *N. Engl. J. Med.* **362,** 1605–1617 (2010).
68. Alam, M. S. *et al.* Selective inhibition of the p38 alternative activation pathway in infiltrating T cells inhibits pancreatic cancer progression. *Nat. Med.* **21,** 1337–1343 (2015).

69. Alam, M. S. *et al.* Counter-regulation of T cell effector function by differentially activated p38. *J. Exp. Med.* **211,** 1257–1270 (2014).
70. Salvador, J. M. *et al.* Alternative p38 activation pathway mediated by T cell receptor – proximal tyrosine kinases. *Nat. Immunol.* **6,** 390–395 (2005).
71. Pentcheva-hoang, T. *et al.* Depletion of Carcinoma-Associated Fibroblasts and Fibrosis Induces Immunosuppression and Accelerates Pancreas Cancer with Reduced Survival. *Cancer Cell* **25,** 719–734 (2014).
72. Beckhove, P., Esposito, I., Herzig, S., Huber, P. E. & Matthias, J. L. Pancreatic cancer microenvironment. *Int.J.Cancer* **121,** 699–705 (2007).
73. Steele, C. W. *et al.* Exploiting inflammation for therapeutic gain in pancreatic cancer. *Br. J. Cancer* **108,** 997–1003 (2013).
74. Hodous, B. L. *et al.* Synthesis , structural analysis , and SAR studies of triazine derivatives as potent , selective Tie-2 inhibitors. *Bioorganic Med. Chem. Lett.* **17,** 2886–2889 (2007).
75. Wu, D. *et al.* Peptides Anti-tumor effects of a novel chimeric peptide on S180 and H22 xenografts bearing nude mice. *Peptides* **31,** 850–864 (2010).
76. Lieu, C., Heymach, J. & Overman, M. Beyond VEGF : Inhibition of the Fibroblast Growth Factor Pathway and Antiangiogenesis. *Clin. Cancer Res.* **17,** 6130–6139 (2011).
77. Françoise, B. *et al.* Article Inhibition of Tumor Angiogenesis and Growth by a Small-Molecule Multi-FGF Receptor Blocker with Allosteric Properties Franc. *Cancer Cell* **23,** 477–488 (2013).
78. Wu, D. *et al.* Peptide-based cancer therapy : Opportunity and challenge. *CANCER Lett.* **351,** 1–10 (2014).
79. Wong, R. S. Y. Apoptosis in cancer: From pathogenesis to treatment. *J. Exp. Clin. Cancer Res.* **30,** 87 (2011).
80. Su, Z., Yang, Z., Xu, Y., Chen, Y. & Yu, Q. Apoptosis, autophagy, necroptosis, and cancer metastasis. *Mol. Cancer* **14,** 1–14 (2015).
81. Millimouno, F. M., Dong, J., Yang, L., Li, J. & Li, X. Targeting Apoptosis Pathways in Cancer and Perspectives with Natural Compounds from Mother Nature. *Cancer Prev. Res.* **7,** 1–28 (2014).
82. Liu, J., Lin, M., Yu, J., Liu, B. & Bao, J. Targeting apoptotic and autophagic pathways for cancer therapeutics. *Cancer Lett.* **300,** 105–114 (2011).
83. Padanilam, B. J. Cell death induced by acute renal injury : a perspective on the contributions of apoptosis and necrosis. *Am J Physiol Ren. Physiol* **284,** 608–627 (2003).
84. Jain, M. V *et al.* Interconnections between apoptotic , autophagic and necrotic pathways : implications for cancer therapy development Signalling pathways regulating apoptosis. *J. Cell. Mol. Med* **17,** 12–29 (2013).
85. Jutooru, I. *et al.* Mechanism of Action of Phenethylisothiocyanate and Other Reactive Oxygen Species-Inducing Anticancer Agents. *MCB* **34,** 2382–2395 (2014).
86. Waris, G. & Ahsan, H. Reactive oxygen species: role in the development of cancer and various chronic conditions. *J. Carcinog.* **5,** 1–8 (2006).
87. Pashkow, F. J. Oxidative Stress and Inflammation in Heart Disease: Do Antioxidants Have a Role in Treatment and/or Prevention? *Int. J. Inflam.* **2011,** 1–9 (2011).
88. Raposo, M. F. D. J., De Morais, A. M. M. B. & De Morais, R. M. S. C. Carotenoids from marine microalgae: A valuable natural source for the prevention of chronic diseases. *Mar. Drugs* **13,** 5128–5155 (2015).
89. Slater, A. F. G., Stefan, C., Nobel, I., Dobbelsteen, D. J. Van Den & Orrenius, S. Signalling mechanisms and oxidative stress in apoptosis. *Toxicol. Lett.* **82,** 149–153 (1995).
90. Lebedeva, I. V *et al.* Strategy for reversing resistance to a single anticancer agent in human prostate and pancreatic carcinomas. *PNAS* **104,** 1–6 (2007).
91. Cheng, X. *et al.* A TrxR inhibiting gold (I) NHC complex induces apoptosis through ASK1-

p38-MAPK signaling in pancreatic cancer cells. *Mol. Cancer* **13,** 1–15 (2014).
92. Jacquemin, G. *et al.* Granzyme B-induced mitochondrial ROS are required for apoptosis. *Cell Death Differ.* **22,** 862–874 (2014).
93. Subramani, R. *et al.* Nimbolide inhibits pancreatic cancer growth and metastasis through ROS-mediated apoptosis and inhibition of epithelial-to- mesenchymal transition. *Nat. Publ. Gr.* **6,** 1–12 (2016).
94. Budram-mahadeo, V., Morris, P. J. & Latchman, D. S. The Brn-3a transcription factor inhibits the pro-apoptotic effect of p53 and enhances cell cycle arrest by differentially regulating the activity of the p53 target genes encoding Bax and p21 CIP1 / Waf1. *Oncogene* **21,** 6123–6131 (2002).
95. Bode, A. M. & Dong, Z. POST-TRANSLATIONAL MODIFICATION OF p53 IN TUMORIGENESIS. *Nat. Rev. Cancer* **4,** 793–805 (2004).
96. Heinrich, M., West, J. E., Montellano, B. R. O. De & Rodriguez, E. ETHNOPHARMACOLOGY OF MEXICAN ASTERACEAE (COMPOSITAE). *Annu. Rev. Pharmacol. Toxicol* **38,** 539–565 (1998).
97. Khoo, B. L. *et al.* Single-cell profiling approaches to probing tumor heterogeneity. *Int. J. Cancer* **139,** 243–255 (2016).
98. Ruiz-Torres, V. *et al.* An updated review on marine anticancer compounds: The use of virtual screening for the discovery of small-molecule cancer drugs. *Molecules* **22,** 1–37 (2017).
99. Katiyar, S. Silymarin and skin cancer prevention : Anti-inflammatory , antioxidant and immunomodulatory effects. *Int. J. Oncol.* **26,** 169–176 (2005).
100. Zhang, X., Eden, H. S. & Chen, X. Peptides in cancer nanomedicine : Drug carriers , targeting ligands and protease substrates. *J. Control. Release* **159,** 2–13 (2012).
101. Vlieghe, P., Lisowski, V., Martinez, J. & Khrestchatisky, M. Synthetic therapeutic peptides : science and market. *Drug Discov.* **15,** (2010).
102. Craik, D. J., Fairlie, D. P., Liras, S. & Price, D. The Future of Peptide-based Drugs. *Chem Biol Drug Des* **81,** 136–147 (2013).
103. Zu, C. & Wang, J. Tumor-colonizing bacteria : A potential tumor targeting therapy. *Crit. Rev. Microbiol.* **40,** 225–235 (2013).
104. Kasinskas, R. W. & Forbes, N. S. *Salmonella typhimurium* Specifically Chemotax and Proliferate in Heterogeneous Tumor Tissue In Vitro. *InterScience* **94,** 710–721 (2006).
105. Cort, A., Ozben, T., Cort, A. & Ozben, T. Natural Product Modulators to Overcome Multidrug Resistance In Cancer Natural Product Modulators to Overcome Multidrug Resistance In Cancer. *Nutr. Cancer* **11,** 37–41 (2015).
106. Ouyang, L. *et al.* Plant natural products : from traditional compounds to new emerging drugs in cancer therapy. *Cell Prolif.* **47,** 506–515 (2014).
107. Malve, H. Exploring the ocean for new drug developments: Marine pharmacology. *J. Pharm. Bioallied Sci.* **8,** 83–91 (2016).
108. Manivasagan, P., Venkatesan, J., Sivakumar, K. & Kim, S. K. Pharmaceutically active secondary metabolites of marine actinobacteria. *Microbiol. Res.* **169,** 262–278 (2014).
109. Stewart, E. J. Growing unculturable bacteria. *J. Bacteriol.* **194,** 4151–4160 (2012).
110. Henrique Rampelotto, P. Extremophiles and Extreme Environments. *Life* **4,** 33–38 (2013).
111. Mashburn, L. M. & Whiteley, M. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* **437,** 422–425 (2005).
112. Kiuru, Paula D'Auria Valeria, M. et. a. Exploring Marine Resources for Bioactive Compounds. *Planta Med* **80,** 1234–1246 (2015).
113. Blunt, J. W., Copp, B. R., Keyzers, R. A., Munro, M. H. G. & Prinsep, M. R. Marine natural products. *Nat. Prod. Rep.* **31,** 237-323 (2013).
114. Hatcher, H., Planalp, R., Cho, J., Torti, F. M. & Torti, S. V. Curcumin: From ancient medicine

to current clinical trials. *Cell. Mol. Life Sci.* **65,** 1631–1652 (2008).
115. Hutzen, B. *et al.* Curcumin analogue GO-Y030 inhibits STAT3 activity and cell growth in breast and pancreatic carcinomas. *Int J Oncol* **35,** 867–872 (2009).
116. Liu, C., Dai, L., Dou, D., Ma, L. & Sun, Y. A natural food sweetener with anti-pancreatic cancer properties. *Oncogenesis* **5,** 1–9 (2016).
117. Gontang, E. A., Gaudêncio, S. P., Fenical, W. & Jensen, P. R. Sequence-based analysis of secondary-metabolite biosynthesis in marine actinobacteria. *Appl. Environ. Microbiol.* **76,** 2487–2499 (2010).
118. Weissman, K. J. Genetic engineering of modular PKSs : from combinatorial biosynthesis to synthetic biology. *Nat. Prod. Rep.* **33,** 1–28 (2015).
119. Marahiel, M. A. A structural model for multimodular NRPS assembly lines. *Nat. Prod. Rep.* **33,** 136–140 (2016).
120. Meier, J. L. & Burkart, M. D. The chemical biology of modular biosynthetic enzymes. *Chem. Soc. Rev.* **38,** 2012–2045 (2009).
121. Agrawal, S., Acharya, D., Adholeya, A., Barrow, C. J. & Deshmukh, S. K. Nonribosomal peptides from marine microbes and their antimicrobial and anticancer potential. *Front. Pharmacol.* **8,** 1–26 (2017).
122. Amoutzias, G. D., Chaliotis, A. & Mossialos, D. Discovery Strategies of bioactive compounds synthesized by nonribosomal peptide synthetases and type-I polyketide synthases derived from marine microbiomes. *Mar. Drugs* **14,** 1–20 (2016).
123. Matsubara, K. Recent advances in marine algal anticoagulants. *Curr. Med. Chem. Cardiovasc. Hematol. Agents* **2,** 13–19 (2004).
124. Gomes, K. M., Duarte, R. S. & Bastos, M. do C. de F. Lantibiotics produced by Actinobacteria and their potential applications (A review). *Microbiology* **163,** 109–121 (2017).
125. Herrmann, J., Fayad, A. A. & Müller, R. Natural products from myxobacteria: novel metabolites and bioactivities. *Nat. Prod. Rep.* **34,** 135–160 (2017).
126. Jackson, S. A. *et al.* Diverse and Abundant Secondary Metabolism Biosynthetic Gene Clusters in the Genomes of Marine Sponge Derived Streptomyces spp . Isolates. *Mar. Drugs* **16,** 1–18 (2018).
127. Reuter, S., Gupta, S. C., Chaturvedi, M. M. & Aggarwal, B. B. Oxidative stress, inflammation, and cancer: How are they linked? *Free Radic. Biol. Med.* **49,** 1603–1616 (2010).
128. Durakova, Z. Some current insights into Oxidative Stress. *Physiolo. Res* **59,** 459–469 (2010).
129. Yu, H. L. *et al.* Structural diversity of antipancreatic cancer capsimycins identified in mangrove-derived S*treptomyces xiamenensis* 318 and post-modification via a novel cytochrome P450 monooxygenase. *Sci. Rep.* **7,** 1–14 (2017).
130. Imhoff, J. F. Natural products from marine fungi - Still an underrepresented resource. *Mar. Drugs* **14,** 1–19 (2016).
131. Brinkmann, C. M., Marker, A. & Kurtböke, D. I. An overview on marine sponge-symbiotic bacteria as unexhausted sources for natural product discovery. *Diversity* **9,** 1–31 (2017).
132. Matsunaga, S., Okada, Y., Fusetani, N. & Van Soest, R. W. M. An antimicrobial C14 acetylenic acid from a marine sponge *Oceanapia* species. *J. Nat. Prod.* **63,** 690–691 (2000).
133. Miyaoka, H. *et al.* Antimalarial activity of kalihinol A and new relative diterpenoids from the Okinawan sponge, Acanthella sp. *Tetrahedron* **54,** 13467–13474 (1998).
134. Pruksakorn, P. *et al.* Trichoderins, novel aminolipopeptides from a marine sponge-derived *Trichoderma* sp., are active against dormant mycobacteria. *Bioorganic Med. Chem. Lett.* **20,** 3658–3663 (2010).
135. Song, F. *et al.* Trichodermaketones A-D and 7-O-methylkoninginin D from the marine fungus Trichoderma koningii. *J. Nat. Prod.* **73,** 806–810 (2010).
136. El-Hossary, E. M. *et al.* Antifungal potential of marine natural products. *Eur. J. Med. Chem.*

126, 631–651 (2017).
137. Pabel, C. T. *et al.* Antimicrobial Activities and Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Bacillus Isolates from the Marine Sponge *Aplysina aerophoba*. *Mar. Biotechnol.* **5,** 424–434 (2003).
138. Kim, S. K., Thomas, N. V. & Li, X. in *Advances in Food and Nutrition Research* **64,** 213–224 (Elsevier Inc., 2011).
139. Guzman A., E. *et al.* Leiodermatolide, a novel marine natural product, has potent cytotoxic and antimitotic activity against cancer cells, appears to affect microtubule dynamics , and exhibits antitumor activity. *Int. J. Cancer* **2126,** 2116–2126 (2016).
140. Nieto, F. R. *et al.* Tetrodotoxin (TTX) as a therapeutic agent for pain. *Mar. Drugs* **10,** 281–305 (2012).
141. Asano, N. Glycosidase inhibitors: Update and perspectives on practical use. *Glycobiology* **13,** 93–104 (2003).
142. Pandey, S. & *et.al*. Diversity of marine bacteria producing beta- glucosidase inhibitors. *Microb. Cell Fact.* **12,** 1–7 (2013).
143. Still, P. C. & *et.al.* Scrutinizing the Scaffolds of Marine Biosynthetics from Different Source Organisms: Gram-Negative Cultured Bacterial Products Enter Center Stage. *J. Nat. Prod.* **77,** 690–702 (2014).
144. Cassier A., P. & *et.al*. Trabectedin and its potential in the treatment of soft tissue sarcoma. *Ther. Clin. Risk Manag.* **4,** 109–116 (2008).
145. Mariottini, G. L. & Grice, I. D. Antimicrobials from cnidarians. A new perspective for anti-infective therapy? *Mar. Drugs* **14,** 1–19 (2016).
146. Ronowicz, M., Kukliński, P. & Mapstone, G. M. Trends in the diversity, distribution and life history strategy of arctic Hydrozoa (Cnidaria). *PLoS One* **10,** 1–19 (2015).
147. Babonis, L. S. & Martindale, M. Q. Old cell, new trick? Cnidocytes as a model for the evolution of novelty. *Integr. Comp. Biol.* **54,** 714–722 (2014).
148. Gravili, C. & et.al. Association between luminous bacteria and Hydrozoa in the northern Ionian Sea. *J. Mar. Biol. Assoc. United Kingdom* **92,** 1317–1324 (2012).
149. Fraune, S. *et al.* Bacteria-bacteria interactions within the microbiota of the ancestral metazoan Hydra contribute to fungal resistance. *ISME J.* **9,** 1543–1556 (2015).
150. Augustin, R., Fraune, S., Franzenburg, S. & Bosch, T. C. G. in *Recent advances on model hosts* **710,** 71–80 (2012).
151. King, A. & et.al. Targeting DnaN for tuberculosis therapy using novel griselimycins. *Science (80-.).* **348,** 1106–1112 (2015).
152. Yin, M. *et al.* A specific antimicrobial protein CAP-1 from *Pseudomonas* sp. isolated from the jellyfish Cyanea capillata. *Int. J. Biol. Macromol.* **82,** 488–496 (2016).
153. Wang, H. *et al*. K. Atlas of nonribosomal peptide and polyketide biosynthetic pathways reveals common occurrence of nonmodular enzymes. *Proc. Natl. Acad. Sci.* **111,** 9259–9264 (2014).
154. Fischbach, M. A. & Walsh, C. T. Assembly-Line Enzymology for Polyketide and Nonribosomal Peptide Antibiotics : Logic , Machinery , and Mechanisms. *Chem.Rev.* **5,** 3468–3496 (2006).
155. Brito, Â. *et al.* Bioprospecting Portuguese Atlantic coast cyanobacteria for bioactive secondary metabolites reveals untapped chemodiversity. *ALGAL* **9,** 218–226 (2015).
156. Charlop-powers, Z. *et al.* Global biogeographic sampling of bacterial secondary metabolism. *Elife* **4,** 1–10 (2015).
157. Yu, D., Xu, F., Zeng, J. & Zhan, J. Critical Review Type III Polyketide Synthases in Natural Product Biosynthesis. *Crit. Rev.* **64,** 285–295 (2012).
158. Tambadou, F. *et al.* Novel nonribosomal peptide synthetase (NRPS) genes sequenced from intertidal mudflat bacteria. *FEMS Microbiol. Lett.* **357,** 123–130 (2014).

159. Amos, G. C. A. *et al.* Designing and Implementing an Assay for the Detection of Rare and Divergent NRPS and PKS Clones in European , Antarctic and Cuban Soils. *PLoS One* **10,** 1–15 (2015).
160. Du, L. & Lou, L. Reports PKS and NRPS release mechanisms. *Nat. Prod. Rep.* **27,** 255–278 (2010).
161. Weber, T. & Marahiel, M. A. Exploring the domain structure of modular nonribosomal peptide synthetases. *Structure* **9,** 3–9 (2001).
162. Rath, C. M., Scaglione, J. B., Kittendorf, J. D. & Sherman, D. H. in *Comprehensive Natural Products II* 454–492 (2010).
163. Selvin, J. *et al.* Ketide Synthase (KS) domain prediction and analysis of iterative type II PKS gene in marine sponge-associated actinobacteria producing biosurfactants and antimicrobial agents. *Front. Microbiol.* **7,** 1–12 (2016).
164. Crosa, J. H. & Walsh, C. T. Genetics and Assembly Line Enzymology of Siderophore Biosynthesis in Bacteria. *Microbiol. Mol. Biol. Rev.* **66,** 223–249 (2002).
165. Rodrigues, L., Banat, I. M., Teixeira, J. & Oliveira, R. Biosurfactants: Potential applications in medicine. *J. Antimicrob. Chemother.* **57,** 609–618 (2006).
166. Gomes, E. S., Schuch, V. & Lemos, E. G. de M. Biotechnology of polyketides: New breath of life for the novel antibiotic genetic pathways discovery through metagenomics. *Brazilian J. Microbiol.* **44,** 1007–1034 (2013).
167. Rutledge, P. J. & Challis, G. L. Discovery of microbial natural products by activation of silent biosynthetic gene clusters. *Nat. Rev. Microbiol.* **13,** 509–523 (2015).
168. Shen, B. Polyketide biosynthesis beyond the type I , II and III polyketide synthase paradigms. *Curr. Opin. Chem. Biol.* **7,** 285–295 (2003).
169. Bruheim, P. *et al.* Chemical diversity of polyene macrolides produced by *Streptomyces noursei* ATCC 11455 and recombinant strain ERD44 with genetically altered polyketide synthase NysC. *Antimicrob. Agents Chemother.* **48,** 4120–4129 (2004).
170. Huczyński, A. Polyether ionophores - Promising bioactive molecules for cancer therapy. *Bioorganic Med. Chem. Lett.* **22,** 7002–7010 (2012).
171. Wang, H., Sivonen, K. & Fewer, D. P. Genomic insights into the distribution , genetic diversity and evolution of polyketide synthases and nonribosomal peptide synthetases. *Curr. Opin. Genet. Dev.* **35,** 79–85 (2015).
172. Staunton, J. & Weissman, K. J. Polyketide biosynthesis : a millennium review. *Milen. Rev.* **18,** 380–416 (2001).
173. Weissman, K. J. Uncovering the structures of modular polyketide synthases. *Nat. Prod. Rep.* **32,** 436–453 (2014).
174. Albarracín, V. H. Ketide Synthase (KS) Domain Prediction and Analysis of Iterative Type II PKS Gene in Marine Sponge-Associated Actinobacteria Producing Biosurfactants and Antimicrobial Agents. **7,** 1–12 (2016).
175. Maiya, S., Grundmann, A., Li, X., Li, S. M. & Turner, G. Identification of a hybrid PKS/NRPS required for pseurotin A biosynthesis in the human pathogen *Aspergillus fumigatus*. *ChemBioChem* **8,** 1736–1743 (2007).
176. Ishikawa, M. *et al.* Pseurotin A and its analogues as inhibitors of immunoglobuline E production. *Bioorganic Med. Chem. Lett.* **19,** 1457–1460 (2009).
177. Nikolouli, K. & Mossialos, D. Bioactive compounds synthesized by non-ribosomal peptide synthetases and type-I polyketide synthases discovered through genome-mining and metagenomics. *Biotechnol. Lett.* **34,** 1393–1403 (2012).
178. Seydlová, G. & Svobodová, J. Review of surfactin chemical properties and the potential biomedical applications. *Cent. Eur. J. Med.* **3,** 123–133 (2008).
179. Marahiel, M. A., Stachelhaus, T. & Mootz, H. D. Modular Peptide Synthetases Involved in Nonribosomal Peptide Synthesis. *Chem. Rev* **97,** 2651–2673 (1997).

180. Mast, Y. *et al.* Synthetic Biology of secondary metabolite biosynthesis in actinomycetes : Engineering precursor supply as a way to optimize antibiotic production Engineering precursor supply as a way to optimize antibiotic production. *FEBS Lett.* **586,** 2171–2176 (2012).
181. Economou, N. J., Cocklin, S. & Loll, P. J. High-resolution crystal structure reveals molecular details of target recognition by bacitracin. *Proc. Natl. Acad. Sci.* **110,** 14207–14212 (2013).
182. Dang, T. & Süssmuth, R. D. Bioactive Peptide Natural Products as Lead Structures for Medicinal Use. *Acc. Chem. Res.* **50,** 1566–1576 (2017).
183. Krastel, P. *et al.* Nannocystin A: An Elongation Factor 1 Inhibitor from Myxobacteria with Differential Anti-Cancer Properties. *Angew. Chemie - Int. Ed.* **54,** 10149–10154 (2015).
184. Hoffmann, H. *et al.* Discovery, Structure Elucidation, and Biological Characterization of Nannocystin A, a Macrocyclic Myxobacterial Metabolite with Potent Antiproliferative Properties. *Angew. Chemie - Int. Ed.* **54,** 10145–10148 (2015).
185. Awakawa, T. *et al.* Reprogramming of the antimycin NRPS-PKS assembly lines inspired by gene evolution. *Nat. Commun.* **9,** 2–4 (2018).
186. Deane, C. in *Nature Medicine* **16,** 1198–1198 (2016).
187. Hertweck, C. The Biosynthetic Logic of Polyketide Diversity Angewandte. *Angew. Chem. Int* **48,** 4688–4716 (2009).
188. Perdicaris, S., Vlachogianni, T. & Valavanidis, A. Bioactive Natural Substances from Marine Sponges: New Developments and Prospects for Future Pharmaceuticals. *Nat Prod Chem Res* **1,** 1–8 (2013).
189. Delgoda, R. & Murray, J. E. in *Pharmacognosy: Fundamentals, Applications and Strategy* 93–100 (2016)
190. Cholewiński, G., Dzierzbicka, K. & Kołodziejczyk, A. M. Natural and synthetic acridines/acridones as antitumor agents: Their biological activities and methods of synthesis. *Pharmacol. Reports* **63,** 306–336 (2011).
191. Bae, J. S. Antithrombotic and profibrinolytic activities of phloroglucinol. *Food Chem. Toxicol.* **49,** 1572–1577 (2011).
192. Boettger, D. & Hertweck, C. Molecular Diversity Sculpted by Fungal PKS – NRPS Hybrids. *ChemBioChem* **14,** 28–42 (2013).
193. Rizzo, C. & Lo Giudice, A. Lo. Marine invertebrates: Underexplored sources of bacteria producing biologically active molecules. *Diversity* **10,** 1–36 (2018).
194. Machado, H., Sonnenschein, E. C., Melchiorsen, J. & Gram, L. Genome mining reveals unlocked bioactive potential of marine Gram-negative bacteria. *BMC Genomics* **16,** 1–12 (2015).
195. Wietz, M., Mansson, M., Gotfredsen, C. H. & Larsen, T. O. Antibacterial Compounds from Marine Vibrionaceae Isolated on a Global Expedition. *Mar. Drugs* **8,** 2946–2960 (2010).
196. Beer, L. L. & Moore, B. S. Biosynthetic Convergence of Salinosporamides A and B in the Marine Actinomycete *Salinispora tropica*. *Am. Chem. Soc.* **9,** 845–848 (2007).
197. Feling, R. H. *et al.* Salinosporamide A: A Highly Cytotoxic Proteasome Inhibitor from a Novel Microbial Source, a Marine Bacterium of the New Genus *Salinospora*. *Angew. Chem. Int* **42,** 355–357 (2003).
198. Flatt, P. M. *et al.* Characterization of the initial enzymatic steps of barbamide biosynthesis. *J. Nat. Prod.* **69,** 938–944 (2006).
199. Chang, Z. *et al.* The barbamide biosynthetic gene cluster : a novel marine cyanobacterial system of mixed polyketide synthase (PKS) -non-ribosomal peptide synthetase (NRPS) origin involving an unusual trichloroleucyl starter unit. **296,** 235–247 (2002).
200. Chang, Z. *et al.* The barbamide biosynthetic gene cluster : a novel marine cyanobacterial system of mixed polyketide synthase (PKS) -non-ribosomal peptide synthetase (NRPS) origin involving an unusual trichloroleucyl starter unit. *Gene* **296,** 235–247 (2002).

201. Udwary, D. W. *et al.* Genome sequencing reveals complex secondary metabolome in the marine actinomycete *Salinispora tropica*. *Proc. Natl. Acad. Sci.* **104,** 10376–10381 (2007).
202. Sheehan, L. S. *et al.* Engineering of the Spinosyn PKS : Directing Starter Unit Incorporation. *J. Nat. Prod.* **69,** 1702–1710 (2006).
203. Ziemert, N. *et al.* Diversity and evolution of secondary metabolism in the marine actinomycete genus *Salinispora*. *Proc. Natl. Acad. Sci.* **111,** 1130–1139 (2014).
204. Frasch, H. J., Medema, M. H., Takano, E. & Breitling, R. Design-based re-engineering of biosynthetic gene clusters: Plug-and-play in practice. *Curr. Opin. Biotechnol.* **24,** 1144–1150 (2013).
205. Fischbach, M. A. Antibiotics from microbes: converging to kill. *Curr. Opin. Microbiol.* **12,** 520–527 (2009).
206. Zhang, L. *et al.* Exploring novel bioactive compounds from marine microbes. *Curr. Opin. Microbiol.* **8,** 276–281 (2005).
207. Giubergia, S. & et.al. in *The Marine Microbiome* 345–376 (2016).
208. Jiang, J. *et al.* Identification of novel surfactin derivatives from NRPS modification of *Bacillus subtilis* and its antifungal activity against *Fusarium moniliforme*. *BMC Microbiol.* **16,** 1–14 (2016).
209. Sieber, S. A. & Marahiel, M. A. Molecular Mechanisms Underlying Nonribosomal Peptide Synthesis : Approaches to New Antibiotics. *Chem.Rev.* **705,** 715–738 (2005).
210. Herdini, C. *et al.* Diversity of Nonribosomal Peptide Synthetase Genes in the Anticancer-Producing Actinomycetes Isolated from Marine Sediment in Indonesia Diversity of Nonribosomal Peptide Synthetase Genes in the Anticancer- Producing Actinomycetes Isolated from Marine Sed. *Indones. J. Biotechnol.* **20,** 34–41 (2015).
211. Solenberg, P. J. *et al.* Production of hybrid glycopeptide *Streptomyces toyocaensis* antibiotics in vitro and in. *Chem. Biol.* **4,** 195–202 (1997).
212. Matsushima, P. & Baltz, R. H. A gene cloning system for 'Streptomyces toyocaensis '. *Microbiology* **142,** 261–267 (1996).
213. Banik, J. J., Craig, J. W., Calle, P. Y. & Brady, S. F. enzymes for generating libraries of unnatural natural products. *J Am Chem Soc.* **132,** 15661–15670 (2010).
214. Baltz, R. H. & Bleomycin, B. Genetic manipulation of secondary metabolite biosynthesis for improved production in Streptomyces and other actinomycetes. *J. Ind. Microbiol. Biotechnol.* **43,** 347–370 (2015).
215. Nikapitiya, C. in *Advances in Food and Nutrition Research* **65,** 363–387 (Elsevier Inc., 2012).
216. Guo, J. P. *et al.* Thermolides, potent nematocidal PKS-NRPS hybrid metabolites from thermophilic fungus *Talaromyces thermophilus. J. Am. Chem. Soc.* **134,** 20306–20309 (2012).
217. Desriac, F. *et al.* Antimicrobial peptides from marine proteobacteria. *Mar. Drugs* **11,** 3632–3660 (2013).
218. Donia, M. S. *et al.* A Systematic Analysis of Biosynthetic Gene Clusters in the Human Microbiome Reveals a Common Family of Antibiotics. *Cell* **158,** 1402–1414 (2014).
219. Zhang, W., Li, Z., Miao, X. & Zhang, F. The screening of antimicrobial bacteria with diverse novel nonribosomal peptide synthetase (NRPS) genes from South China sea sponges. *Mar. Biotechnol.* **11,** 346–355 (2009).
220. Yu, Z. *et al.* Targeted Delivery of Bleomycin: A Comprehensive Anticancer Review. *Curr. Cancer Drug Targets* **16,** 509–521 (2016).
221. Samak, M. El, Solyman, S. M., Hanora, A. & Sea, R. Antimicrobial activity of bacteria isolated from Red Sea marine invertebrates. *Biotechnol. Reports* **20,** 1–8 (2018).
222. Piel, J. in *Comprehensive Natural Products II* 475–510 (2010).
223. Safari Fitriani, W., Chasanah, E. & Wahyudi Tri, A. Antibacterial and anticancer activities of marine bacterial extracts and detection of genes for bioactive compounds synthesis. *Int. J.*

Pharm. Pharm. Sci. **8,** 55–59 (2016).
224. Frank, J. A. *et al.* Critical Evaluation of Two Primers Commonly Used for Amplification of Bacterial 16S rRNA Genes. *Appl. Environ. Microbiol.* **74,** 2461–2470 (2008).
225. V. Berridge, M., Herst M., P. & Tan S., A. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. *Biotechnol. Annu. Rev.* **11,** 127–151 (2016).
226. Radjasa, O.K., S.I.O. Salasia, A. Sabdono, J. Weise, J.F. Imhoff, C. L. and M. J. R. Antibacterial Activity of Marine Bacterium *Pseudomonas sp*. Associated with Soft Coral Sinularia polydactyla against Streptococcus equi Subsp. zooepidemicus. *International Journal of Pharmacology* **3,** 170–174 (2007).
227. Stabili, L. *et al.* Aglaophenia octodonta (Cnidaria, Hydrozoa) and the Associated Microbial Community: a Cooperative Alliance? *Microb. Ecol.* **76,** 258–271 (2018).
228. Boero, F., Bucci, C., Colucci, A. M. R., Gravili, C. & Stabili, L. Obelia (Cnidaria, Hydrozoa, Campanulariidae): A microphagous, filter-feeding medusa. *Mar. Ecol.* **28,** 178–183 (2007).
229. Costa, S. & Rugiero, I. Synthesis of Hydroxy-Androstane-1,4-Diene-3, 17-Dione Derivatives by Biotransformations of Bile Acids with *Pseudomonas alcaliphila*. *Org. Chem. Curr. Res.* **4,** 1–4 (2015).
230. Kim, M., Oh, H.-S., Park, S.-C. & Chun, J. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. *Int. J. Appl. Eng. Res.* **64,** 346–351 (2014).
231. Oves M, N. F. Red Sea Microbial Diversity for Antimicrobial and Anticancer Agents. *J. Mol. Biomark. Diagn.* **07,** 1–14 (2016).
232. Chau, R., Kalaitzis, J. A., Wood, S. A. & Neilan, B. A. Diversity and biosynthetic potential of culturable microbes associated with toxic marine animals. *Mar. Drugs* **11,** 2695–2712 (2013).
233. Caboche, S., Leclère, V., Pupin, M., Kucherov, G. & Jacques, P. Diversity of monomers in nonribosomal peptides: Towards the prediction of origin and biological activity. *J. Bacteriol.* **192,** 5143–5150 (2010).
234. Tang, G.-L., Cheng, Y.-Q. & Shen, B. Polyketide Chain Skipping Mechanism in the Biosynthesis of the Hybrid Nonribosomal Peptide-Polyketide Antitumor Antibiotic Leinamycin in *Streptomyces atroolivaceus* S-140. *J Nat Prod.* **69,** 387–393 (2008).
235. Zhang, X. G. *et al.* Antitumor activity of a Rhodococcus sp. Lut0910 isolated from polluted soil. *Tumor Biol.* **39,** 1–9 (2017).
236. Abuderman, A. A. Anti-Cancer and Antimicrobial Activity of Novel Bacterium *Brevibacterium* Sp . from Saudi Arabia Peninsula. *J Res Med Dent Sci* **6,** 25–30 (2018).
237. Sagar, S. *et al.* Cytotoxic and apoptotic evaluations of marine bacteria isolated from brine-seawater interface of the Red Sea. *BMC Complement. Altern. Med.* **13,** 1–8 (2013).
238. El-Moneam, N. M. A., El-Assar, S. A., Shreadah, M. A. & Nabil-Adam, A. Isolation, identification and molecular screening of *Pseudomonas sp*. metabolic pathways NRPs and PKS associated with the Red Sea Sponge, Hyrtios aff. Erectus, Egypt. *J. Pure Appl. Microbiol.* **11,** 1299–1311 (2017).
239. Zheng, L. *et al.* Identification of norharman as the cytotoxic compound produced by the sponge (Hymeniacidon perleve)-associated marine bacterium *Pseudoalteromonas piscicida* and its apoptotic effect on cancer cells. *Biotechnol. Appl. Biochem.* **44,** 135–142 (2006).
240. Sannino, F. *et al. Pseudoalteromonas haloplanktis* TAC125 produces 4-hydroxybenzoic acid that induces pyroptosis in human A459 lung adenocarcinoma cells. *Sci. Rep.* **8,** 1–10 (2018).

Annexes

Potential of marine bacteria to synthesize bioactive compounds for medical applications

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IV Postgrad Symposium in Biomedicine

Introduction

- Current available drugs to treat several diseases are loosing their efficacy.
- New and alternative compounds/approaches are needed.
- Marine natural products have been isolated and identified from different marine sources like algae, cnidarians and microorganisms.
- Polyketides (PKs) and non-ribosomal peptides (NRPs) are groups of natural compounds with a variety of relevant biological activities for medical purposes [10].
- **Goal:** screening of NRPS and PKS genes in microorganisms isolated from cnidarian species.

Material and Methods

- Extraction and isolation of bacteria in association with 6 cnidarian species collected in the Atlantic ocean (20-38 m depth).
- Long term storage of pure bacterial isolates.
- Extraction of gDNA from bacterial suspension by heat lysis
- PKS and NRPS genes PCR-screened with degenerate primers (ketosynthase and adenylation, respectively).

Results and Discussion

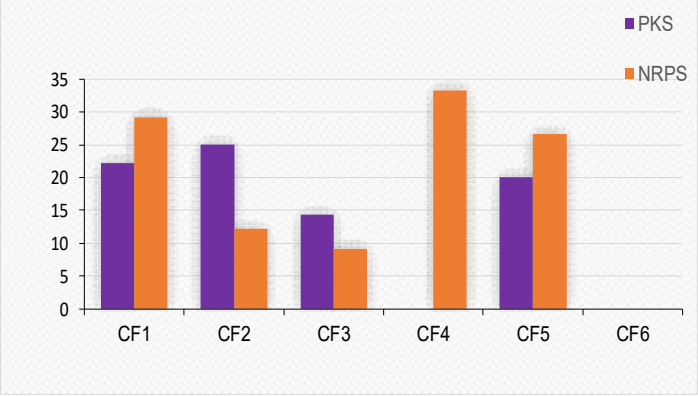

Fig. 2: Percentage of bacteria presenting PKS and NRPS genes in each cnidarian species tested.

Table 1: Bacterial clones isolated and screened for PKS and NRPS genes. (+) stands for positive and (-) for negative presence of the genes; CF – cnidarian species; ns - not screened.

CF1			CF2						CF3			CF4			CF5			CF6		
Clones	PKS	NRPS	Clones	PKS	NRPS	Clones	PKS	NRPS	Clones	PKS	NRPS	Clones	PKS	NRPS	Clones	PKS	NRPS	Clones	PKS	NRPS
MT1	ns	(-)	MT25	(-)	(-)	MT41	ns	(-)	MT58	(-)	(-)	MT69	(-)	(+)	MT81	(-)	(-)	MT96	ns	(-)
MT2	ns	(-)	MT26	ns	(-)	MT42	(-)	(+)	MT59	(-)	(-)	MT70	ns	(-)	MT82	(-)	(+)	MT97	ns	(-)
MT3	ns	(-)	MT27	ns	(-)	MT43	ns	(-)	MT60	(+)	(+)	MT71	ns	(+)	MT83	(-)	(+)	MT98	ns	(-)
MT4	ns	(-)	MT28	ns	(-)	MT44	(-)	(-)	MT61	ns	(-)	MT72	(-)	(+)	MT84	ns	(-)	MT99	(-)	(-)
MT5	ns	(+)	MT29	ns	(-)	MT45	ns	(-)	MT62	ns	(-)	MT73	(-)	(-)	MT85	ns	(-)	MT100	ns	(-)
MT6	ns	(+)	MT30	ns	(-)	MT46	(-)	(-)	MT63	ns	(-)	MT74	ns	(-)	MT86	ns	(-)	MT101	ns	(-)
MT7	ns	(-)	MT31	ns	(-)	MT47	ns	(-)	MT64	ns	(-)	MT75	(-)	(-)	MT87	ns	(-)			
MT8	ns	(-)	MT32	(-)	(-)	MT48	ns	(-)	MT65	(-)	(-)	MT76	ns	(-)	MT88	ns	(-)			
MT9	ns	(-)	MT33	ns	(-)	MT49	ns	(-)	MT66	(-)	(-)	MT77	(-)	(-)	MT89	(+)	(-)			
MT10	ns	(+)	MT34	ns	(-)	MT50	ns	(-)	MT67	(-)	(-)	MT78	(-)	(-)	MT90	(+)	(-)			
MT11	(-)	(-)	MT35	ns	(-)	MT51	(+)	(+)	MT68	(-)	(-)	MT79	(-)	(+)	MT91	(-)	(+)			
MT12	ns	(-)	MT36	ns	(-)	MT52	ns	(-)				MT80	ns	(-)	MT92	(-)	(-)			
MT13	ns	(-)	MT37	ns	(-)	MT53	ns	(+)							MT93	(-)	(+)			
MT14	(-)	(-)	MT38	ns	(-)	MT54	ns	(-)							MT94	(-)	(-)			
MT15	ns	(+)	MT39	(-)	(-)	MT55	ns	(-)							MT95	(-)	(-)			
MT16	(-)	(-)	MT40	(+)	(+)	MT56	ns	(-)												
MT17	ns	(-)				MT57	ns	(-)												
MT18	(-)	(-)																		
MT19	(-)	(+)																		
MT20	ns	(-)																		
MT21	(-)	(+)																		
MT22	(-)	(-)																		
MT23	(+)	(+)																		
MT24	(+)	(-)																		

Table 2: Examples of compounds associated with PKS and NRPS genes and PKS-NRPS hybrids, as well as their biological activities.

Genes	Compounds	Activities	References
PKS	Biosurfactants	Antimicrobial; Antibiofilm	1 2
	Aromatic polyketides	Antibiotic; Antioxidant; Anticancer; Immunosuppressive Anti-inflammatory	3
NRPS	Surfactin	Antibiotic; Antifungal; Antiviral; Anti-tumour; Hemolytic	4 5
	Bacitracin	Antibiotic, Antifungal	4 6
Hybrids	Tetramic acids (ex.: Equisetin, Pseurotin A)	Toxicity; Neuritogenic properties; Antifungal;	7 8 9

- Bacterial strains isolated from CF6 did not present PKS or NRPS genes (Table 1, Fig. 2)
- Cnidarians harbouring bacteria with these biosynthetic genes can be ordered as:

  PKS ► CF2 > CF1> CF5> CF3> CF4;

  NRPS ► CF4> CF1> CF5> CF2> CF3;
- Some bacterial strains extracted from CF1, CF2, CF3 and CF5 are PKS-NRPS hybrids because these groups exhibit both genes (Fig. 2, Table 1).
- The screening shows a general tendency of a higher representation of NRPS-positive strains than PKS-positive strains (Table 1). Other authors, however, obtained more PKS representation in marine cyanobacteria [11].
- The bacteria presenting PKS and NRPS genes will be further studied as to certain the exact compounds they potentially express, as well as it will be tested their biological activity for different medical applications (Table 2).

Conclusions

- The results of this screening shows a greater occurrence of NRPS genes in bacteria isolated from cnidarian species.
- Further studies on the molecular structure, expression/biosynthesis and activity of these genes on these bacteria will help to unravel potentially new natural compounds.
- Screening for NRPS and PKS genes as indicators of the capacity of bacteria to produce clinically significant bioactive metabolites constitutes a beneficial and reliable approach.

References

[1] Selvin, J. *et al. Front. Microbiol.* **7**, 1–12 (2016).
[2] Rodrigues, L. *et al. J. Antimicrob. Chemother.* **57**, 609–618 (2006).
[3] Rutledge *et al. Nat. Rev. Microbiol.* **13**, 509–523 (2015).
[4] Tambadou, F. *et al. FEMS Microbiol. Lett.* **357**, 123–130 (2014).
[5] Ongey *et al. Microb. Cell Fact.* **15**, 1–16 (2016).
[6] Economou *et al. Proc. Natl. Acad. Sci.* **110**, 14207–14212 (2013).
[7] Boettger *et al.ChemBioChem* 28–42 (2013).
[8] Maiya, Sel. *et al. ChemBioChem* **8**, 1736–1743 (2007).
[9]Ishikawa, M. *et al. Bioorganic Med. Chem. Lett.* **19**, 1457–1460 (2009).
[10] Machado, H., et al. *BMC Genomics*, 1–12 (2015).
[11] Brito, Â. *et al. ALGAL* **9**, 218–226 (2015).

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