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Chapter 8

Drug Discovery from Natural Products for Pancreatic Cancer

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Abstract

Since ancient times, natural products (NPs) have been used as anti-infectives, anti-inflammatories, antioxidants, analgesics and antitumorals and many compounds derived from NPs are in clinical use. The use of plants in traditional medicine for multiple purposes is well known, and throughout recent history, metabolites of microbial origin have had an extraordinary impact on the welfare of humanity. There is an outstanding diversity of chemical structures that nature, and especially microorganisms, are able to produce, due to millenniums of evolution. Since only a small amount of the world's biodiversity has been evaluated for potential biological activity, many more useful natural lead compounds await discovery, the challenge being how to access this natural chemical diversity. However, the validation and selection of primary screening assays, both phenotypic and target-based, are vital to guaranteeing a selection of extracts or molecules with relevant pharmacological action. The screening of antitumor agents against pancreatic cancer (PC) involves the use of established cell lines, cancer stem cells and spheroids that mimic the patient's tumor. Improvements in the discovery of natural products along with the emergence of new technologies in cancer screening assays, promise the discovery of new and valuable drugs to tackle pancreatic cancer in the coming years.

Keywords: natural products, pancreatic cancer, fungi, bacteria, plant, dereplication, phenotypic-based screening, target-based screening, high-throughput screening, spheroids, organoids

1. Introduction

Pancreatic cancer (PC) is the fourth leading cause of cancer mortality in the United States and accounts for about 3% of all newly diagnosed cancers each year. The American Cancer

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Society estimated that 43,090 people would die from this disease in 2017 [1]. PC is a multifactorial disease, making it difficult to treat with conventional antitumoral therapy. The poor survival rate is likely due to the lack of early diagnosis, rapid disease progression, high metastasis rate and unsuccessful outcome of treatment. Although there are different types of treatment available for PC, most patients have no recognizable symptoms, making early diagnosis difficult [2], and chemotherapies or radiation therapies are often ineffective. While surgery is generally considered the best treatment option, only 10–20% of patients with PC are surgical candidates. Radiation therapy and chemotherapy are two other common methods for treating PC. However, the biochemical and physiological characteristics of PC appear to limit the effectiveness of these standard forms of therapy, in part due to genetic alterations.

Furthermore, recent studies have shown that PC is highly enriched with cancer stem cell (CSC) subpopulations that are resistant to current chemotherapeutic drugs and therefore promote tumor recurrence [3]. CSCs, also called tumor-initiating cells, share many characteristics with normal stem cells, such as asymmetric cell division, where each CSC generates one daughter cell with self-renewal capacity and another cell destined to differentiate. The self-renewal capacity helps to maintain the number of CSCs within the tumor, and its descendent progeny generate the mass of the tumor [4]. CSCs also exhibit unique features, such as their metastasis ability and the ability to remain in a quiescent state, which protect them from the chemotherapeutic drugs developed to target actively dividing cells. Another phenomenon frequently seen in PC is the cancer cell epithelial to mesenchymal transition (EMT), associated with metastasis, CSC generation and treatment resistance [5].

Although many efforts have been made to find a cure, there is much work left to be done because PC is still an unmet medical need affecting an increasing number of patients every year. Current research categorizes anticancer agents into two major groups based on their mechanisms of action and origins: cytotoxic anticancer agents and molecular targeted therapeutic agents. But the failure of current drugs and treatments can be attributed, in part, to our limited understanding of the targets of the disease and to a lack of reliable disease-relevant screening methods that mimic the key pathophysiological features of cancer. Therefore, efforts now should be addressed to developing new models and assay formats, innovative screening technologies that better summarize *in vivo* physiology [6], and new, effective and safe compounds or combinations.

NPs have been, and still are, one of the main sources of drug discovery. According to the data from Newman and Cragg [7], most new FDA-approved drugs between 1981 and 2014 were derived from NP structures. Natural constituents are widely distributed in various natural sources, including plants, microorganisms and invertebrates. Plant-derived molecules continue to make up a large portion of the pharmaceuticals in the clinic, and the production of antibiotics by microorganisms was one of the biggest breakthroughs in the history of drug discovery in the twentieth century. Bacteria and fungi represent two of the most important sources for novel therapeutic agents exhibiting the most diverse biological actions. Since only a small amount of the world's biodiversity has been evaluated for potential biological activity, many more useful natural lead compounds await discovery, the challenge being how to access this natural chemical diversity.

In the last decades, despite the difficulty of finding novel scaffolds, an increasing number of research groups have dedicated numerous efforts to exploring alternative sources, such as the marine environment, which has become an extraordinarily rich source of new drugs. At present, plants, microorganisms and marine invertebrates are major sources of NPs for discovering novel drugs.

To better understand the huge impact of NPs on cancer pharmaceuticals, it is worth mentioning that out of 155 small molecules used as chemotherapeutics, 73 are directly NPs and another 40 are derivatives or synthetic NP mimetics [8]. Furthermore, current research trends in the field suggest an optimistic future for NPs in cancer prevention and new therapeutics drug discovery. Because of the complex chemistry generated by centuries of evolution of NPs, more success is expected in drug discovery with NPs than with synthetic molecules. However, that complexity of the natural molecules requires a coordinated effort from the interaction of multidisciplinary research areas with new and more sophisticated analytical and technical expertise in order to extract, isolate, identify and turn them into promising leads.

This chapter provides insights into the advances in cancer drug discovery from NPs using high-throughput screening (HTS) technologies, with a special emphasis on the biological tools and cell-based assay platforms implemented to untap new NP scaffolds with novelty in their mode of actions, and the most promising natural molecules under development today.

2. Natural products in drug discovery

The use of plants in traditional medicine is well known for multiple purposes. Over the millennia, plants have been used as anti-infectives, anti-inflammatories, antioxidants, analgesics and antitumorals, and there are many compounds derived from plants used in the clinic. The most famous example to date is probably the synthesis of the anti-inflammatory agent acetylsalicylic acid (aspirin), derived from salicin and isolated from the bark of the willow tree *Salix alba* L [9]. Other examples are morphine, codeine, digitoxin, quinine and the antitumorals paclitaxel, vincristine and vinblastine, and a long list of other drugs. Metabolites of plants still constitute a major area of research but the microbial secondary metabolism of bacteria and fungi has been intensely explored in industrial screening programs in the last decades.

The biosynthesis and breakdown of proteins, fats, nucleic acids and carbohydrates, which are essential to all living organisms, is known as primary metabolism, while the mechanism by which an organism biosynthesizes other compounds is known as secondary metabolism. These secondary metabolites are known as NPs and are often found to be unique to an organism or species [10]. Generally, secondary metabolites are not essential for the growth of an organism and are produced either as a result of the organism adapting to its surroundings or to act as a possible defense mechanism [11]. The biosynthesis of secondary metabolites derived from the fundamental processes of photosynthesis, glycolysis and the Krebs cycle which generate limited building blocks, but the formation of novel secondary metabolites is infinite. The most important building blocks employed in the biosynthesis of secondary metabolites are those derived from the intermediates:

acetyl coenzyme A, shikimic acid, mevalonic acid and 1-deoxyxylulose-5-phosphate, which are involved in innumerable biosynthetic pathways. The catabolic systems using these secondary metabolites are directed by the polyketide synthase (PKS) and the non-ribosomal peptide synthetase (NRPS), which catalyze the elongation of polyketides and synthesis of oligopeptides and also the biosynthetic pathways of terpenoids and alkaloids; these systems are really responsible for ensuring the diversity of the NPs. The evolution in the biosynthetic pathways may be due to natural causes (e.g., viruses, horizontal transfer or environmental changes) or unnatural causes (e.g., chemicals or radiation), in an effort by the microorganism to adapt to the environment. These modifications and alterations have resulted in a huge library of chemical structures optimized by natural selection that possess a broad array of biological activities. The challenge is how to access this chemical diversity and find the appropriate assays to test these biological activities.

In the 2000s, the traditional natural products screening was gradually abandoned because of frequent re-discovery of previously isolated compounds, the inherent technical difficulties associated to the isolation of active constituents of extracts, the incompatibility of NP extracts with HTS campaigns, and the structural complexity and low titer production of NPs, which required total synthesis and derivatization, sometimes economically and synthetically challenging. NP discovery was therefore replaced by molecular target-based drug discovery using large synthetic combinatorial libraries. However, the success of these combinatorial libraries in cancer have been brought into question since only one compound from this origin has been approved by the FDA, in 2005, for treatment of renal cell carcinoma, and in 2013, for treating thyroid cancer. This antitumor compound is sorafenib, co-developed and co-marketed by Bayer and Onyx Pharmaceuticals. Combinatorial libraries lack the structural diversity and complexity given by nature to NPs. In a further step, the diversity-oriented synthesis (DOS) approach was developed to mimic NPs and the resulting compounds are currently being tested in a large number and variety of biological screens in order to determine their role as a promising *hit* [12].

Nonetheless, recent advances in technology and sensitive instrumentation for the rapid identification of novel bioactive NPs and structure elucidation have opened up a new era and greatly improved the NP discovery process [13]. NPs, their semi-synthetic derivatives and natural product-inspired compounds still represent one of the most important sources of chemical diversity and bioactive novel structures ever described [8, 14].

The extremely prolific production of novel molecules by some groups of microorganisms, especially some taxa of actinomycetes (a phylum of Gram-positive bacteria) and fungi, did not require the use of an unlimited number of cultivation conditions to ensure that novel molecules were produced. In fact, for decades researchers have agreed on the application of a maximum of three to four production media at a time are sufficient to exploit the production of new bioactive molecules [15]. Microbial extracts have been largely exploited in antibiotic discovery, but new applications of these secondary metabolites are emerging, such as their relevant activity as antitumor agents. In recent years, increasing numbers of complete annotated genomes have been confirming the presence of a huge biosynthetic potential in bacteria and fungi, in many cases only detected as cryptic pathways from genome mining of biosynthetic pathways [16]. We still do not know the most important factors conditioning the nutritional requirements and secondary metabolism regulatory factors of most of the species screened which might be producing molecules quite below the detection threshold. Further

studies are in progress to modulate and heterologously express the secondary metabolism of microorganisms, opens up an emerging vast field of research in synthetic biology.

Furthermore, the difficulty of discovering novel molecules and the recurrent re-discovery problem of old, well-known molecules has required in parallel moving away from traditional approaches challenging the secondary metabolism of these species from quite different culture-based perspectives. The challenge of finding new molecule classes from libraries of secondary metabolites produced by microorganisms has required a change of paradigm with a shift in the number of new extracts tested and an improvement in the strain selection conditions and the nutritional conditions required for the production of novel molecules.

Access to the microbial diversity in the environment has traditionally been focused not only on intensive sampling from widely diverse geographical locations and habitats, but also other novel approaches were introduced in the 2000s. One of these approaches for drug discovery from microbial strains involves the application of the One Strain Many Compounds (OSMAC) method, which attempts to induce silent biosynthetic clusters leading to the accumulation of compounds by a combination of cultivation and nutritional conditions. OSMAC helps to determine the modulating effect that altered culture conditions (i.e., media composition, temperature, osmolarity and pH) may have on the secondary metabolite production of microorganisms [17]. Examples of such culture variations include the use of different liquid or solid media, such as solid beans or rice medium or the mimicry of extreme habitats by cultivating at colder temperatures or using highly saline media [18]. The OSMAC approach has been routinely used at Fundación MEDINA, a reference center for natural products drug discovery, to exploit our microbial strain collection of 190,000 strains (in part inherited from the Merck & Co. Inc. and Cubist Collections) to generate a NP Library of more than 180,000 extracts. MEDINA has introduced small-scale bacterial and fungal fermentations in tubes and deep well microplates that can be readily adapted to automated liquid handling equipment for further extraction and processing. Thus, the reduction of the fermentation volumes opens up the possibility of testing multiple nutritional conditions while offering the possibility of exploring minor groups of isolates and understanding their requirements up to as many as 20 different media. An average of the best eight fermentation media covering the largest metabolic space of the producing strains was shown to ensure an increase in the numbers and diversity of the strains tested [19]. All crude extracts are obtained from these cultures, using organic solvents to collect most of the secondary metabolites generated both, inside the microbial cells and excreted to the culture media. In the case of extracts from plants or invertebrates, different parts of the plant or animal are crushed and then extracted, also with organic solvents.

Screening of NPs, just as synthetic compounds, is performed following different approaches depending on the paradigm chosen for each application. In the case of antitumor screening, both target- and phenotypic-based assays are normally used. The screening methods are discussed below in Section 2.1 of this chapter, and the process of identifying the new molecules is summarized in **Figure 1**.

Once the active extracts in screening are detected, chemical identification of the novel compounds is necessary. Although diverse strategies are followed for isolating the active compounds from microorganisms, here we describe one of the most exhaustive approaches (**Figure 1**). The process of identifying known compounds responsible for the activity of an

extract prior to bioactivity-guided isolation is referred to as dereplication, and must be done as soon as possible in drug discovery process [20]. An early dereplication of known molecules may be performed through different systematic analysis techniques. One of the most successful methods combines liquid chromatography with high resolution mass spectrometry (LC-HRMS). Identification of known NPs with no chemical or pharmacological interest is inevitable and should be detected early through the comparison of analytical data against proprietary LC-MS libraries of microbial metabolites in research groups with long experience, or against public and commercial libraries of NPs, such as Chemspider or the Chapman & Hall Dictionary of NPs.

Active extracts containing novel components are of great interest and the molecular identification and isolation of these novel compounds are required. To do this, first, the microbial strain is regrown in the same conditions on a medium scale (150 mL), and bioassay-guided extract fractionation is carried out. Enriched fractions are generated through semipreparative HPLC method using proper separation columns and solvent gradient. The fractions are then tested for activity following the screening paradigm. In some cases, LC/HRMS and NMR dereplication allow identify the bioactive components at this stage, but in most cases, regrowth on

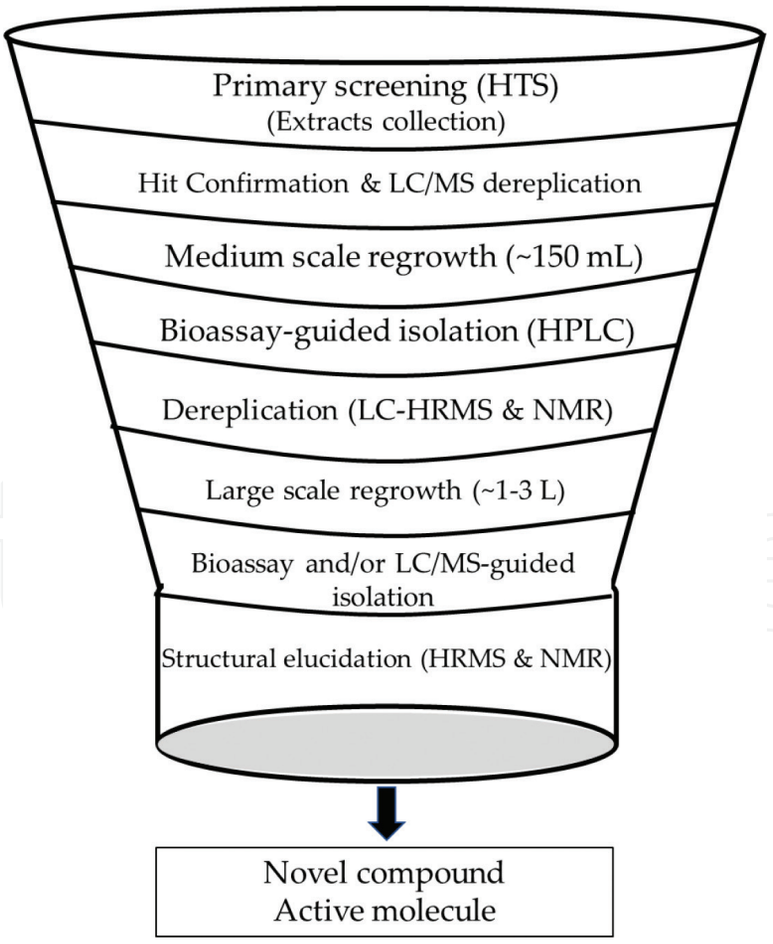

Figure 1. Schematic representation of the high-throughput screening and integrated dereplicating chemistry platform used to discover molecules from microbial extracts.

a large scale (1–3 L) is needed to have enough quantity of the bioactive compounds, whose structure elucidation is eventually performed using LC-HRMS and nuclear magnetic resonance (NMR) [21]. In the case of NPs from plants or invertebrates, similar bioassay-guided fractionation, dereplication, elucidation and chromatographic purification steps are followed [22, 23].

Undoubtedly, the discovery of drugs from natural products requires a multidisciplinary team: a group of experienced microbiologists or biologists working on plants or invertebrates to generate a collection of samples with the greatest possible biodiversity, researchers with experience in screening and with skills to work with HTS and robotic equipment, and lastly, a group of chemists with extensive knowledge about the chemistry of natural products.

2.1. Screening in pancreatic cancer

Screening has played a critical role in the discovery of leads that are further optimized for their properties, eventually leading to clinical candidates and drugs. The tremendous progress made in life sciences has resulted in the definition of many pathological processes and mechanisms of drug action. Drug discovery for cancer is carried out using both, target-based and phenotypic-based approaches. Target-based approaches to drug discovery are extensively used in the pharmaceutical industry but there are very few fully validated drug targets in cancers that are dependent on the tumor microenvironment, such as pancreatic cancer [24]. Due to this lack of avowed target in PC and the advances in cell culture, the most widely used approaches are phenotypic.

The latest advancements have led to the establishment of various molecular and cellular bioassays in conjunction with HTS methods. HTS decreases the amount of testing compound required so that only microgram quantities are needed. This is advantageous for certain NPs that are difficult to isolate and purify, and permits assaying compounds that are difficult to synthesize. Fluorescent methods are probably the classic choice for HTS, as they allow the best discrimination of the signal of interest from the background, though luminescent methods or other new technologies like AlphaScreen™, fluorescence resonance energy transfer (FRET) technology, time-resolved fluorescence (TRF) and fluorescence polarization are becoming more relevant. The current move is away from the traditional 96-well plate to 384- or 1536-well plates, where reagent costs are typically 100 times lower, and assay volumes decrease from 200 to 5–10 μl, and the quantity of compound or crude extract assayed drops to nanoliters. The use of such a low volume in assays leads to the need for liquid handling equipment, robotic platforms and new technology advancements such as Acoustic Droplet Ejection Technology [25].

Furthermore, image acquisition using robotic fluorescent microscopy and automated image analysis, generally referred to as high-content screening (HCS), has become an essential tool in early drug discovery programs, especially in cancer research. High-content cellular imaging is increasingly meeting the challenges of high-throughput needs and facilitating the integration of disease-relevant screens in cancer models such as three-dimensional (3D) cultures. NP screening has been adapted to HTS technologies, and a huge effort has been made to adapt the classical NP research laboratories to centralized HTS facilities.

The increase of chemodiversity, together with HTS methods and novel assay models in cancer research, make the use of NPs a promising source of anticancer drugs. The two main approaches to drug discovery for PC, target- and phenotypic-based screening, are described.

2.1.1. Target-based screening in pancreatic cancer

A target for a disorder is only fully validated when there is a registered drug for which it can be shown that the principle mode of action is by modulation of the target. For decades, PC was commonly treated with 5-fluorouracil (5-FU), also used in other types of cancer. The suicide inhibitor 5-FU works through irreversible inhibition of thymidylate synthase [26]. The cytidine analog Gemcitabine (2′,2′-difluoro 2′-deoxycytidine), which replaced 5-FU, was approved by the Food and Drug Administration (FDA) in 1996 [27], becoming the standard first-line treatment for PC. In 2005, the FDA approved the combination of gemcitabine and erlotinib [28]. Erlotinib is a receptor tyrosine kinase inhibitor, which acts on the epidermal growth factor receptor (EGFR). Other combination clinical trials have been conducted since then.

A combination of oxaliplatin, irinotecan, 5-FU and leucovorin, called FOLFIRINOX [29], is chemotherapy regimen confined to patients with good performance status because of its high toxicity and severe side effects. Oxaliplatin is a platinum-based antineoplastic agent, irinotecan prevents DNA from unwinding by inhibition of topoisomerase 1 and leucovorin (5-formyltetrahydrofolate) is an adjuvant in cancer chemotherapy.

Nab-paclitaxel, a nanoparticle albumin-bound paclitaxel, which is natural product from extract of *Taxus brevifolia* (Pacific yew) [30], targets tubulin and destroys cancer cells by preventing the normal breakdown of microtubules during cell division. In September 2013, the FDA-approved nab-paclitaxel for use in treating advanced PC. The last therapy approved for PC, in 2015, was the combination of Onivyde (irinotecan liposome injection), 5-FU and leucovorin. Despite great efforts, many years of research and numerous studies, these chemotherapeutic options for treating PC are far from satisfactory at present [31].

In recent years, further attempts have been made to discover new chemotherapeutics directed to new targets which may provide an approach for PC prevention and treatment. Pathways with relevant novel targets are: K-ras (Raf [32], MAPK, Erk, PI3Ks, PDK-1 [33]; p53 [34]; growth factor (EGF, EGFR [35], FGF, FGFR [36], VEGF [37], IGF [38]) and the pathway of epithelial to mesenchymal transition (Wnt/β-catenin [39], TNFα [40], Notch [41], Snail-1, Slug, E-cadherin [42]). Clinical trials have been developed to evaluate some of these targets but the results have been disappointing. Studies testing the antibody bevacizumab, which blocks angiogenesis by inhibiting vascular endothelial growth factor A (VEGF-A) [43] and cetuximab, which blocks ligand-binding domain of EGFR [44], have been negative.

Although these experimental targets are widely used for target-based screening in PC, there is an urgent need to identify innovative therapeutic targets. Current approaches to the discovery of new biomarkers and targets range from the use of microarrays for gene expression profile of PC patients versus healthy controls [45], to proteomic [46] or secretome profiles [47].

Few examples of NPs have been reported for PC with different target-directed mechanisms. Some of the more representative ones are described below and summarized in **Table 1**.

NP compound or derivate	Ref.	Source	Target
Nab-paclitaxel (derivated from paclitaxel)	[30, 48]	*Taxus brevifolia* (tree)	Tubulin (microtubules)
Hispidulin	[49]	Artemisia and salvia (plants)	VEGF receptor 2-mediated PI3K/Akt/mTOR signaling pathway
Betulinic acid	[51]	*Betula pubescens* (tree)	Lamin B1
Ixabepilone	[52]	*Sorangium cellulosum* (Gram-negative bacterium)	Microtubule stabilizer
Apigenin	[54, 55]	Widely distributed in plants	COX-2, IKK-β-mediated NF-κB activation
Baicalein	[56]	*Scutellaria baicalensis* and *S. lateriflora* (plant)	Lipoxygenases (LOXs)
Ellagic acid	[57]	Fruits, nuts and vegetables	Arachidonic acid (AA) pathway
[6]-Gingerol	[58]	*Zingiber officinale* (plant)	Arachidonic acid (AA) pathway
Thymoquinone	[59]	*Nigella sativa* (plant)	Arachidonic acid (AA) pathway
Triptolide	[60]	*Tripterygium wilfordii* (plant)	Arachidonic acid (AA) pathway

Table 1. Summary of experimental NPs and derivatives for PC treatment with known target.

The most advanced, which has been approved by FDA, is nab-paclitaxel, derived from natural paclitaxel [48], mentioned above in the chapter. Another example, reported by He et al. [49] is hispidulin, a flavone found in some plants including artemisia and salvia, which targets the VEGF receptor 2-mediated PI3K/Akt/mTOR signaling pathway in endothelial cells, leading to the suppression of pancreatic tumor growth and angiogenesis.

Other class of secondary metabolites of plants with effectiveness in PC is terpenoids. The mechanism of the terpene antitumor effects is the inhibition of posttranslational isoprenylation of proteins regulating cell growth [50]. For instance, betulinic acid, a triterpenoid obtained from the bark of *Betula pubescens*, exhibits potent antitumor activities and can downregulate lamin B1; knockdown of lamin B1 significantly attenuates the proliferation, invasion and tumorigenicity of PC cells [51]. Moreover, the epothilone B lactam ixabepilone, a microtubule stabilizer produced by a Gram-negative bacterium *Sorangium cellulosum*, which was approved by the FDA for breast cancer treatment in 2007, was assayed for PC in a Phase II trial in patients with advanced pancreas cancer [52], showing encouraging activity in the patients.

Additionally, the arachidonic acid (AA) pathway plays a key role in carcinogenesis. The AA pathway metabolic enzymes phospholipase A2s (PLA2s), cyclooxygenases (COXs) and lipoxygenases (LOXs), and their metabolic products, such as prostaglandins and leukotrienes, have been considered novel preventive and therapeutic targets in cancer [53]. AA pathway inhibitory NPs have been developed as chemopreventive and therapeutic agents against several cancers. For example, apigenin, a flavonoid widely found in plants, suppresses inducible COX-2 expression and inhibits the growth of PC cells *in vitro* and *in vivo* by IKK-β-mediated NF-κB activation [54]. Epidemiologic studies suggested that apigenin is related to a decreased risk of certain cancers, including PC [55]. Another is baicalein (5,6,7-trihydroxyflavone), found

in the roots of *Scutellaria baicalensis* and *S lateriflora*, which inhibits LOXs and in turn down-regulates Bcl-2, increases Bax, increases cytosolic cytochrome c, and activates caspase-9, promoting apoptosis and exhibiting anticancer activity against PC cells [56]. Ellagic acid, a hydrolyzed metabolite of ellagitannin found in certain fruits, nuts and vegetables, has been reported to possess anti-pancreatic cancer properties, targeting AA pathway. Zhao et al. [57] described that ellagic acid inhibited PC growth in PANC-1 xenografted mice, by suppressing various pro-tumorigenic mediators. Other natural compounds with described activity for this pathway are [6]-gingerol, a phenol constituent of the plant *Zingiber officinale* Roscoe (ginger) [58]; thymoquinone [59], derived from black cumin *Nigella sativa* and triptolide [60], a diterpenoid isolated from *Tripterygium wilfordii*. Many more NPs that modulate AA pathway are under research for other cancer types and could also be active against PC.

2.1.2. Phenotypic-target screening in pancreatic cancer

While all of the early drugs were discovered by phenotypic screening, the past three decades have given rise to new technologies for performing HTS that have since dominated the pharmaceutical industry. Development of patient-derived cell cultures, three-dimensional (3D) culture, organotypic systems, advances in cell imaging, microfluidics and nanotechnologies are the future trends in drug discovery and development.

Patient-derived cell cultures offer a more clinically relevant model for testing novel gene and cell-based therapies. These models are decidedly valuable in cancer research, where highly selective drugs targeted at genetically defined clinical subtypes are needed to support a more patient-centric approach to drug development [61]. Potential drugs have been tested against patient-derived primary cancer subtypes for various cancers, with promising results in glioblastoma [62] and leukemia [63]. The problem in PC research is that there are few *in vitro* models of exocrine pancreas development and primary human pancreatic adenocarcinoma (PDAC), and the models are just starting to be established. The use of surgically resected pancreatic cancer tissue is difficult because of the limited amount of residual pancreatic cancer tissues remaining after the large amount of cancer tissue has been used in the histopathologic examination for diagnosis. To overcome this limitation, researchers have tried to establish cancer cell lines from patient-derived cancer tissues, but this approach has not been very successful due to specific histopathologic characteristics of PC, such as low cancer cellularity and the extensive desmoplastic reactions by the associated fibroblasts. Consequently, the number of established patient-derived PC cell lines is currently much lower than that of other cancers [64–66], there currently being only 11 cell lines from the American Type Culture Collection and another 4 from the European Collection of Authenticated Cell Cultures (ECACC). These are widely used despite the limitations, such as the *in vitro* cell culture conditions which modify cells over time, losing the expression of markers or enriching specific cell populations. Patient-derived tumor xenograft mice models (PDXs) have been used recently for predicting patient responses for patient-selection strategies [67], but this animal model cannot be used for massive drug discovery for obvious reasons.

Recent evidence suggests the existence of small populations of CSC, which are believed to be responsible for tumor initiation and progression as well as resistance to chemotherapy and

radiation. Identification of the regulatory mechanisms and signaling pathways involved in CSC are expected to help researchers identify and design novel agents that target this resistant cell population in PC. Pancreatic CSC can be allowed to divide and grow in ultra-low binding tissue culture dishes to form multicellular spheroids that will favor the formation of multicellular tissues with the appropriate cell-cell and cell-matrix (ECM) interactions necessary for full functionality. Natural and synthetic biomaterials and nanomaterials are used to build these 3D cultures, providing a robust architecture in 96- and 384-well formats. This technology is being successfully applied in cancer models [68], although the culture medium and materials used still need to be improved.

Coupled with 3D cultures, HCS imaging systems have been developed, with huge advances in microscopy and image-informatics solutions [69]. Image acquisition using robotic fluorescent microscopy and automated image analysis has become an essential tool in early drug discovery programs. HCS cellular imaging has increasingly met the challenges of high-throughput needs and facilitates the integration of disease-relevant models and screens at early stages of the drug discovery process [70]. Although various PC cell lines can grow as spheroids in 3D cultures, it is unclear how well they reflect the properties of the original human tumor [71, 72].

There are some examples of promising NPs against pancreatic cancer obtained and described through phenotypic-based screening and nutritional studies, which have been reported in scientific publications and patents and are summarized in **Table 2**.

One of the main currents for treating cancer using natural plants is traditional Chinese medicine. Chinese medicine is an old form of medicine built on a foundation of more than 2500 years of Chinese medical practice. In recent times, new studies have been made and new patents have been registered in relation to this ancient science with the aim of modernizing traditional Chinese medicine [73]. Some patents of PC treatment have been registered on a combination of tens of herbal and other NPs, based on a secret prescription handed down from ancestors and on traditional Chinese medical theory [74, 75]. These patents report a very high efficacy in clinical studies and have shown a total recovery of up to 90% in 2–5 years. This percentage is incredibly high so a confirmation in other populations would be necessary to validate the results, but there is no record of the patents being tested in other countries. Additionally, the principal components of the flower *Paeonia lactiflora*, albiflorin and paeoniflorin, which are a functional food ingredient widely used for more than 2000 years in traditional Chinese medicine, have been patented for pancreatic cancer prevention and treatment [76]. *In vitro* and *in vivo* experiments show that albiflorin has antitumoral activity and may provide a new option for the clinical treatment of tumors, although these trials are only in the first stage of drug development.

Furthermore, dietary proanthocyanidins mostly present in apples, pears and pulses, has been suggested to reduce the risk of pancreatic cancer by 25% [77]. Another example is *Chelidonium majus* L. (Papaveraceae), a medicinal herb with antitumoral activity that is widely found in Europe. High cytotoxic activity against pancreatic cancer has been reported by an extract of *C. majus* [78]. Also, Sarcaboside B was isolated from the whole plant of *Sarcandra glabra* and shows *in vitro* results of antineoplastic activity in PC cells according to a patent posted by Ding Shengyu [79]. Usnic acid is extracted from lichen and has been used for its antimicrobial activities. Its effect against cancer cells was first reported over 30 years ago, and specifically its effect has been observed in PC cells [80].

NP compound or derivate	Ref.	Source	Approach
Albiflorin and paeoniflorin	[76]	*Paeonia lactiflora* (flower)	*In vitro* and *in vivo*
Proanthocyanidins	[77]	Apples, pears and pulses (fruit)	Epidemiological study
Extract from *Chelidonium majus L.*	[78]	*Chelidonium majus L.* (plant)	*In vitro* and *in vivo*
Sarcaboside B	[79]	*Sarcandra glabra* (plant)	*In vitro*
Usnic acid	[80]	Lichen	*In vitro*
Extract from *Spirulina platensis*	[81]	*Spirulina platensis* (blue-green alga)	*In vitro* and *in vivo*
Aplidine	[82]	*Aplidium albicans* (ascidian, invertebrate marine)	*In vitro* and *in vivo*
Manzamenone O	[83]	*Plakortis sp.* (marine sponge)	*In vitro*
Polysaccharide-K	[84, 85]	*Trametes versicolor* (mushroom)	*In vitro*
Antroquinonol	[86]	*Antrodia camphorate* (mushroom)	*In vitro*
MMH01	[87]	*Antrodia cinnamomea* (fungi)	*In vitro*
Beauvericin	[88]	*Fusarium oxysporum* (fungi)	*In vitro*
Globosumones	[89]	*Chaetomium globosum* (fungi)	*In vitro*
MDN-0090	[90]	*Onychola* sp. (fungi)	*In vitro*

Table 2. Summary of experimental NPs and derivatives for PC treatment from phenotypic-based screening and epidemiological studies.

Marine samples, from marine microorganisms, algae or invertebrates, are increasingly more relevant as sources for cancer chemoprotectives. The lead compound, Ecteinascidin-743 (Yondelis®), is the first marine anticancer agent approved in the European Union for patients with Soft Tissue Sarcoma (STS) and for the treatment of Relapsed Ovarian Cancer. In the case of PC, marine samples still have a long way to go before they can be used in treatment but there is a whole universe of compounds waiting to be discovered. One example is *Spirulina platensis*, which is a blue-green alga used as a dietary supplement because of its hypocholesterolemic properties. Among other bioactive substances, it is also rich in tetrapyrrolic compounds closely related to bilirubin molecule, a potent anti-proliferative agent. The anti-proliferative effects of *S. platensis* were observed against PC cells and were also shown *in vivo* where inhibition of PC growth was evidenced from the third day of treatment in a mice model [81]. Another compound of marine origin, Aplidine (Dehydrodidemnin B), extracted from the invertebrate ascidian *Aplidium albicans*, shows dose-dependent cytotoxic activity against PC cells as well as significant activity against mice bearing human cancer xenografts. Aplidine's mechanisms of action seem to be mediated by the AKT pathway and the reduction in ERK activation [82]. Also, Manzamenone O was isolated from a marine sponge and has been patented against PC [83].

From the fungi kingdom, polysaccharide-K (PSK, krestin) is one of the most commonly used medicinal mushroom extracts, with a long history in cancer therapy in Asia, especially in Japan [84]. Zhang et al. [85] have reported that PSK decreases the invasiveness of a human PC cell line. Also, antroquinonol, a ubiquinone derivative isolated from the mushroom *Antrodia camphorata*, induced a concentration-dependent inhibition of cell proliferation in PC [86].

Among the microbial NPs, very few are described in bibliographies. One of those published is the compound MMH01, which is isolated from the parasitic fungus *Antrodia cinnamomea* on aromatic tree *Cinnamomum kaneirai Hay.* (Lauraceae), which markedly inhibited growth of a PC cell line [87]. Other examples are the compound beauvericin, extracted from the fungal strain *Fusarium oxysporum*, which inhibits migration of the metastatic PC cell [88], and globosumones, from the Sonoran Desert endophytic fungus *Chaetomium globosum*, with inhibitory activity of cell proliferation in PC cells [89]. Also noteworthy is MDN-0090, a compound patented by our research group at Fundacion MEDINA, from a fungus identified as *Onychola* sp. [90], with *in vitro* activity against PC. This compound was obtained through a phenotypic-based screening of more than 90,000 microbial extracts from Medina's NP Library following a cytotoxicity screening in 2D cancer cell culture and a dereplication and identification of the novel compound. Promising results with this novel compound have been obtained from an *in vivo* mice model of disease (unpublished data).

2.2. Future trends in screening in pancreatic cancer

Although many efforts have been made to improve the technology, research models and sources for finding a cure, there is much work left to be done in pancreatic cancer research. Here, we summarize the future trends in this research.

One solution that more closely mimics tumor properties is the use of an alternative 3D model for tissues, termed organoids, which go one step further. 3D organotypic models have potential for bridging the gap between cell-based discovery and complex animal models. Adult stem cells are prepared from human adult tissues and embedded in a three-dimensional matrix, where they self-organize into epithelial structures that resemble the original organ. One example for PC was developed by Huang et al. [91], who differentiated human pluripotent stem cells (PSCs) into exocrine progenitor organoids that formed ductal and acinar structures in culture, expressing the mutations frequently found in patients. These organoids would recapitulate the properties of the original tumor, maintaining the differentiation status, histoarchitecture, phenotypic heterogeneity and retaining patient-specific physiologic changes. The publication of Boj et al. [92] describes the obtaining of PC tissues from patients undergoing surgical resection. The tissue is minced, digested with enzymes, embedded in matrigel and culture with propagation until 20 passages (~6 months) or is cryopreserved, which makes it a very useful model. Related to this is the new creation of living organoid biobanks [93], which consist of a collection of cryopreserved organoids from patients. The ability to create organoids from individual tumors and the enormous clinical diversity of these specimens can be extremely useful for drug discovery. One large collection of these cultures is the Hubrecht Organoid Technology (HUB) "living" biobank [94]. The HUB is part of the Human Cancer Models Initiative in collaboration with The National Cancer Institute, Cancer Research UK, and the Wellcome Trust Sanger Institute. This "living" biobank stores approximately 1000 cancer cell models that best represents the hallmarks and diversity of human breast, colorectal, lung, pancreatic and prostate cancer. The organoids generated have been assayed to analyze drug sensitivity to a vast array of anticancer drugs. This well-characterized library of cultures, with genome sequenced and clinical data from patients, has been created to aid in basic research and explore novel therapeutic strategies and drugs, being accessible to industry and academia [95].

Therefore, tumor organoids can be used in PC models and for drug screening to identify precision therapy strategies, but nowadays only a few laboratories have enough equipment, facilities and access to patient samples to develop them. One step further is the organ-on-a-chip, still under development. This technology is essentially miniaturized microfluidic perfusion systems which allow long-term *in vitro* growth of primary cells and tissues in a format that is viable for scaling up for high-throughput discovery campaigns. These systems model the complex tissue microenvironment and communication, reproducing *in vivo* tissue and organ functionality. One example reported by Maschmeyer et al. is a four-organ-chip system that mimics human liver, skin, intestine and kidney [96]. Furthermore, the use of microfluidic perfusion chambers in these systems permits the homeostatic function of the organ as if it were the blood flow, supplying nutrients and discharging catabolic metabolites [97]. We look forward to seeing how these promising advances develop in the new era of drug discovery in PC research.

3. Conclusion

In this scenario, PC is still an unmet medical need and new therapies need to be discovered. The ancestral use of natural products in medicine continues in force, but with novel approaches. The increase of chemodiversity together with HTS methods and novel assay models in cancer research make the use of NPs a promising source of novel anticancer drugs. Many more useful natural lead compounds await discovery and the challenge is how to access this natural chemical diversity. More and more multidisciplinary teams are needed to access the world's biodiversity, identify novel compounds and evaluate their potential biological activity.

Promising compounds are currently being tested and many more are expected to be found from diverse natural product sources, with the help of the new trends in cancer research.

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Conflict of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject-matter or materials discussed in the manuscript apart from those disclosed.

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