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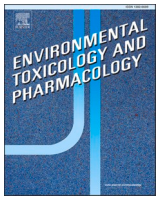

Emerging pharmaceutical therapies of Ascidian-derived natural products and derivatives

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ABSTRACT

In a growing multidrug-resistant environment, the identification of potential new drug candidates with an acceptable safety profile is a substantial crux in pharmaceutical discovery. This review discusses several aspects and properties of approved marine natural products derived from ascidian sources (phylum Chordata, subphylum Tunicata) and/or their deduced analogues including their biosynthetic origin, (bio)chemical preclinical assessments and known efficacy-safety profiles, clinical status in trials, but also translational developments, opportunities and final conclusions. The review also describes the preclinical assessments of a large number of other ascidian compounds that have not been involved in clinical trials yet. Finally, the emerging research on the connectivity of the ascidian hosts and their independent or obligate symbiotic guests is discussed. The review covers the latest information on the topic of ascidian-derived marine natural products over the last two decades including 2022, with the majority of publications published in the last decade.

1. Introduction

The increased importance of marine natural products (MNPs) research is reflected by an increasing number of manuscripts, reviews and book chapters since 2000 (Table S1), as well as a multiplication of databases on natural products to over 120 active databases. The review of Sorokina and Steinbeck (2020) provides a complete overview of the databases on natural products. One open-access single dataset on natural products named Coconut, available on COCONUT: the COlleCtion of Open NatUral producTs. | Zenodo) contains structures and annotations of over 400,000 nonredundant natural products which makes this resource an immense database on natural products available today. Carroll et al. (2021) describes 1490 new compounds from the literature published in 2019 of which new structures are highlighted from a wide variety of sources. Within this diversity, the marine ecosystem is a specific but opulent source of chemical and biological innovation, with nearly 1 000 new MNPs per year reported in recent years (Alves et al., 2018). The Clinical Pipeline Marine Pharmacology (Midwestern University, 2022), initiated by A.M.S. Mayer in 1998, consolidates in a structured way the information on the bioactivity of MNPs. In these two decades, over 60 marine-derived products from different sources have been approved for medical use and/or are considered promising in anticancer research. In general, anticancer treatment gets primary attention in the screening of MNPs before treatment of viral, microbial, parasitic and other metabolic disorders and diabetic, neuroprotective and repurposed therapies. As of October 2020, the Clinical Pipeline Marine Pharmacology provides an updated list of marine-derived compounds which have either been approved or are subject to drug development in Phase III, II or I clinical studies in the USA, Europe, Australia and China (Clinical Pipeline Marine Pharmacology). No ongoing, terminated or completed clinical studies have been reported from China.

This review focuses on the MNPs originating from marine Ascidiacea and discusses in detail the available information from a pharmaceutical preclinical and clinical translational viewpoint; hence complementing the many reviews on structural, (bio)synthetical and biochemical preclinical aspects already available (Table S2). Ascidiacea represent three orders in a class of the phylum Chordata, subphylum Tunicata. Common names are ascidians, tunicates or sea squirts. They have attracted enormous scientific interest not only because of their worldwide distribution, environmental-ecological and evolutionary impact, but also

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by the discovery of novel bioactive metabolites with considerable pharmaceutical promise in different therapeutic areas with unmet medical needs.

The literature search for the review began with the exploration of the most recent information on the topic via search platforms including Academia, Researchgate, Publisher sites and the 8 governmental and public websites mentioned in section 3 using the general keywords tunicates, ascidians, marine natural products, bioactive compounds, secondary metabolites, symbionts followed by the more in-depth technical search terms including marine anticancer, antiviral, antibacterial, antiparasitic etc. drugs, bioactivities, cytotoxicity, topoisomerase inhibition, kinase inhibition, biosynthesis, MoAs etc. and consultation of older publications.

2. Product source identification and the role of symbionts

Logically, the initial research on MNPs targeted large marine organisms as collecting sources: sponges, tunicates, corals and algae (Gerwick and Moore, 2012). The first steps in the search for bioactive compounds are initiated by in vitro tests on crude or fractionated extracts and subsequent structural elucidations of the active compounds and the biosynthetic pathways that lead to the production of these compounds, termed as the top-down approach. The research on the biosynthetic origin of natural pyridoacridines revealed important information on the presence of similar or analogous metabolites in different phyla. Carroll and Scheuer (1990) suggested the production of similar alkaloids by the same microorganisms in different hosts. The structural resemblance between the natural products in these marine macroorganisms and products produced by microorganisms raised questions on the biosynthetic origin of these compounds (Piel, 2004). Evidence on the direct involvement of microscopic producers was found in the discovery of identical compounds or close analogs in microorganisms of marine and terrestrial origin or after semisynthesis. By the turn to the 21st century, advanced technologies generated large clonal libraries of entire genomes to identify biosynthetic gene clusters, post-translational modifications and pathways of interest (Piel, 2009; Donia et al., 2011; Chen et al., 2021). Key information on the molecular biosynthetic pathways confirmed the central importance of associated microorganisms in the production of thousands of unique molecular species in a symbiotic lifestyle (Chen et al., 2017). Metagenomic research unequivocally confirmed the symbiosis hypothesis of Haygood (1999) in 1999. As a result, research groups focused on culturing mainly marine bacterial organisms from various symbiotic hosts (Williams, 2009). Nowadays, large-scale phylogenomic mining has identified numerous gene clusters as biosynthetic sources of bioactive natural compounds (Yuan et al., 2022; Malit et al., 2022). Yuan et al. (2022) describes 56 biosynthetic gene clusters from cultured ascidian-derived fungus *Amphichorda felina* SYSU-MS7908 and Malit et al. (2022) provides an excellent overview on the traditional natural product discoveries in the top-down approach that do not require prior knowledge of a producer's genome nor its biosynthesis capability and the alternative bottom-up approach searching for microbial genomes for the natural product biosynthesis machinery. The number of microbial species has been estimated at around 10^6 while only a few thousand species have been isolated in pure cultures (Kaeberlein et al., 2002). Sequestration of metabolites from dietary sources by predation was also demonstrated in a study where the pentacyclic kuanoniamines A, B, C, and D and shermilamine B, found in the mollusk *Chelynotus semperi* were clearly produced from an unidentified ascidian dietary source.

Tianero et al. (2015), Buedenbender et al. (2017), Chen et al. (2017), Evans et al. (2017), Matos and Antunes (2021), Chen et al. (2021) and Ramesh et al. (2021) describe comprehensively the microbial diversity associated with ascidians. Unique direct research on the gut-associated microbiota in the cold water solitary ascidian *Ciona intestinalis* indicated a rich and diverse gut microbiota of 61 bacterial and 40 fungal strains affiliated to 33 different microbial genera, dominated by the gammaproteobacteria class (Utermann et al., 2020). In vitro, screening of the crude microbial extracts revealed antibacterial, anticancer and/or antifungal potential and recent discoveries in crude extracts of the ascidian *Didemnum bistratum* exhibited prominent larvicidal acute toxicity against the Zika vector mosquitoes *Aedes aegypti* and *Cluex quinquefasciatus*. The presence of anthraquinone and indole derivatives, substances from associated actinomycetes (Chen et al., 2021), are held responsible for the eco-friendly biocontrol against the mosquitoes (Arumugam et al., 2019).

At the moment, strong evidence shows that approx. 8 % (around 80 products in total) of the known secondary metabolites from ascidians have identified symbiotic organisms, and indirect evidence implicates microorganisms in the synthesis of many more (Schmidt, 2015; Chen et al., 2017). Many of the known metabolites from ascidian sources are likely to be synthesized by bacterial (actinomycete) strains of *Streptomyces* or *Micromonospora* (Tianero et al., 2015; Buedenbender et al., 2017; Watters, 2018). The sources of trabectedin and plitidepsin discovered in ascidians are also bacterial-derived from *Streptomyces stauroporeus*. However, the actual synthetic origin of a majority of metabolites in ascidians remains unknown (Tianero et al., 2015). Also, the importance of the environmental and biological functions with high ecological relevance of the association between host and symbiont is not yet well understood and subject to thorough investigations. Microorganisms may be very host-specific while others are widespread and range from free-living in seawater to host associations with different phyla: sponges, tunicates, sea squirts, coral, jellyfish and algae (Choudhary et al., 2018; Romano, 2018; Ruocco et al., 2021).

Evolutionary genome reduction has also been observed in ascidian symbiotic lifestyles. Lopera et al. (2017) described a verrucomicrobial symbiont of an ascidian *Lissoclinum* sp. in a transition status of genome reduction and silent genes while active copies of a large polyketide synthase pathway for mandelalide compounds were maintained. Mandelalides are cytotoxic compounds that likely exert a chemical defense function for the host (Lopera et al., 2017). 'Secondary' metabolites are considered essential for the defense, survival, shelter, color morphs, nutrient exchanges and adaptations to other adverse conditions and likely facilitate the adaptation of ascidians to new environments. Species-specificity and even tissue-specificity have been demonstrated and the intracellular or extracellular lifestyles of obligate symbionts result in vertical or horizontal transmission among the host colonies and the respectively low or high phylogenetic diversity of the symbiont. Cultures in laboratory conditions of these symbionts in strong ascidian species-specific associations remained unsuccessful (López-Legentil et al., 2005; Donia et al., 2011; Schmidt et al., 2005, 2012; Schmidt, 2015; Tianero et al., 2015; Evans et al., 2017; Buedenbender et al., 2017; Chen et al., 2017; Morita and Schmidt, 2018; Longo et al., 2020; Chen et al., 2021).

The biosynthetic pathways of MNPs in these symbiotic lifestyles are complex. Initial confusion on citations of confirmed producers has inevitably been projected in the literature in the course of the research developments. Initial citations evidently referred to the collected macroorganismal sources and were later followed by citations of the actual associated microorganismal producers and/or acquired by predation. Also, citations on semisynthetic analogs produced under changed fermentation conditions, changed tunicate feeding conditions, precursor replacements in steps of the biosynthesis or combinatorial biosynthesis often refer to the producer of the product that served as a lead compound in the changed conditions although these new compounds are extra marked as (semi)synthetic analogs. The most recent literature more and more cites the actual producers which is evident for free-living microorganisms. However, mentioning the connectivity with the larger host organisms in symbiotic lifestyles in manuscripts is preferable to facilitate the correct source identification and connectivity when known.

3. The pharmaceutical potential and clinical status of ascidian-derived MNPs

During the period since 1998, the global marine pharmaceutical preclinical pipeline involved research with more than 1000 marine chemicals with a variety of bioactivities and Modes of Action (Clinical Pipeline Marine Pharmacology). All these preclinical developments have been precisely documented in the reports of Mayer et al. (2010) (see references in Clinical Pipeline Marine Pharmacology). Also, the most recent literature or governmental public health organizations reported on several discontinued or terminated research activities for biomedical use or health risk alerts on clinical developments of several promising MNPs and/or terminated clinical developments for reasons of lack of efficacy, toxic or strategic. Since 2017 the clinical status of some of the following drugs has been under investigation or changed by US FDA and EU EMA. The following section is an updated version on these events and authorization and marketing issues on ascidian MNPs and derivatives based on information in the literature and governmental, public and private (supplier) databases: the US Food and Drug Administration (US Food and Drugs Administration, 2023a), the European Medicines Agency (European Medicines Agency, 2023a), The National Cancer Institute (European Medicines Agency, 2023a), the US National Library of Medicine (Clinical Trails, 2023c), the Clinical Pipeline Marine Pharmacology (Midwestern University, 2022), Drugbank (online, 2023), MedChemExpress (MedChemExpress, 2023a) and PR Newswire (Newswire, 2023b).

3.1. Ecteinascidine 743 (ET-743, trabectedin, Yondelis©)

3.1.1. Preclinical assessments

Ecteinascidine 743 is the most potent antitumor antibiotic of a group of related bioactive compounds (Le et al., 2015). Ecteinascidine 743 was initially detected in extracts of the ascidian *Ecteinacidia turbinate* and later identified as a product of the bacterial symbiont *Candidatus Endoecteinascidia frumentensis* (Rinehart et al., 1990; Wright et al., 1990; Rath et al., 2011). Ecteinascidine 743 or trabectedin represents a new class of antineoplastic drugs. D'incalci et al. (2014) outlined in detail the Mode of Action (MoA) of trabectedin. One of the unique features of its MoA is its potential to modulate gene expression in a promotor and gene-dependent way in pharmaceutical concentrations. Ecteinascidine 743 is a tetrahydroisoquinoline alkaloid and shares a common pentacyclic core with compounds from terrestrial bacteria. However, the isoquinolines cover over 20 subclasses in the plant kingdom exhibiting a diverse spectrum of pharmacological activities (Singh et al., 2021). A comprehensive review on the chemistry and biology of other ecteinascidines was published by Le et al. (2015). Recently, 14 new isoquinoline tyrosine phosphatase inhibitors were isolated from a free-living deep-sea fungus *Aspergillus puniceus* (Liu et al., 2022). To our knowledge, tetrahydroisoquinolines have not been reported in plants.

The biosynthetic class of ecteinascidine 743 is a nonribosomal peptide synthetase-derived (NRPS-derived) alkaloid. The product development became successful by semisynthesis from cyanosafracin B, a natural fermentation product of *Pseudomonas fluorescens* due to extremely low yields in ecteinascidine 743 supply from the ascidian source (10^{-4} to 10^{-5} % w/w) (Rath et al., 2011; Cuevas et al., 2000). This need was met by semisynthesis from the microbial natural product cyanosafracin B, a fermentation product of *Pseudomonas fluorescens* (Cuevas et al., 2000). In cells, the MoA of ecteinascidine 743 interacts with DNA, and results in a cell cycle checkpoint G2/M block to repair damaged DNA after replication and ensuing tumor suppressor protein p53-independent apoptosis. The molecular target is the minor groove of DNA. Once bound, it interferes with the functioning of the nucleotide Excision Repair System so as to bring about a cytotoxic effect (Soares et al., 2011; Gordon et al., 2016). The work of Amaral et al. (2015) demonstrated that trabectedin is able to inhibit binding of the Ewing sarcoma (EWS) transcriptional factor EWS-FLI1, the potent oncogene of EWS to DNA but also increases the attachment of EWS-FLI1 to the target gene insulin-like growth factor receptor 1 (IGF1R) resulting in upregulation of IGF1R. Trabectedin showed limited efficacy in the monotherapeuthic treatment of the bone and soft tissue Ewing sarcoma (EWS) in clinical settings. The work of Amaral et al. (2015) further reports on the increase of trabectedin efficacy in EWS in combination with the anti-IGF signaling agent linsitinib (OSI-906). On the other hand, the EU EMA Committee for Medicinal Products for Human Use (EU EMA CHMP) reported highly effective trabectedin in cisplatin-resistant sublines of osteosarcoma U-2OS and Ewing's sarcoma Saos-2 cells and in methotrexate-resistant osteosarcoma cells (European Medicines Agency, 2007).

3.1.2. Clinical status

Ecteinascidine 743 was commercialized by PharmaMar under the trade name Yondelis© in 2001. The product received orphan designation in the EU for the treatment of soft tissue sarcoma, a highly heterogeneous, rare group of mesenchymal malignancies often resulting in metastasis, in May 2001 and the treatment of ovarian cancer in October 2003 (European Medicines Agency, 2022d). Several clinical trials showed a favorable toxicity profile of trabectedin as an alternative therapeutic option in adult patients with advanced soft tissue sarcoma who had not responded to treatment with the known anthracycline liposomal chemotherapy drug doxorubicin and ifosfamide (Nakamura et al., 2016). The EU EMA CHMP concluded differently that the benefit/risk profile was too low, mainly due to inadequate proof of clinical efficacy (European Medicines Agency, 2007). In 2006, PharmaMar S.A. submitted a marketing authorization application for the treatment of patients with unresectable advanced soft tissue sarcoma after failure of doxorubicin and ifosfamide, or for patients who are unsuited to receive these drugs. Efficacy data were mainly based on the treatment of the distinct histological specific subtypes soft tissue fat cell sarcoma (liposarcoma) and smooth muscle cell sarcoma (leiomyosarcoma) (European Medicines Agency, 2022d). In the EU the marketing authorization for trabectedin/Yondelis© was first authorized in September 2007 for the treatment of soft tissue sarcoma and ovarian neoplasms/cancer. An update of the European Public Assessment Report (EPAR) in 2022 confirmed the authorization for use in the EU.

In October 2015, Yondelis© (applicant Janssen) was approved by US FDA as a novel drug to treat cancer-type soft tissue sarcoma including liposarcoma and leiomyosarcoma that cannot be removed by surgery or advanced (metastatic) or patients who had already been treated with doxorubicin chemotherapy (Novel Drug Approvals for 2015 | FDA). The US FDA based its approval on the results of a randomized clinical Phase III study (ET743-SAR-3007) on trabectedin or dacarbazine in patients with advanced liposarcoma or leiomyosarcoma, conducted between May 2011 and January 2015 (Patel et al., 2019). This study ET743-SAR-3007 is described in full detail in Patel et al. (2019). The final analysis and Kaplan-Meier plots in Patel et al. (2019) demonstrate comparable survival between liposarcoma and leiomyosarcoma patients at risk receiving trabectedin or dacarbazine although both liposarcoma and leiomyosarcoma patients demonstrated improved disease control with trabectedin (in months) after an overlapping dramatic drop in percent survival to, respectively, approx. 60 % and 70 % vs 50 % within two months after the start of the treatments. There was, however, no statistically significant difference in overall survival. This study was connected with clinical trial NCT01343277 (Clinical Trails, 2023c). For both sarcomas, there are only a few chemotherapeutic options available (doxorubicin, ifosfamide and dacarbazine), and the expected median survival after diagnosis of metastasis is about a year.

A type II variation on the use of trabectedin to extend the indication of ovarian cancer to include relapsed platinum-sensitive ovarian cancer in combination with pegylated doxorubicin was approved by EU EMA in 2009. This study was mainly based on a pivotal phase III clinical trial OVA301 with primary endpoint progression-free survival showing the

superiority of the trabectedin/doxorubicin combination of 21 % risk reduction for disease progression compared to single agent doxorubicin treatment. Also, overall response rates were higher with the trabectedin/doxorubicin combination (27.6 % vs. 18.8 %). Results for overall survival were compatible with a risk reduction for death but without significance.

Then, a follow-up EU-authorized phase III study coded OVC-3006 compared the trabectedin/doxorubicin combinatory therapy with single-agent doxorubicin therapy in patients with advanced relapsed ovarian cancer after two-line platinum-based chemotherapy with overall survival as a primary endpoint. This study was prematurely discontinued based on an interim futility analysis indicating a lack of overall survival superiority as a primary endpoint as well as progression-free survival as a secondary endpoint in the combination arm over the doxorubicin arm. This triggered a formal re-evaluation of the efficacy/safety balance in 2020 to answer the question of whether the marketing authorization of Yondelis should be maintained, varied, suspended or revoked. Based on design arguments and statistical justifications of the obtained interim data, EU EMA concluded that study 3006 did not permit to conclude on the effects of Yondelis + doxorubicin in third-line platinum-sensitive ovarian cancer. Finally, the positive benefit-risk balance in terms of progression-free survival, as previously established in the trial 301 study, remained unchanged. It was nevertheless recommended that the marketing authorization information should be updated to reflect the results from study OVC-3006 (European Medicines Agency, 2020e). Since its marketing, postapproval pharmacovigilance activities have regularly updated the safety profile of Yondelis in the marketing authorization information, e.g. cardiac toxicity and interstitial lung disease, together with measures to minimize those risks (European Medicines Agency, 2007; European Medicines Agency, 2022d, 2020d, 2020c). US FDA also alerted concern on potential signals of serious risks on interstitial lung disease and mucositis in 2019 and is currently evaluating the need for regulatory action (US Food and Drugs Administration, 2019a).

Since December 1999 approx. 70 clinical studies have been issued (MedChemExpress, 2023a). Trabectedin, alone or in combination, is currently still actively being investigated for its efficacy-safety profile in several disease indications or using protocols different from the currently approved ones. In the ClinicalTrials.gov database, 26 clinical trials in active or recruiting status involving trabectedin are reported in October 2022.

3.2. Plitidepsin (dehydrodidemnin B; Aplidine©)

3.2.1. Preclinical assessments

Plitidepsin is an active anticancer substance discovered in the ascidian genus *Tridemnum*. Plitidepsin is a cyclic depsipeptide, a peptide in which one or more of its amides (–CONH–R–) are replaced by its corresponding ester (–C(O)OR–). Plitidepsin was expected to be used to treat adults with multiple myeloma. The MoA blocks the protein eEF1A2 involved in breaking down wrongly folded proteins which are toxic to myeloma cells. By blocking eEF1A2, plitidepsin causes the accumulation of these proteins in multiple myeloma cells, damaging them and ultimately leading to their death. This information has been thoroughly reviewed by Alonso-Álvarez et al. (2017). Recently, intravenously administered plitidepsin demonstrated highly effective preclinical activity against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and might become a repurposed drug as a promising alternative COVID-19 therapeutic. Its antiviral activity was 27.5-fold more potent than that of the anticorona drug remdesivir (Martinez, 2021; White et al., 2021).

3.2.2. Clinical status

Plitidepsin has also been commercialized by PharmaMar under the commercial name Aplidine©. The drug received orphan designations in the EU for the treatment of acute lymphoblastic leukemia in July 2003 (European Medicines Agency, 2020b), multiple myeloma in November 2004 (European Medicines Agency, 2023d), primary myelofibrosis (European Medicines Agency, 2023g) and post essential thrombocythaemia myelofibrosis in February 2011 (European Medicines Agency, 2023f). Numerous clinical trials have been conducted during the past two decades (Alonso-Álvarez et al., 2017). In 2010, Aplidine© had the clinical status phase III in the disease area of cancer. However, single-agent Aplidine© has shown limited antitumor activity and activities in other hematological malignancies have been disappointing so far (Alonso-Álvarez et al., 2017). Further research has been discontinued for reasons of concerns about toxicity despite its strong antiviral and in vivo cytotoxicity. Aplidine©, as well as the closely related didemnin B, have been withdrawn from marketing after numerous clinical trials due to significant toxicity, including cardiotoxicity (Newman and Cragg, 2016). Aplidine© was therefore already not listed in the 2009 US FDA cumulative list of all compounds that have received orphan designation (Food, 2023). No further updates were found in the US FDA database.

This product was withdrawn from the Community Register of designated orphan medicinal products in June 2011 for the treatment of acute lymphoblastic leukemia (update in European Medicines Agency, 2020b), primary myelofibrosis (update in European Medicines Agency, 2023g) and post essential thrombocythaemia myelofibrosis (update in European Medicines Agency, 2023f) on request of the sponsor. In July 2018, the EU EMA CHMP (European Medicines Agency, 2018) finally adopted a refusal of the marketing authorization for Aplidine©. The Committee based its refusal on the modest increase in time of around one month without the disease getting worse and insufficient improvement in overall survival. However, on 26 October 2020, a judgment of the General Court of the EU annulled the Commission Implementation Decision refusal of the marketing authorization (European Medicines Agency, 2020a; Europe, 2023).

3.3. Lurbinectedin (PM01183; Zepzelca©)

3.3.1. Preclinical assessments

Lurbinectedin is a synthetic trabectedin analog having similar MoAs as those described for trabectedin: covalent binding to DNA, causing double-strand breaks, disruption of DNA/protein interactions and RNA transcription, induction of apoptosis in tumor microenvironments, reduced expression of the inflammatory chemokine ligand 2 and reducing tumor angiogenesis. DNA adducts in vitro living cells also triggered double-strand breaks, S-phase accumulation and apoptosis (Patel et al., 2021). Tumor growth inhibition by lurbinectedin is associated with reduced proliferation, increased rate of aberrant mitosis and subsequent induced apoptosis (Vidal et al., 2012). The potent mean GI_{50} cytotoxicity in a 23-cell line panel was 2.7 nM (Leal et al., 2010).

Significant in vitro antitumor activity against chemosensitive and chemoresistant human ovarian clear cell carcinoma (CCC) cells was demonstrated and in vivo tumor growth inhibition of mouse CCC cell xenografts, in a combinatory treatment with the antineoplastic drug 7-ethyl-10-hydroxycamptothecin (SN-38) initiated a significant synergistic effect (Takahashi et al., 2016). The strongest synergistic effect in combinatory treatments has been observed in cisplatin-resistant tumors. Single lurbinectedin or cisplatin-combined therapies are effective in treating cisplatin-sensitive and cisplatin-resistant preclinical ovarian tumor models Soares et al. (2011). The results of (Takahashi et al., 2016) are in line with previous experiments by Leal et al. (2010). The authors demonstrated strong tumor growth inhibition in four murine xenograft models of human cancer without loss of weight of treated animals.

3.3.2. Clinical status

Lurbinectedin has also been commercialized by PharmaMar under the commercial name Zepzelca©. Numerous clinical trials against multiple diverse cancers are ongoing. The first clinical trial on the effects of lurbinectedin started in March 2009 (MedChemExpress, 2023a). Lurbinectedin has received EU orphan designation (EU/3/12/1053) for the

treatment of ovarian cancer in 2012 (European Medicines Agency, 2023e) and EU orphan designation (EU/3/18/2143) for the treatment of small cell lung cancer (SCLC) in 2019 (European Medicines Agency, 2023h). In 2020, US FDA approved lurbinectedin for the treatment of metastatic SCLC with disease progression on or after platinum-based chemotherapy in adult patients (Food, 2020). Other clinical trials on single agent lurbinectedin and in combination with doxorubicin are currently ongoing in phases II and III (PharmaMar, 2023). Lurbinectedin was positively tested based on its overall response and acceptable safety /risk balance as second-line treatment for patients with SCLC after the failure of first-line platinum-based chemotherapy or in the event of a relapse in a phase II basket trial (Trigo et al., 2020). A follow-up on this study in the form of a randomized phase III trial of reduced doses of trabectedin in combination with doxorubicin as second-line therapy of SCLC did not meet the primary endpoint for superiority, as was reported in a press release, compared to topotecan, a synthesized analog of the natural drug camptothecin or the sequentially administered cyclophosphamide/doxorubicin/vincristine (VAC) (Patel et al., 2021).

3.4. The staurosporine family

3.4.1. Staurosporine

Staurosporine was the first representative of a large group of indolo (2,3-α)carbazoles in the bacterium/actinomycete *Streptomyces staurosporeus* in 1977 (Omura et al., 1977) and is considered a lead compound of the natural or synthetic analogs midostaurin, edotecarin, lestaurtinib, becatecarin, edotecarin, 7-Hydroxystaurosporine, enzastaurin, CEP-1347, etc. CEP-1347 was also found in the terrestrial fungus *Xylaria* sp. and the marine bacteria *Nocardiopsis* sp. (Christy and Shankari, 2020). Ascidian sources are among other the genus *Eudistoma* sp. (Jimenez et al., 2012; Palanisamy et al., 2017; Zenkov et al., 2020). The ascidian genus *Eudistoma* was also the provider of several other alkaloids including eudistomins, eudistines, methyleudistomins and pibocin, a natural acetylcholinesterase inhibitor (Jimenez et al., 2012). Natural staurosporine is the antibiotic AM-2282. The history, diversity, biological properties and applications in antitumor therapy of Indolo[2,3-α] carbazoles have recently been thoroughly reviewed by Zenkov et al. (2020). At least 55 compounds of natural origin have been discovered in ascidians, clams, slugs and bacteria (Streptomycetes, Actinobacteria and Cyanobacteria) (Zenkov et al., 2020). The description of staurosporine and important derivatives is therefore here as much as possible directed on new information or information which has not been included in Zenkov et al. (2020).

The following compounds, midostaurine, lestaurtinib, becatecarin and edotecarin, are all (semi)synthetic derivatives of the natural lead compound staurosporine.

3.4.2. Midostaurine ((PKC-412; CGP41251; Rydapt©)

3.4.2.1. Preclinical assessments. Midostaurine or N-benzoylstaurosporine is a multi-target kinase inhibitor blocking the action of many receptor tyrosine kinases with IC_{50}s ranging from 22 to 500 nM. Mutations in the class-III receptor tyrosine kinase fms-like tyrosine kinase 3 (FLT3) are common in acute myeloid leukemia (AML) (Baer, 2019). Fms is an integral transmembrane glycoprotein that exhibits ligand-induced tyrosine-specific protein kinase activity which triggers a signaling cascade (MedChemExpress, 2023b). An abnormal form of the mutated FLT3 tyrosine kinase gene stimulates the survival and growth of acute myeloid leukemia cells. Midostaurine blocks the abnormal FLT3 enzyme thereby stimulating the death of the abnormal cells and control of the spread of the cancer. Midostaurine also blocks a mutated form of another enzyme, KIT kinase, the receptor tyrosine-protein kinase encoded by the KIT-gene CD117, which plays an important role in stimulating the abnormal growth of mast cells in patients with mast cell disorders.

3.4.2.2. Clinical status. In 2006 and 2010, respectively, the EC granted orphan designations EU/3/04/214 and EU/3/10/765 to Novartis for the treatment of acute myeloid leukaemia (European Medicines Agency, 2023b) and mastocytosis (European Medicines Agency, 2023c). In 2017 the EU EMA and US FDA granted approval for use and marketing authorization for midostaurine as the medicine name Rydapt© following successful phase III trials since 2008, in combination with the chemotherapeutic drugs daunorubicin and cytarabin for the indications acute myeloid leukemia and/or aggressive systemic mastocytosis, systemic mastocytosis with associated hematologic neoplasms or mast cell leukemia (European Medicines Agency, 2022c; US Food and Drugs Administration, 2017). The European authorization and EPAR were last positively updated in 2022 (European Medicines Agency, 2023i). Rydapt© is only given for treatments of identified FLT3 mutation-positive acute myeloid leukemia. Midostaurin became the first approved FLT3 inhibitor Baer (2019). Midostaurine is also being tested for the treatment of myelodysplastic syndrome and further showed strong activity in patients with mutations of CD135, an fms-like tyrosine kinase 3 receptor (Millward et al., 2006). The clinical trials of midostaurine showed alternating success since midostaurine proved ineffective against metastatic melanoma in another phase II trial (Millward et al., 2006).

3.4.3. Lestaurtinib (CEP-701)

3.4.3.1. Preclinical assessments. Lestaurtinib also blocks several receptor multi-kinases, with specificity for TrkA, TrkB and TrkC and FLT3 (Beljanski, 2009). A recent update on first and second generation FLT3-inhibitors is provided by Baer (2019). The drug has potent activity against receptor tyrosine kinases at very low doses (IC_{50}s of 0.9 nM to less than 25 nM). It binds to a protein that is present on the surface of some types of cancer cells and stops them from dividing (National Cancer Instituut, 2023). Lestaurtinib is a so-called dirty drug since it inhibits everything in the genome (US Food and Drugs Administration, 2019b). Another potent bioactivity of lestaurtinib in a research stage revealed potent activity in mouse satellite cells in vitro and in vivo. In vivo, subcutaneous administration of lestaurtinib to acutely injured animals increased both the number of satellite cells and muscle repair (Buchanan et al., 2020).

3.4.3.2. Clinical status. Lestaurtinib was in multiple clinical uses for the treatment of acute myeloid leukaemia (Faderl and Kantarjian, 2018). The drug received orphan designation in the EU in 2006 (European Medicines Agency, 2022a), however, the tests were discontinued in 2012 (Williams, 2013) and the product was withdrawn from the Community Register of designated Orphan Medicinal Products in April 2015 on request of the sponsor (European Medicines Agency, 2009b). Lestaurtinib failed to meet its primary endpoint in a phase 3 trial evaluating it as salvage therapy after chemotherapy in patients with FLT3-activating mutations (Schiller et al., 2016).

3.4.4. Becatecarin (NSC 655649)

3.4.4.1. Preclinical assessments. Becatecarin is the N-monoethylated analog of rebeccamycin, a chlorinated indolocarbazole isolated from the actinomycetes *Nocardia aerocolonigenes*, *Saccharothrix aerocolonigenes* and *Lechevalieria aerocolonigenes* obtained from or unique to the ascidian species *Symplegma rubra*, *polyclinum vasculosum* and to a lesser extent *Aplidium solidum* (Buedenbender et al., 2017; Zenkov et al., 2020). Table 1 in Buedenbender et al. (2017) summarizes the de novo operational taxonomic units of the actinomycetales symbionts in ascidian species. Becatecarin acts as an intercalator into DNA, topoisomerases I and II inhibitor and antitumor drug against multiple diverse cancer types (van Pée, 2012; Elshahawi et al., 2015; Zenkov et al., 2020). However, in general, the effectiveness of becatecarin in preclinical and

clinical assessments was estimated lower than the effectiveness of existing drugs (Zenkov et al., 2020). Becatecarin is a more water-soluble synthetic rebeccamycin analog purposed to improve the bioavailability (Denny, 2013). Becatecarin and rebeccamycin differ in bioactivities. Becatecarin is a dual topoisomerase I and II inhibitor and a DNA-intercalator (Nock et al., 2011; Schwandt et al., 2012) while rebeccamycin is a weak topoisomerase I inhibitor. Topoisomerase inhibitors are mostly DNA intercalators (Denny, 2013), thereby interfering with the topoisomerase I-catalyzed DNA breakage-reunion reaction and initiating DNA cleavage and apoptosis. A recent update on structural and bioactive similarities and differences between rebeccamycin and its derivative becatecarin are described in Huang et al. (2020).

3.4.4.2. Clinical status. Becatecarin is an anticancer compound for the treatment of hepatobiliary duct tumors, a rare and aggressive form of cancer with a high medical need and very limited survival. A total of 13 completed or terminated clinical studies on the use of single agent becatecarin or in combinatory treatment of a variety of cancer types, including biliary tree cancer, acute myeloid leukemia, neuroblastoma, solid tumors, colorectal cancer, lung and breast cancer have been reported in the US National Library of Medicine between 2003 and 2014 (Clinical Trails, 2023a). No results of these studies have been posted or evaluated by the US Federal government. Orphan designation of becatecarin was granted by the EC EMA in 2006 for the treatment of the biliary cancer tract (European Medicines Agency, 2022a). The last EU EMA update on 21 February 2019 reported the withdrawal of becatecarin from the Community Register of designated Orphan Medicinal Products in October 2018 at the request of the sponsor (update in European Medicines Agency, 2009a). The tests have been discontinued for the reason of 'not superior to existing therapies'. The source of this information on PR Newswire 'Exelixis Helsinn to Discontinue Becatecarin Trial Program' (Newswire, 2023a) was no longer accessible since 15 September 2021.

3.4.5. Edotecarin (PHA-782615)

3.4.5.1. Preclinical assessments. Edotecarin is also a synthetic derivative of rebeccamycin and staurosporin analog having an introduced $(CH_2)_2NEt_2$ substituent at the imide nitrogen atom of the rebeccamycin molecule (Zenkov et al., 2020). Edotecarin is a strong topoisomerase I inhibitor causing single-strand DNA breaks (PubChem, 2023; Zenkov et al., 2020) and subsequent inhibition of DNA replication and decreased tumor cell proliferation. The MoA of staurosporin differs from that of edotecarin in the absence of protein kinase inhibitory properties. In vitro and in vivo inhibition of tumor growth has been observed in breast, cervix, pharynx, lung, prostate, colon, gastric, and hepatic cancer models (Saif and Diasio, 2005; Saif et al., 2010) and was effective in cells with acquired P-glycoprotein resistance. Increased toxicity in vitro and/or synergistic effects have been observed in combination with cisplatin, 5-fluorouracil, etoposide, paclitaxel, doxorubicin, vincristine, camptothecin and gemcitabine (Drugbank online, 2022b; MedChemExpress, 2023b; Sigmond et al., 2010).

3.4.5.2. Clinical status. Five clinical trials are reported in Clinical Trials (Clinical Trails, 2023b). Antitumor responses have been reported in patients with refractory metastatic colon cancer and gastric, breast and esophageal cancers and in patients with irinotecan- or 5-fluorouracil (5-FU)-refractory colorectal cancer (Denny, 2013). Only one study was completed in phase III in 2008 on the effects of edotecarin in patients with glioblastoma multiforme (Clinical Trails, 2008). The studies have not been evaluated by EU EMA or US FDA.

3.4.6. 7-Hydroxystaurosporine (UCN-01)

3.4.6.1. Preclinical assessments. The MoA of 7-hydroxystaurosporine is involved in the inhibition of 3-phosphoinositide-dependent protein kinase 1 which acts as a master phosphorylating and activating serine/threonine kinase of a subgroup of the family of protein kinases (Sendorowicz AM, 2002; Imming et al., 2006; Overington et al., 2006; Mull et al., 2020; Drugbank online, 2022a). The conventional cyclin-dependent protein kinase C (cPKCα) was the main target for inhibition at an extremely low Ki of 0.44 nM (Mizuno et al., 1995). However, the insolubility of 7-hydroxystaurosporine in water is an expected disadvantage in cellular processes although recent research of Mull et al. (2020) showed that treatment of mice with low-dose 7-hydroxystaurosporine induced a reversible post-mitotic G1 arrest in normal cells and improved tolerance to bolus 5-FU in no tumor-bearing mice. In addition, pre-treatment of tumor-bearing mice with lower-dose 7-hydroxystaurosporine before 5-FU treatment decreased chemotoxicity allowing higher 5-FU doses for enhanced therapeutic efficacy.

3.4.6.2. Clinical status. Drugbank online (2022a) and Clinical Trails (2010) reported multiple completed clinical trials on the treatment of different cancers. A last update on clinical progress was posted on 9 September 2010. No active trials or approvals have recently been reported.

3.4.7. Enzastaurin (LY317615)

3.4.7.1. Preclinical assessments. Enzastaurin acts as a specific inhibitor of protein kinase Cβ (PKCβ). The enzyme is involved in both the protein kinase B and mitogen-activated protein kinase signaling pathways that are active or overexpressed in many cancers (Hill and Hemmings, 2002; Bourhill et al., 2017). Preclinical data on inhibition of protein kinase B may indicate reduced cell proliferation and increased apoptosis in a range of mammalian cells and inhibited angiogenesis in tumors (Hill and Hemmings, 2002; Bourhill et al., 2017). Combination therapy of enzastaurin and the drug ibrutinib showed synergistic regulation of whole-transcriptome expression resulting in induction of antitumor effects, including reduction of proliferation, promoting apoptosis, inducting G1 phase arrest, preventing cell invasion and migration, and down-regulating activation of downstream signaling in diffuse B cell lymphoma (He et al., 2019).

3.4.7.2. Clinical status. Enzastaurin was used as a single agent or in combination with other drugs or radiotherapy in 50 clinical studies since 2002 (Clinical Trails, 2023c). Three completed trials in phase III for the treatment of relapsed glioblastoma multiform, grade 4 gliomas (an aggressive and malignant form of brain cancer), non-Hodgkin's lymphoma and diffuse large B-cell lymphoma and many other mostly completed and 4 terminated phase I and II studies on the treatment of several other tumor types, including colorectal cancer, non-SCLC, pancreatic cancer, and mantle cell lymphoma. EU EMA granted orphan designations in 2005, 2009 and 2022 for, respectively, the treatment of glioma (European Medicines Agency, 2005), diffuse large B-cell lymphoma (European Medicines Agency, 2009c) and Ehlers-Danlos syndrome (European Medicines Agency, 2022b). Both studies on treatments of glioma and diffuse large B-cell lymphoma have been positively updated by EU EMA in October 2020 (updates in European Medicines Agency, 2005; European Medicines Agency, 2009c). However, Williams (2015) reported the discontinuation of phase III tests on the treatment of diffuse large B-cell lymphoma in 2013 for reasons of lack of efficacy. The tests failed to meet the endpoints.

3.4.8. CEP-1347 (KT7515)

3.4.8.1. Preclinical assessments. CEP-1347 is a selective and potent inhibitor of the stress-activated protein kinase pathway, an intracellular signaling pathway that is an essential component of the stress response leading to neuronal programmed cell death or apoptosis. CEP-1347 was

originally developed for the treatment of Parkinson's disease due to its selective inhibition of the growth of retinoblastoma cell lines expressing murine double minute 4 (MDM4), a tumor suppressor protein P53 inhibitor (Togashi et al., 2020). In vitro cell culture systems and in-vivo mouse and nonhuman primate models of Parkinson's disease have shown that CEP-1347 protects dopamine neurons in the substantia nigra, the area of the brain affected by Parkinson's disease. CEP-1347 reduces the expression of MDM4 and activates the P53 pathway in MDM4-expressing retinoblastoma cells, which is required for growth inhibition (Togashi et al., 2020). Togashi et al. (2020) see CEP1347 as a promising candidate for the treatment of retinoblastomas, where functional inactivation of the P53 protein as a result of MDM4 activation is reportedly common. In an earlier study CEP-1347 directed against two identified key targets involved in the apoptotic process, the mixed lineage kinase (MLK) family and glyceraldehyde phosphate dehydrogenase (GAPDH) demonstrated robust neuroprotective activity in multiple in vitro and in vivo models of neuronal cell death but did not prove effective in patients affected with Parkinson's disease (Waldmeier et al., 2006).

CEP1347 is also an inhibitor of the oncogenic P21-activated kinase 1 (PAK1) pathway. Mixed lineage kinase 3 (MLK3) was reported to function in the tumorigenesis of breast cancer cells by activating PAK1 (Das et al., 2019). Suppression of PAK1 also reduced in vitro and in vivo growth of ovarian cancer cells. These inhibitory effects were dependent on the activation of the P53 protein and subsequent increase in P21 (Prudnikova et al., 2016). According to Togashi et al. (2020) these studies demonstrate a CEP1347-regulated MLK-PAK1 pathway, independent of MDM4. The authors forward the hypothesis on the antiproliferative effects of CEP1347 in retinoblastoma due to the activation of the P53 protein via the MLK3-PAK1 pathway. Another study demonstrated knockdown of survivin overexpression in ovarian cancer stem cells resulting in increased sensitivity to paclitaxel, an anticancer chemotherapeutic plant alkaloid. Overexpression of surviving plays a key role in the chemoresistance of ovarian cancer stem cells (Togashi et al., 2018; Das et al., 2019). CEP-1347 also showed anti-inflammatory properties by lowering the level of inflammatory cytokines during ischemia and has been proposed as antistroke therapy (Christy and Shankari, 2020). The CEP-1347 used in this study was a natural analog isolated from the terrestrial fungus *Xylaria* sp. and the marine bacteria *Nocardiopsis* sp.

3.4.8.2. Clinical status. One completed and one terminated study on the use of CEP-1347 in the treatment of Parkinson's disease is reported in Clinical Trails (2023c). The status of the completed study has not been verified so far. No new active trials have been reported.

4. Ascidian MNPs without clinical history so far

4.1. Peptides

4.1.1. Preclinical assessments

Besides the natural bioactive therapeutic drugs plitidepsin and didemnin B, numerous other ascidian-derived linear and cyclic peptides, depsipeptides and thiazole-, thiazoline- and quinolone-based peptides (mollamides, ulithiacyclamides, sistratamide, didmolamides, clavanins, patellins, keenamide, trunkamide A, lissoclinolide, styelin, clavanin, clavaspirin, plicatamide, dicynthaurin, halocidin, halocyamines) have been discovered and reported (Tincu and Taylor, 2004; Rawat et al., 2006; Teruya et al., 2008; Sivonen et al., 2010; Fang et al., 2016; Lee et al., 2017; Negi et al., 2017; Palanisamy et al., 2017; Arumugam et al., 2018; Kang et al., 2018; Gogineni and Hamann, 2018; Watters, 2018; Dahiya et al., 2020; Cegłowska et al., 2020; Zhang et al., 2021). A recent review of Ahmed et al. (2022) describes the effects of peptides as therapeutic agents on various physiological pathways of microtubule balance disturbances, suppression of angiogenesis, cell migration/invasion, and cell viability/apoptosis for the treatment of cervical and ovarian cancers. Techniques for the total synthesis of bioactive peptides have been compared and evaluated by Arumugam et al. (2018). The authors concluded that the recombinant production in *Escherichia coli*, reviewed by Li (2011), might be the most cost-effective process and a good alternative for large-scale peptide production compared to solid and solution phase techniques. However, despite enormous potential, a major drawback in the utilization of these bioactive peptides is the limited stability and bioavailability issues (Dahiya et al., 2020) although, with exceptions e.g. talaropeptide A. Ascidian-derived talaropeptides, the talarolides A-D are a new class of nonribosomal peptide synthetase (NRPS) mediated N-methylated linear peptides and one cyclic heptapeptide hydroxamate analog (talarolide A). The actual producer source was finally identified as the associated symbiont Ascomycete fungus *Talaromyces sp* (Dewapriya et al., 2017, 2018). *Talaromyces* is a ubiquitous genus found in both terrestrial and marine ecosystems. Cultures of *Talaromyces* sp. in micro bioreactors enabled the scale-up of the production of the talaropeptides. The talaropeptides displayed pronounced selective antibacterial activities at IC_{50} values in the low μM-range with a clear structure-activity relationship requirement built around the N-terminal acetylation against the growth of the Gram-positive bacterium *Bacillus subtilis* and exhibited low mammalian cell cytotoxicity. Additionally, compound talaropeptide A showed high plasma stability against various rat proteases (Dewapriya et al., 2018). Talarolide A is the first reported cyclic peptide hydroxamate from a fungus. Talarolide A showed poor cytotoxicity in tests on the growth of human cancer cells, the fungus *Candida albicans* and the bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* (Dewapriya et al., 2017).

Two cysteine-rich peptides, turgencin A and B, and their methionine-oxidized derivatives were found in the Arctic ascidian *Synoicum turgens* (Hansen et al., 2020). Turgencin A was most potent against bacteria with a minimum inhibitory concentration (MIC) of 0.4 μM. In addition, the peptide inhibited the growth of the melanoma cancer cell line A2058 and the human fibroblast cell line MRC-5 at IC_{50} values of 1.4 μM and 4.8 μM, respectively (Hansen et al., 2020). The MoA of these small partly cationic and amphipathic antimicrobial peptides (AMPs) facilitates binding to the bacterial surface and destabilizes the anionic part of the cell membrane in microorganisms (Pasupuleti et al., 2012). Marine-derived AMPs seem to enable stronger cationic interactions with the bacterial anionic membranes leading to membrane disruption and cell death, compared to terrestrial AMPs (Hansen et al., 2020).

Another interesting antibacterial ionizing peptide from ascidian origin targeting the integrity of bacterial membranes is clavanin A (Tincu and Taylor, 2004). Clavanin A is as effective as the human cathelicidin antimicrobial peptide LL-37. Clavanin A showed significant inhibition of *Staphylococcus aureus* colony forming in an experimental wound model (Silva et al., 2015). Also, the activity of clavanin A against the bacteria *Escherichia coli* and *Listeria monocytogenes* and the fungus *Candida albicans* was substantially greater in acidic conditions. Clavanin A permeabilized the outer and inner membranes of *Escherichia coli* more effectively at pH 5.5 than at pH 7.4. This pH effect might be attributed to the high net positive charge of clavanin A at pH 5.5 (Tincu and Taylor, 2004). Enhancement of the pharmaceutical potential of ionizing compounds in acidic conditions such as clavanin is an interesting observation for intracellular processes since Carter's research in 1972 demonstrated compartments with varying intracellular pH (pH_i) values between 6.12 and 7.50 in large muscle cells of the crustacean giant barnacle (*Balanus nubilus*). The assumption of compartmentalization of the pH_i in cells is in contrast to the reported higher integrated pH_i values of e.g. the homogenate technique of (Pörtner et al., 1990). A similar approach was used in the description of a bioavailability model on the ionizing contaminant tributyltin to explain the enhanced bioaccumulation and adverse effects of this contaminant in biota (Parmentier et al., 2019).

The induction of AMPs is an essential innate immune response.

Recently, a lipopolysaccharide (LPS)-induced chemo-attractive peptide was identified and characterized from the ascidian *Ciona robusta* (formerly *Ciona intestinalis* type A; Longo et al., 2020). Tunic tissue infiltrated LPS induced an inflammatory response of physiological defense pathways starting fighting the pathogenic organisms, removing damaged and dead tissue, producing growth factors and promoting extracellular matrix remodeling. In a study, the ability of the LPS-induced chemo-attractive peptide was tested in a wound-healing experiment on physically scratched human dermal cells. After 16 h post incubation, the damaged cells showed improved cell motility and enhanced ability to reduce the physical damage (Longo et al., 2020).

Many complex natural ascidian-originated thiazole-, thiazoline- or oxazoline-based cyclic hexa-, hepta- or octapeptides with alternating hydrophobic amino acids possess exceptional structural and bioactive properties (Dahiya et al., 2020). The ascidian *Didemnum molle* is a well-known producer of many of these peptides. Several mollamides are thiazole-based cyclohexapeptides from this ascidian source (Dahiya et al., 2020; Zhang et al., 2021). IC_{50} cytotoxicities of mollamide analogs tested in several cancer cell lines ranged from high to marginal between 1.2 and 100 μM (Arumugam et al., 2018). Although mollamide B showed significant cell growth inhibition, evaluation by the National Cancer Institute (NCI) in the 60-cell-line panel showed no improved sensitivity above the mean (Donia et al., 2008). Antiviral, antibacterial and antiparasitic inhibitory values in tests with mollamides differed a lot too. The antiviral IC_{50} activities of mollamide F against HIV infection in two bioassays, an HIV integrase inhibition assay and a cytoprotective cell-based assay with human cells prone to apoptosis upon HIV infection, were 39 and 78 μM, respectively (Lu et al., 2012). Another compound from the same source *Didemnum molle*, the benzene derivative molleurea was active only in the cytoprotective cell-based assay (IC_{50}: 60 μM) (Ji et al., 2018). Another in vitro assay on the toxicity of mollamide B against HIV-1 in human peripheral blood mononuclear (PBM) cells resulted in an EC_{50} value of 48.7 μM. Mollamide B also exhibited activity against the parasitic protozoans *Leishmania donovani* causing leishmaniasis (IC_{50} and IC_{90} values of 21.6 μM and 42 μM) and *Plasmodium falciparum* causing falciparum malaria (IC_{50}: approx. 2.4 μM) (Donia et al., 2008). On the other hand no antibacterial activity of mollamide B and another peptide from *Didemnum molle* was demonstrated against MRSA, *Mycobacterium*, *Candida albicans*,*Candida glabrata*, *Candida krusei*, and *Cryptococcus neoformans* (Donia et al., 2008).

Cyanobactins refer to a group of over 100 diverse ribosomal cytotoxic cyclic peptides of 6–8 amino acids (Sivonen et al., 2010; Watters, 2018; Cegłowska et al., 2020). As the name suggests, the name cyanobactin has been derived from a cyanobacterium, *Prochloron didemni*, an obligate symbiont in ascidian Didemnidae, source *Lissoclinum* sp. (Schmidt et al., 2005). Some of these cyanobactins, e.g. bistratamides, lissoclinamides and patellamides are known cytotoxic and metal binding.

The gene clusters and pathways involved in the formation of the thiazoline and oxazoline rings in the cyanobactins, expressed in *Prochloron didemni*, have been well studied and the biotransformations involve several complex enzymatic steps: amino acid heterocyclization, peptide cleavage, peptide macrocyclization, heterocycle oxidation, and epimerization of a diastereomer to its chiral counterpart. Some closely related products are also prenylated (Koehnke et al., 2014; Czekster et al., 2016). The molecular targets are unknown although patellamides inhibit the multidrug resistance gene 1 (MDR1) (Lopez and Martinez-Luis, 2014; Watters, 2018).

Cytotoxic linear thiazole-containing tripeptides, virenamides A, B and C (ascidian source *Diplosoma virens*) exhibited topoisomerase II inhibition against various cultured cells mouse leukemia P388, A549, HT-29 and CV1 at approx. IC_{50} values 1.3, 2.7 and 5.2 μM; recalculated from μg/mL (Zhang et al., 2021). Total synthesis of virenamide A and G are described in Gan et al. (2008).

While the IC_{50} cytotoxicities of many ascidian-derived peptides range in the low μM values, one peptide possessing striking IC_{50} cytotoxicity in the low nM-range is the thiazoline-based prenylated cyclopeptide trunkamide A against the cell lines DU-145 (7.08 nM), IGROV (7.31 nM), SK-BR-3 (5.44 nM) and Hela (3.90 nM) (McKeever and Pattenden, 2003). The full synthesis of trunkamide A has been achieved (McKeever and Pattenden, 2003).

Four iodobenzene-containing dipeptides and a related bromotryptophan-containing dipeptide, named apliamides A to E and an iodophenethylamine apliamine A were isolated from the ascidian *Aplidium* sp. These novel compounds exhibited moderate but selective in vitro cytotoxicity against several cancer cell lines and a very potent apliamide D displayed significant inhibition against Na^+/K^+-ATPase (Won et al., 2015). All compounds exhibited moderate cytotoxicity at IC_{50} values 7.8–21.1 μM against the K562 leukemia cell line. Similar trends were found for the A549 lung cancer cell-line (IC_{50} 8.3–22.8 μM) although apliamides A and E were inactive ($IC_{50} > 100$ μM). All compounds exhibited weak cytotoxicity in the MRC5 human lung fibroblast cell-line ($IC_{50} > 37.0$ μM). Apliamide C and apliamine A showed no inhibition of a normal cell line (MRC5, $IC_{50} > 100$ μM) and moderate cytotoxicity against the cancer cell lines K562 and A549 (IC_{50} 7.8–22.8 μM). Remarkable, apliamide D is the strongest inhibitor against Na^+/K^+-ATPase at an IC_{50} value of 3.2 μM while this compound showed poor cytotoxicity on the normal cell line (MRC5, $IC_{50} > 100$ μM). None of these compounds exhibited significant antibacterial activities against diverse Gram-positive and Gram-negative strains (MIC > 100 μg/mL) or inhibition against the key enzymes in bacterial metabolism sortase A and isocitrate lyase (Won et al., 2015).

Botryllamides A–H, K, L are a group of natural analogs of dehydrotyrosine and brominated tyrosine derivatives obtained from the ascidians *Botryllus* sp. and *Aplidium altarium*. Botryllamides inhibit multidrug-resistant cells and block selectively the ATP-binding cassette G2 multidrug transporter (ABCG2) (Takada et al., 2010; Cherigo et al., 2015). In a mouse model lacking the aka P-glycoprotein (ABCB1)-mediated efflux from the brain, botryllamide G was shown to in vivo increase brain exposure to the breast cancer-approved central kinase inhibitor lapatinib by blocking the ABCG2 drug efflux transporter in the brain (Strope et al., 2020), thereby reducing the blood-brain barrier. Lung cancers and especially breast cancers cause a higher occurrence of brain metastases (references in Strope et al., 2020). Surprisingly, the presence of uninhibited Abcb1 in wild-type mice proved effective in lapatinib efflux despite ABCG2 inhibition. Botryllamide A, C and E are strong inhibitors of the human colon tumor cells HCT-116 at IC_{50} concentrations between 28 and 33 μM (Rao and Faulkner, 2004). On the other hand, the botryllamides A, B, C, G, K and L did not deliver significant growth inhibition in tests on the cancer cells lines MCF-7 (breast), H460 (lung) and SF268 (central nervous system) (Yin et al., 2010).

The biogenetically brominated arginine/tryptophan-derived eusynstyelamide and its natural analogs have originally been isolated from the ascidians *Eusynstyela latericius*, *Eusynstyela misakiensis* and/or *Didemnum candidum* (Tapiolas et al., 2009; Barykina, 2010). Ent-eusynstyelamide B, an enantiomer of eusynstyelamide B and the eusynstyelamides D, E, and F have also been found in the Arctic bryozoan *Tegella cf. spitzbergensis*. The finding of eusynstyelamides in different phyla from different geographical areas fed the hypothesis of biosynthesis by different bacterial strains (Tadesse et al., 2011). Tests against the bacteria *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Corynebacterium glutamicum*, and methicillin-resistant *Staphylococcus aureus* (MRSA) and the fungus *Candida albicans* showed poor antibacterial and antifungal activity and weak activity against the melanoma cell line A-2058 was observed as well (Tadesse et al., 2011). Liberio et al. (2015) investigated the MoA of eusynstyelamide B in LNCaP prostate and MDA-MB-231 breast cancer cell lines. The compound caused cell growth inhibition at an IC_{50} of 5 μM, G2 arrest, apoptosis and was identified as a novel non-intercalating topoisomerase II poison that activates DNA damage response pathways and induced double-strand breaks. The compound showed similar potency to the anticancer drug etoposide. In contrast to

MDA-MB-231 cells, eusynstyelamide B did not induce cell death in LNCaP cells when treated for up to 10 days of exposure. It should be noted that a previous study on the effects of eusynstyelamides A, B and C on three human tumor cell lines of breast MCF-7, central nervous system SF-268 and lung H-460 reported nontoxic (Tapiolas et al., 2009). Eusynstyelamides B and C inhibit neuronal nitric oxide synthase, a producer of arginine-derived NO, an important cellular signaling molecule, at IC_{50} values of 4.3 and 5.8 μM (Tapiolas et al., 2009). Eusynstyelamide A was much less potent with an IC_{50} value of 41.7 μM.

The pharmacophore model of marine antimicrobial eusynstyelamides inspired Paulsen et al. (2021) to develop a series of synthetic cationic amphipathic barbiturates having an N,N'-dialkylated-5, 5-disubstituted structure. Minimum inhibitory concentrations of approx. 2.5–10 μM (recalculated from μg/mL based on the MW of the precursor eusynstyelamide B) were achieved against 30 multi-resistant bacterium isolates, including strains with extended-spectrum β-lactamase-carbapenemase production able to effectively hydrolyze the antibiotics carbapenems and β-lactams. Further, strong in vivo efficacy in mice was demonstrated. The MoA studies showed a strong membrane-disrupting effect (Paulsen et al., 2021). The structural elucidation and synthesis of eusynstyelamides have been reported by several groups (Tapiolas et al., 2009; Barykina, 2010; Tadesse et al., 2011).

4.1.2. Clinical status

No clinical information on ascidian-derived peptides was reported in the cited databases nor in the governmental databases US FDA and EU EMA (accessed on 31 July 2022). A possible reason for the lack of clinical developments might be the instability of the peptides in vivo test conditions.

4.2. Pyridoacridine alkaloids

4.2.1. Preclinical assessments

Since the first discovery of the natural pyridoacridine alkaloid amphimedine in *Xestospongia* sponges in 1983, the literature discloses comprehensively the structural chemistry, biosynthesis and biological activities of over 75 marine-derived pyridoacridine compounds and their isomeric forms that have been isolated from four phyla (Porifera, Chordata-Subphylum Tunicata, Mollusca and Cnidaria) and the total synthesis of almost every natural pyridoacridine is available. All this information is, among many others, available in the manuscripts and reviews of Bishop and Ciufolini (1992); Steffan et al. (1993); Molinski (1993); Copp et al. (2003); Marshall and Barrows (2004); Marshall et al. (2009); Sharma et al. (2010), (2013); Kijjoa (2015); Sandjo et al. (2015); Sharma et al. (2015); Ibrahim and Mohamed (2016); Khalil et al. (2016); Joule and Álvarez (2019); Jiang and Wang (2021). The total synthesis of pyridoacridines in the 21st century is presented in Joule and Álvarez (2019).

Natural pyridoacridines have a common planar tetracyclic pyrido [4,3,2-*m*,*n*]acridine structure. Marine pyridoacridines are tetracyclic, pentacyclic, hexacyclic, heptacyclic or octacyclic. The structural elucidation of pyridoacridines is detailed in Ibrahim and Mohamed (2016). Pyridoacridines display diverse biological activities including cytotoxicity, fungicidal, bactericidal and anticancer activities via different MoAs such as binding with DNA, inhibition of the DNA/RNA/protein synthesis, production of reactive oxygen species (ROS), inhibition of DNA repair system topoisomerase, cleavage/catenation/damage of DNA and/or cell-cycle arrest or apoptosis. However, the biological potential of many known natural pyridoacridines is low. Analogs may have an altered heterocyclic ring structure. Heterocyclic derivatives are well known for their biological activities (Marshall et al., 2009; Sharma et al., 2010, 2013). Because of continued interest in pyridoacridine analogs, a new class of pyridoacridine metal complexes was developed to improve the affinity towards nucleic acid binding (Sharma et al., 2015).

In ascidian species the pyridoacridine subclasses shermilamines, kuanoniamines, amphimedines, ascididemins, dercitins, diplamines and eilatins have been collected from *Cystodes dellechiajei*, *Eudistoma* sp., *Eudiste* sp. and *Tridemnum* sp. Major pyridoacridines and related analogs were reported in purple, blue, green and brown chromotypes of *Cystodes dellechiajei* as well as in the sponge *Xestospongia* sp. (Joule and Álvarez, 2019).

The first identified sponge-derived amphimedine is relatively inactive compared to its natural sponge analogs neoamphimedine, the positively charged deoxyamphimedine and the ascidian analogs 13-didemethylaminocycloshermilamine D 914 and demethyldeoxyamphimedine (Marshall et al., 2003; Bry et al., 2011). Both ascidian compounds as well as other related compounds shermilamine B, kuanoniamine D, N-deacetylshermilamine B, N-deacetylkuanoniamine D and styelsamines C and D were found in the Mediterranean purple ascidian species *Cystodytes dellechiajei* (Bry et al., 2011).

Sharma et al. (2015) argued that neoamphimedine is one of the most effective metabolites of the marine pyridoacridine family since its antitumor efficacy equals the well-known anticancer drug etoposide in tumor cell line tests and acted as effective as 9-minocamptothecin against the resistant human colorectal cancer cell line HCT116 (Marshall et al., 2003; Khazir et al., 2014). Deoxyamphimedine damages DNA via the production of ROS and facilitated DNA cleavage in vitro, unlike amphimedine or neoamphimedine (Marshall et al., 2009). The action of 'pyridoacridine-metal complexes' is also based on the affinity towards nucleic acid binding.

In general, most of the ascidian pyridoacridines exert intercalator-induced inhibition of topoisomerase II in the low μM IC_{50} values (Kijjoa, 2015). Various subclasses of natural pyridoacridines are used as lead compounds in the development of derivatives (Molinski, 1993; Marshall and Barrows, 2004; Sharma et al., 2015; Ibrahim and Mohamed, 2016). Ascididemin, a natural chemical defense and feeding deterrent in the ascidian *Didemnum* sp. (López-Legentil et al., 2005), and its synthetic derivatives display multiple biological activities such as antibacterial/antifungal activities, topoisomerase II enzyme inhibition, antituberculosis activity, antitrypanosomal activity and in vitro and in vivo tumor cytotoxicity (Matsumoto et al., 2003; Appleton et al., 2010; Feng et al., 2010). The antibacterial activities of ascididemin against *Escherichia coli* and *Micrococcus luteus* are also superior with respective MIC values of 0.2 μM and 0.3 μM compared to its lesser active tested pharmacore analogs in the MIC range below 17.4μM (Sandjo et al., 2015). However, the bactericidal activity of ascididemin tested selective: ascididemin significantly inhibited the growth of the bacterium *Bacillus subtilis* but was completely inactive against *Pseudomonas aeruginosa* (Lindsay et al., 1995). Similarly, the toxicity against fungi is also selective: inhibition zone radii against *Candida albicans* and *Cladisporium resinae* were between 10 and 14 mm while no activity was observed against *Trichophyton mentagrophytes* (Lindsay et al., 1995).

Ascididemin and 12-deoxyascididemin (ascidian source: *Polysyncraton echinatum*) were highly toxic against *Trypanosoma brucei brucei* at IC_{50} values of 77 and 32 nM, respectively (Feng et al., 2010). Another pyridoacridine eilatin from the same source was less potent (IC_{50}: 1.33 μM). Synthesized ascididemin-related pyridoacridone alkaloids, including ascididemin, were most active against *Plasmodium falsiparum* followed by *Trypanosoma cruzi* and *Trypanosoma brucei brucei* with intracellular and extracellular *Leishmania donovani* being the least susceptible (Copp et al., 2003). However, when tested in vitro against *Plasmodium falsiparum* strains K1 and NF54, none of the ascididemin-like analogs reached the activities of the antimalarial drugs artemisinin (IC_{50}: 4.6 nM) and chloroquine (IC_{50}: 15.6 nM) (Copp et al., 2003), both values recalculated from μg/mL. It should also be noted that several of the synthesized pyridoacridone analogs of ascididemin possess much higher activity (up to 2000-fold) compared to ascididemin against extracellular *Trypanosoma cruzy* and *Trypanosoma brucei brucei* (*Trypanosoma brucei rhodesiensei*) (Copp et al., 2003). Moderate IC_{50} cytotoxicity activities of these synthesized pyridoacridone alkaloids, including ascididemin, against the mammalian cell lines rat skeletal muscle myoblast L6 cells and murine macrophage-like RAW cells have been

noted between 8.8 and > 35 μM and between 7 and > 353μM, respectively. Most potent in vitro IC_{50} tumor cytotoxicity was reported for ascididemin against mouse leukemia P388, human colorectal carcinoma HCT116, human breast MCF7 and two DNA single-strand break repair-deficient Chinese hamster ovary cell-lines EM9 and xrs-6 at 0.3–0.4 μM and the highest cytotoxicity at 30 nM, respectively (Lindsay et al., 1995). In general, most of the ascidian pyridoacridines, with some exceptions, exert intercalator-induced inhibition of topoisomerase II and antimicrobial activities in the low μM IC_{50} values (Kijjoa, 2015). Ascididemin and another pyridoacridine, meridine, have been shown to stabilize G4-quadruplexes and thereby inhibit telomerase (Guittat et al., 2005).

Comparison of the biological activities of ascididemin and its natural or synthetic derivatives also showed peculiar structure-activity relationships. Lindsay et al. (1995) reported the presence of N-8 in ring A and a completed ring E as an essential requirement for the bioactivity of this compound. Antibacterial and antifungal properties of ascididemin seemed modulated by the presence or absence of ring A N-8. While N-8 is a requirement for *Escherichia coli* and *Cladisporium resinae* growth inhibition, its absence was essential for the inhibition of *Trichophyton mentagrophytes*. On the other hand, the findings of Copp et al. (2003) revealed the arrangement of the four linear rings essential for the highest activity against *Plasmodium falsiparum* and not necessarily including the N in ring A and the additional ring E. The absence of the N-8 in ring A in AK37, a closely ascididemin-related analog, created a new function by stabilizing the DNA-topoisomerase I cleavable complex and ring D did not exhibit any important role in AK37's topoisomerase I activity (Marshall et al., 2004; Sharma et al., 2015). An important remark here is the confusion created by the nonconform use of the ring skeleton identification and numbering of ascididemin and its analogs in reports: e.g. the essential moieties N-8 in ring A and a completed ring E in Lindsay et al. (1995) and Sharma et al. (2015) are, respectively, N in ring E and ring C in Sandjo et al. (2015) and N-8 is N-1 in Copp et al. (2003). Structural alterations tend to reduce bactericidal, fungicidal and trypanocidal activities. The antibacterial potency decreased 10-fold for *Escherichia coli* and 30-fold for *Micrococcus luteus* when the E ring in Sandjo et al. (2015) contained an OH-group and also no further improvement in the antibacterial activity was noted when the E ring of ascididemin is replaced with a thiazole or an oxazinole ring. The replacement of the entire pyridine ring E in Sandjo et al. (2015) by the ethylthio-moiety to 4-(ethylthio)-6H-pyrido[2,3,4-kl]acridin-6-one exerted potent (MIC: 0.34 μM) toxicity against *Mycobacterium tuberculosis* H37rv, similar to the toxicity (MIC: 0.35 μM) of ascididemin. The most interesting compounds of a series of synthetic pyrido[2,3,4-kl] acridin-6-one analogs showed less antituberculosis activity against the growth of *Mycobacterium tuberculosis* H37Rv strain (MIC: 2μM) but negligible cytotoxicity towards Vero cells and mouse leukemia P388 cells (IC_{50} > 25 μM) compared to the antituberculosis active but cytotoxic (IC_{50} <140 nM) ascididemin (Appleton et al., 2010).

The natural substance eilatin is a highly symmetrical alkaloid isolated from the Red Sea ascidian *Eudistoma* sp. and the Australian ascidian *Polysyncraton echinatum* (Rudi et al., 1988; Feng et al., 2010; Sharma et al., 2015). Eilatin showed antitrypanosomal activity at an IC_{50} of 1.33 μM against *Trypanosoma brucei brucei* although much less potent than the ascididemins 12-deoxyascididemin (IC_{50}: 77 nM) and ascididemin (IC_{50}: 32 nM; Feng et al., 2010). A new family of dinuclear eilatin-type complexes of the general formula M(bpy)2- (Lig)]2 + (M = Ru, Os; Lig = eilatin or its synthetic isomers dibenzoeilatin or isoeilatin) is under investigation (Gut et al., 2003; Bergman et al., 2005). The eilatin-Ruthenium(II) complex is interesting for its unusual nucleic acid binding specificity (Luedtke et al., 2002; Zeglis and Barton, 2008). Eilatin-ruthenium(II) complexes intercalate to nucleic acids (Luedtke et al., 2003) although the selectivity in binding destabilized single-base mismatches is lower compared to the smaller mismatch-specific metalloinsertor $Rh(bpy)_2$(chrysene-5,6-quinonediimine)$^{3+}$ (Zeglis and Barton, 2008). Eilatin is characterized by a width, named W1, intercalating the opening of the phosphate backbone. Another larger size width W2 does not allow intercalation with DNA (Vargiu and Magistrato, 2012). A novel divergent approach for synthesizing eilatin and its synthetic isomer isoeilatin has been reported with yields of 9.6 % and 5.1 %, respectively (Plodek and Bracher, 2015). In the search for new active derivatives of eilatin an octacyclic analog, 9-aminopyridoacridine, with μM cytotoxicities against two cancer cell lines was synthetized (Bouffier et al., 2009).

A group of pharmaceutical active thiazole-containing pyridoacridines has been found in the ascidian, sponge and mollusk species (Taraporewala, 1991; Bishop and Ciufolini, 1992; Kijjoa, 2015). Many of these compounds from different taxa are structurally related. Nordehydrocyclodercitin from the ascidian *Aplidium cratiferum* is structurally related to the sponge metabolites stellettamine and cyclodercitin (Gunawardana et al., 1992; Agrawal and Bowden, 2007). The pentacyclic shermilamines and kuanoniamines detected in the ascidian source *Cystodytes dellechiajei* are structurally closely related to the sponge-derived dercitin from *Dercitus* sp. the putative bioactive pharmacophores of the pentacyclic and hexacyclic structures have been identified as thiazolo[5,4-*b*]acridine and the isomeric thiazolo[4,5-*b*] acridine nucleus (Taraporewala, 1991). Research on the cytotoxic potential of dercitin on mammalian cells showed the importance of the four basic nitrogen moieties capable of binding to acidic amino acid residues. The cytotoxic potential was lowered by (progressive) removal of the basic nitrogen atoms. Also, the presence of a fused thiazole ring contributed to the cytotoxic effect while the pyridine ring E seemed redundant (Taraporewala, 1991; Agrawal and Bowden, 2007). The sulfur atom seemed also essential for antiviral activities, anti-HIV included (Sharma et al., 2015).

The ascidian *Cystodytes dellechiajei* contains mainly shermilamine B and kuanoniamine D (Steffan et al., 1993). In silico docking information on interactions between shermilamine B and the binding sites of two key proteins of bacterial cell wall construction, β-ketoacyl acyl protein synthase I (MtKasA) and decaprenylphosphoryl-β-D-ribose 2'-epimerase 1 (MtDprE1) and a third protein, essential for the catalyzation of cofactor Coenzyme A biosynthesis of *Mycobacterium tuberculosis*, type I pantothenate kinase (MtPanK), proved promising as antibacterial/antituberculosis drug, especially with MtKasA as the target (Siam et al., 2020).

The biosynthesis of shermilamine B, involving the labeled precursors tryptophan and dopamine administered via the diet of *Cystodytes dellechiajei* and the total synthesis of kuanoniamines and dercitines have been described in Steffan et al. (1993); Bishop and Ciufolini (1992). The natural pyridoacridines diplamine (the N-acetyl analogue of lissoclin B), diplamine B, isodiplamine, lissoclinidine, cystodytin K and cystodytin J were isolated from the ascidians *Lissoclinum notti*, *Lissoclinum cf.dadum* and/or *Diplosoma* sp. (Charyulu et al., 1989; Kijjoa, 2015). Although these compounds are related, their structures are quite distinct and differ in stability. Diplamine is unstable. Diplamine, diplamine B, isodiplamine and cystodytin J possess the iminoquinone portion while lissoclinidine B has an extra 1,3-oxathiolane ring. Lissoclinidine B and diplamine B were able to stabilize and increase the cellular p53 protein and the ubiquitin ligase activity in the human double minute 2 (Hdm2) test in a dose-dependent treatment with the greatest increase at 10 μM, similar to the increase at 50 μM of N-acetyl-leucyl-leucyl-norleucinal, a potent inhibitor of proteolysis catalyzed by proteasomes (Kijjoa, 2015). Cystodytin J and diplamine are potent DNA intercalators and (dose--dependent) inhibition of topoisomerase II-catalyzed kinetoplast DNA decatenation in vitro at IC_{90} of 8.4 and 9.2 μM, respectively, and thus more potent than etoposide (IC_{90}: 68 μM). Lissoclinidine B and diplamine B were found equally bioactive, despite their structural differences. Kijjoa (2015) therefore suggested essential functionality of the aromatic ring system and sidechain. The great number of preclinical research reports on pyridoacridines did not result in clinical developments.

4.3. Acetylcholinesterase inhibitors

4.3.1. Preclinical assessments

Several marine toxins, e.g. pibocin, varacin, pictamine, lepadine, pulmonarins A & B, irenecarbolines, from the ascidian sources *Eudistoma* sp., *Lissoclinum* sp., *Clavelina picta* and *Clavelina lepadiformis* are acetylcholinesterase inhibitors (Jimenez et al., 2012; Palanisamy et al., 2017; Watters, 2018).

4.3.2. Clinical status

These compounds are considered cytotoxic antagonists seemingly without pharmaceutical potential though a few none ascidian-derived acetylcholinesterase inhibitors are being used in the treatment of Alzheimer's disease in the EU although admittedly with varying success (Kabir et al., 2021; Silva et al., 2021).

4.4. Meridianins

4.4.1. Preclinical assessments

Ascidian meridianins are brominated indole alkaloids connected to aminopyrimidine. The sources are *Aplidium meridianum*, *Amphicarpa meridiana* and *Synoicum* sp. Ascidian meridianins seem unique as potent inhibitors of many kinases and signaling pathways. Meridianin C, a natural bromo-derivative, has an IC_{50} of 1.44 μM against PIM-1 kinase (Gompel and Leost, 2004). Meridianin C and modified analogs at the pyrimidine scaffold, are also selective inhibitors of the so-called tau-specific protein kinases glycogen synthase kinase 3β (GSK3β), dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A) and CLK1 kinases at sub-μM concentrations (Giraud et al., 2011; Han et al., 2021; Silva et al., 2021). GSK-3β and DYRK1A are considered master regulators in neurodegenerative pathways (Demuro et al., 2021). GSK-3β is also known to regulate glycogen metabolism. A new set of natural analogs of meridianins A-G has been detected in *Aplidium meridianum* and *Aplidium falklandicum* from Antarctica (Núñez-Pons et al., 2015). Inhibition of overexpression of kinases is part of the key strategies to combat chronic diseases such as Alzheimer's disease, diabetes, antimalarial activity, antituberculosis activity, inflammation, cancer and bipolar disorders (Núñez-Pons et al., 2015; Han et al., 2021; Martins et al., 2020; Lima and Medeiros, 2022). GSK3β is an intermediate multifunctional serine/threonine kinase having diverse physiological pathways (Palomo et al., 2017) and is involved in numerous chronic diseases. Meridianins for treatment therapies of Alzheimer disease and diabetes have been comprehensively described in the manuscripts/reviews 'Kinase Inhibitors of Marine Origin' (Bharate et al., 2013), 'Structural-based optimizations of the marine-originated meridianin C as Glucose Uptake Agents by Inhibiting GSK-3β' (Han et al., 2021) and 'Marine organisms as alkaloid biosynthesizers of potential anti-Alzheimer and diabetes agents' (Lima and Medeiros, 2022) but not in the 2020 review on 'Marine natural products, multitarget therapy and repurposed agents in Alzheimer's disease' (Martins et al., 2020). The meridians A, B, C and G displayed weak cancer cell growth inhibition in four cancer cell lines (Zhang et al., 2022). The antitumor activity and kinase inhibition efficacy of newly designed derivatives of meridians improved at the sub-μM range (Giraud et al., 2011; Yadav et al., 2015; Zhang et al., 2022; Kruppa et al., 2022). In contrast, several other kinase inhibitors known as ATP-competitive inhibitors are potent in the nM range (Demuro et al., 2021). This might explain why the meridianin scaffold has not been used in the structure-activity-based study in the drug design paper 'Application of fragment-based de novo design to the discovery of selective pM inhibitors of Glycogen Synthase Kinase-3 Beta' (Park et al., 2016) despite the importance of the indole group as a target molecule in this study. For some reason, this could indicate doubts about the use of meridianin scaffolds in quantitative structure-activity relationship research on novel drugs. However, one meridianin analogue displayed strong DYRK1A inhibition with an IC_{50} of 34 nM (Giraud et al., 2011).

Several metabolites from ascidian species such as, e.g. halocynthiaxanthin and fucoxanthinol from *Halocynthia roretzi* (Konishi et al., 2006), meridianins, brominated 3-(2-aminopyrimidine)-indole alkaloids, from *Aplidium meridianum* (Gompel and Leost, 2004), and other compounds from *Diplosoma virens* (Ogi et al., 2008) and *Polyclinum indicum* (Pusphabai Rajesh et al., 2010) have novel MoAs of inducing apoptosis and cell-cycle arrest (Wali et al., 2019).

4.5. Ritterazines

4.5.1. Preclinical assessments

Ritterazines are dimeric steroidal pyrazine alkaloids originally isolated from the ascidian source *Ritterella tokiada*. Ritterazines are strong tumor inhibitors at sub- nM concentrations with ritterazine B as the most potent at an IC_{50} of 0.17 nM (Imperatore et al., 2014). Ritterazines and cephalostatins were also discovered in the marine worm *Cephalodiscus gilchristi* and share a common MoA. A microbial origin of both compound groups is suspected (Lee et al., 2009; DCM and Clinton, 2015).

The MoA of the ritterostatin-cephalostatin hybrid ritterostatin GN1N is immunoprecipitation of HSP70s (Ambrose et al., 2017). In the cell the compound binds predominantly to the GRP78 gene and/or protein that play a crucial role in protein folding and protein quality control in the endoplasmic reticulum (ER), activating an unfolded protein response and subsequent apoptosis (Ambrose et al., 2017). Tahtamouni et al. (2018) demonstrated mediation of the ER stress-induced apoptotic pathway by two cephalostatin analogs.

The very limited yields from natural sources have necessitated chemical synthesis. A total synthesis of cephalostatin was achieved (Shi et al., 2011; Menna et al., 2011). In 2004, Nawasreh and Winterfeldt (2004) reported on the synthesis of cephalostatin analogs with anti-lymphoma's activity.

4.6. Polyandrocarpamines

4.6.1. Preclinical assessments

Polyandrocarpamines A and B are 2-aminoimidazolone alkaloids isolated from the ascidian source *Polyandrocarpa* sp. 2-aminoimidazolone analogs have been synthesized from the sponge natural product leucettamine B (Loaëc et al., 2017). The ascidian polyandrocarpamines A and B are potent inhibitors of cell division cycle 2-kinases such as CLK1, CLK2 and DYRK1A. DYRK1A is overexpressed in neurodegenerative diseases including Down syndrome and Alzheimer's disease (Menna et al., 2011; Silva et al., 2021).

4.7. Lamellarins and ningalins

4.7.1. Preclinical assessments

Lamellarins and ningalins are a wide variety of DOPA/TOPA-derived pyrrole-polyphenol alkaloids, often sulfated, acetated or diacetated. This class of compounds is being tested in various preclinical applications against central nervous system disorders, cancer, microbes and parasites, and as therapeutic antioxidants with multiple molecular targets: topoisomerase I, kinases and drug efflux pumps (Watters, 2018; Schilf et al., 2021). The first lamellarin (D) was found in the mollusk *Lamellaria* sp. and later identified in various ascidian *Didemnum* sp. which are presumed to be the dietary sources of the molluscs Facompré et al. (2003). Lamellarin D accumulates rapidly in mitochondria where it induces mitochondrial target topoisomerase I cleavage complexes leading to mtDNA damage (Khiati et al., 2014). Plisson et al. (2012) detected and characterized the lamellarins Z, G, A6 and the ningalins B to G in extracts of the Australian ascidian *Didemnum* sp. Especially ningalins showed selective broad-spectrum inhibition properties against the neurodegenerative molecular targets CK1d, CDK5 and GSK3b while low cytotoxicity was demonstrated against a range of human cell lines (Plisson et al., 2012). This interesting combination might be seen as a new promising tool to inhibit kinases and target the ATP-binding pocket.

Lamellarin D is a potent topoisomerase I inhibitor but did not inhibit topoisomerase II. The compound displayed also high cytotoxic activities against multidrug-resistant tumor cell lines with the highest toxicities against prostate cancer cells at GI_{50} values in the range of 10–20 nM (Facompré et al., 2003). Lamellarin D and Ningalin B showed treatment options for central nervous system disorders (Karami et al., 2021). Several papers describe the total synthesis of lamellarins (references in Facompré et al., 2003; Schilf et al., 2021) and sulfated analogs Ridley et al. (2002). Especially the sulfated lamellarin α 20-sulfate is a unique selective inhibitor of HIV-1 integrase while other sulfated lamellarins and lamellarin α are not (Ridley et al., 2002). It should be noted that not all synthetic analogs possess cytotoxicity, e.g. lamellarin 501 was inactive against topoisomerase I and poorly selective against prostate cancer cells (Facompré et al., 2003).

4.8. Mandelalides

4.8.1. Preclinical assessments

Mandelalides derived from the South African colonial ascidian didemnid *Lissoclinum* sp. were first reported in 2012 (Sikorska et al., 2012). These compounds are complex glycosylated polyketide macrolides. Mandelalides A and B exhibited in vitro potent cytotoxicity in the low nM range against lung cancer NCI-H460 cells and mouse Neuro-2A neuroblastoma cells (Sikorska et al., 2012). These compounds may be toxic to normal mammalian cells due to their direct inhibition of mt-ATP synthase. On the other hand, mandelalide A-induced depletion of cellular ATP indirectly activated the AMP-mediated protein kinase (AMPK) as a possible protective response (Mattos et al., 2022).

The latest version of a series of systematic reviews by Mayer et al. (2020) describes the novel findings on the preclinical pharmacology of over 90 polyketides from diverse sources. Three of the reported natural polyketides are ascidian-derived: oxazinin A, iejimalide C and forazoline A. A fourth bacterial symbiont has recently been associated with mandelalide-containing *Lissoclinum* sp. (Lopera et al., 2017).

Oxazinin A is a racemic, prenylated polyketide dimeric combination of benzoxazine, isoquinoline, and a pyranring (Lin et al., 2014). Interestingly, oxazinin A exhibits unique structural features and originates from a lesser-known taxonomic group of fungi. The preclinical pharmacology pipeline of oxazinin A is also restricted at the moment. Oxazinin A only showed strong selective antimycobacterial activity against *Mycobacterium tuberculosis* (IC_{50} 2.9 μM) in a panel of bacteria. Oxazinin A exhibited cytotoxicity against the human CEM-TART T-cell leukemia line in the low μM concentration range (LC50 4.7 μM) and modest potency to inhibit several human transient receptor potential channels (Lin et al., 2014). The recently reported total synthesis of oxazinin A is another verified example of the complexity of biosynthetic pathways in producing organisms involving nonenzymatic chemistry (Aniebok et al., 2022).

4.9. Iejimalides

4.9.1. Preclinical assessments

Natural iejimalides from the ascidian source *Eudistoma cf. rigida* act in vitro and in vivo against various cancer cells at low nM concentrations (Fürstner et al., 2007; Kazami et al., 2014). The gene and protein pathways regulating the downstream effects of iejimalides have been characterized by McHenry et al. (2010). Identified MoAs are sequential pH neutralization in lysosomes, induction S-phase cell-cycle arrest and initiation of apoptosis. The iejimalides inhibit vacuolar H^+-ATPase activity in epithelial tumor cells at an IC_{50} of 0.12μM. This inhibition may indirectly lead to the disorganization of actin and a lysosome-interfered cell death process. It is known that V-ATPase subunits B and C are directly bound to actin filaments with high affinity (Kazami et al., 2014). Here again, the total synthesis pathways of iejimalide A to D are known (Fürstner et al., 2007; Kazami et al., 2014).

4.10. Forazoline A

4.10.1. Preclinical assessmens

The main interests in orazoline A are its highly unusual and unprecedented skeleton with two thiazoline-type moieties and in vitro and in vivo efficacy against the fungal pathogen *Candida albicans* (Wyche et al., 2014). The MIC of the in vitro activity was 17.7 μM, recalculated from μg/mL. Forazoline A is water-soluble at 5 μg/mL that may promote better in vivo bioavailability. After eight hours, *Candida albicans*-infected mice treated in an immunocompromised mouse model showed a significant decrease of 1.5 $\pm$ 0.12 cfu/kidney body burden compared to controls without toxic effects (Wyche et al., 2014). The efficacy of forazoline A is comparable to that of the antifungal medicine amphotericin B. There is more: combinatory treatment of forazoline A and amphotericin B had a synergistic effect in vitro. Forazoline A damages the membrane integrity of the fungus in a dose-dependent way and may be an alternative against the rising drug resistance of pathogens (Wyche et al., 2014).

4.11. Spiroketals

4.11.1. Preclinical assessmens

Spiroketals or spiroacetals comprise a large group of bistramides and didemnaketals common in many organisms. Their structures contain two or more oxacyclic rings where the oxygen atoms of different rings share a common spiro-carbon atom. The bistramides A, B, C, D and K were the first discovered in the ascidian species *Lissoclinum bistratum* (Biard et al., 1994). The ascidian-derived bistramide A is cytotoxic in the IC_{50} nM-range and possesses antitumor activity, activates PKC-δ and blocks sodium channels (Watters, 2018). The primary molecular target of bistramide A is G-actin filaments. The MoA induces filament disassembly and prevents filament formation (Lenci, 2020). A bistramide A analog was able to reversibly bind actin in vitro and in vivo, thereby inhibiting A549 non-small cell lung tumor cell growth (Watters, 2018).

Another group of spiroketals, the didemnaketal analogs A to G, were discovered in the ascidian *Didemnum* genus (Zhang et al., 2012; Mohamed et al., 2014; Shaala et al., 2014). The bioactivities of this class of spiroketals are moderate and diverse: D and E exhibited moderate activity against the kinases CDK5, CK1, DyrK1A, and GSK3. Didemnaketals F and G displayed moderate activity against HeLa cells with IC_{50} values of 49.9 and 14.0 μM, respectively. All analogs displayed low cytotoxicity. In addition, didemnaketal F displayed potent antimicrobial activity against *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 14053 with GI-zones of 20 and 24 mm at 100 μg/disc (Mohamed et al., 2014; Shaala et al., 2014). The didemnaketals A and B demonstrated effective HIV 1-protease inhibition (IC_{50}: 2 and 10 μm, respectively; (Zhang et al., 2012).

Bioactivities and biosynthesis issues of diverse spiroketals, including ascidian compounds, have been reviewed in Zhang et al. (2018) and Watters (2018) and Lenci (2020) presented a library of first and second generation synthesis of spiroketal-containing molecules.

4.12. Terretriones

4.12.1. Preclinical assessmens

In 2015, the novel compound terretrione D with a 1,4-diazepane skeleton and the known terretrione C were detected in the ascidian *Didemnum* sp.-derived fungus *Penicillium* sp. CYE-97 (Shaala and Youssef, 2015). Both compounds demonstrated excellent anti-migratory activities in a wound healing assay against the highly metastatic triple-negative human breast cancer cells MDA-MB-231 with IC_{50} values of approx. 17 μM compared to the control drug S-ethyl (IC_{50} 43.4 μM). On the other hand, antiproliferation activity against HeLa cells at an IC_{50} of 50 μM was extremely modest compared to the IC_{50} of 1.7 nM of another positive control paclitaxel. Antibacterial activities were negative against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli*

ATCC 25922. Antifungal activities of terretrione D and C in the disc diffusion assay at 100 μg/disc were modest against *Candida albicans* ATCC 14053 showing GI-zones of 17 and 19 mm, respectively, and a MIC of 32 μg/mln (Shaala and Youssef, 2015).

4.13. Lepadins

4.13.1. Preclinical assessmens

Recently a new decahydroquinoline lepadin, named lepadin L, was found in the ascidian *Clavelina lepadiformis* (Casertano et al., 2022). Lepadins express moderate to strong toxicity against cancer cell lines, tyrosine kinase and butyrylcholine esterase activity, as well as parasites. Lepadin A is, in contrast to B and L, a strong cytotoxic drug against human melanoma, breast, colon and colorectal cancer cell types and mouse myoblast cancer cells (Casertano et al., 2022). The MoA of Lepadin A on the human melanoma A375 cells suggests impairment/inhibition of the self-renewing capacity of the cells due to strong inhibition of cell migration, induction of G2/M phase cell cycle arrest, dose-dependent decrease of cell clonogenicity and cell death endpoint (Casertano et al., 2022).

4.14. Thiaplidiaquinones

4.14.1. Preclinical assessmens

Thiaplidiaquinones (ascidian source *Aplidium conicum*) or thiazinoquinones are highly unusual meroterpenoid alkaloids. The pharmaceutical interest in the bioactivity of thiaplidiaquinones lies in improved intracellular ROS production inducing apoptotic cancer cell lines (Carbone et al., 2012) although the natural products seem weak inducers of ROS in Jurkat cells (Harper et al., 2015). The thiaplidiaquinone A and B also showed antimalarial activities against the deadliest Trypanosoma species *T. falciparum*, targeting the DNA stabilizing enzyme topoisomerase II, however, the two isomers seem to have different MoAs: thiaplidiaquinone A and its synthetic dioxothiazine isomer caused necrotic cell death while thiaplidiaquinone B and its similar dioxothiazine isomer mediated cell death via apoptosis. Harper et al. (2015) were able to connect these different MoA properties to the structural activity relationships of both structures. Recently, thiaplidiaquinones A and B and their respective analogs were identified as strong inhibitors of mammalian and protozoan farnesyltransferases in the nM range thereby disturbing membrane integrity (Cadelis et al., 2017). Thiaplidiaquinones A and B and their dioxothiazine analogs have been synthesized (Khalil et al., 2012). A concise total synthesis of thiaplidiaquinone A was published in Carbone et al. (2012). Structure-activity relationships between synthesized prenyl and farnesyl analogs showed the most potent farnesyltransferase inhibition by the geranyl and farnesyl series (Cadelis et al., 2017). On the other hand, both the prenyl and farnesyl analogs performed better than the geranyl series in antiplasmodial assays against the chloroquine-sensitive *Plasmodium falciparum* NF54 strain (Cadelis et al., 2017).

4.15. Eudistidines

4.15.1. Preclinical assessmens

Naturally occurring eudistidine A, B, C from ascidian *Eudistoma* sp. are also very interesting structurally as well as biologically. Eudistidine A inhibits the interaction of the transcription factor hypoxia-inducible factor 1, known as HIF1, with the co-activator protein p300. Tumors often grow under oxygen-deprivation conditions. The HIF1/p300 complex is required for the transcription of hypoxia-responsive genes and is therefore an attractive therapeutic target (Chan et al., 2015).

Eudistidines contain a unique tetracyclic fused structure of two pyrimidine rings and an imidazole ring. All eudistidines can be synthesized. Eudistidine C has another molecular framework (Chan et al., 2016). The natural and the synthetic eudistidine A equally inhibited the binding of the p300 protein and the HIF-1α factor with an IC_{50} of 75 μM while eudistidine C was much less interfering with the complex (Chan et al., 2015). Eudistidine C, A and five synthetic eudistidine C analogs eudistidine C 3-methylindole, eudistidine C N-methyl pyrrole, eudistidine C p-phenol, eudistidine C resorcinol and eudistidine C phloroglucinol exhibited significant antimalarial activity against the chloroquine-sensitive D6 strain and the chloroquine-resistant W2 strain of *Plasmodium falciparum* with eudistidine A and the phloroglucinol analog of eudistidine C as the most potent inhibitors (Chan et al., 2016).

4.16. Tamandarins

4.16.1. Preclinical assessments

Tamandarins A and B are depsipeptides found in an unidentified ascidian species of the Didemnidae family and seem related to ascidian-derived didemnin B although it should be noted that didemnin B was also produced by the free-living α-proteobacterium Tistrella mobilis in marine sediment (Xu et al., 2012). Tamandarin A reduced 50 % overall cell survival of severe human cancer cell lines of pancreas, prostate and head and neck carcinomas at low concentrations of 1.7, 1.3, and 0.94 μM, respectively, recalculated from ng/mL (Arumugam et al., 2018). The molecular target is unknown and the hypothesis is a similarity between the MoA of tamandarins and didemnin B (Vera and Joullié, 2002; Lee et al., 2012; Watters, 2018).

4.17. Eudistomins

4.17.1. Preclinical assessments

Eudistomins (A, C, E, F, I, K, L, N, O, T, U, X and the series Y1-Y13) are a large family of unique β-carboline alkaloid structures. Structurally, these compounds and related analogs form a tricyclic pyrido[3,4-*b*] indole nucleus. Some, e.g. eudistomin C, include a rather unique oxathiazepine ring. Eudistomins were mainly isolated from the ascidian species *Eudistoma olivaceum* but are also present in the ascidian genera *Lissoclinum*, *Pseudodistoma*, *Ritterella*, and *Synoicum* (Galvis and Kouznetsov, 2017). In general, eudistomins are bioactive against tumors, viruses, bacteria, and malaria. The MoA targets protein synthesis, and binding of 5-hydroxyserotonin, monoamine oxidase and benzodiazepine receptors in the central nervous system. Protein synthesis suppression in yeast cells has been suggested as an antiviral cellular response. The molecular target of eudistomin C is the 40S ribosomal us11 protein (Ota et al., 2016). The series of β-carbolines known as eudistomins Y showed a range of moderate to significant antimicrobial activities and weak cytotoxicity (Galvis and Kouznetsov, 2017).

5. Opportunity and limitation highlights

Interestingly to know is that approx. 1 % of the MNPs show antitumor cytotoxicity as against 0.01 % amongst their terrestrial counterparts (Palanisamy et al., 2017). This finding explains the growing interest in research on MNPs. Ascidians provide rich sources of MNPs and bioactive primary and secondary metabolites. There is however increasing proof that a majority of the actual or predicted sources of these natural products and so-called intermediate metabolites in these microorganisms are associated symbionts: bacteria, actinomycetes, and cyanobacteria. The symbiotic lifestyle is complex since these microorganisms are free or obligate, often species-specific and even tissue-specific and are phylogenetically diverse through horizontal transmission.

The free or obligate symbiotic nature of the mutual lifestyles may result in complex biosynthetic pathways of marine natural compounds depending on the individual or mutual involvement of the symbiotic lifestyles in the biosynthesis of marine natural drugs, e.g. the microbial biosynthesis might result in precursors which are subsequently finalized by posttranslational modifications by the host. The natural drugs or their synthetic analogs exhibit a wide variety of Modes of Action like binding

with DNA, inhibition of DNA/RNA/protein synthesis, production of ROS, inhibition of topoisomerase and kinase, cleavage/catenation/damage of DNA, cell-cycle arrest and/or apoptosis.

Numerous clinical trials with marine natural compounds are ongoing in the last two decades. However, many potential marine drug developments have failed for reasons of toxicity, lack of efficacy (not better than existing drugs) or strategic and oftentimes low product availability. Clinical developments have oftentimes been hampered by problems in the scale-up production process of these drugs (Martins et al., 2014; Van Andel et al., 2018). In addition, new pharmaceutical drugs should preferably be more effective than existing approved drugs or at least improve the target efficacy in mixtures with existing drugs. The latter

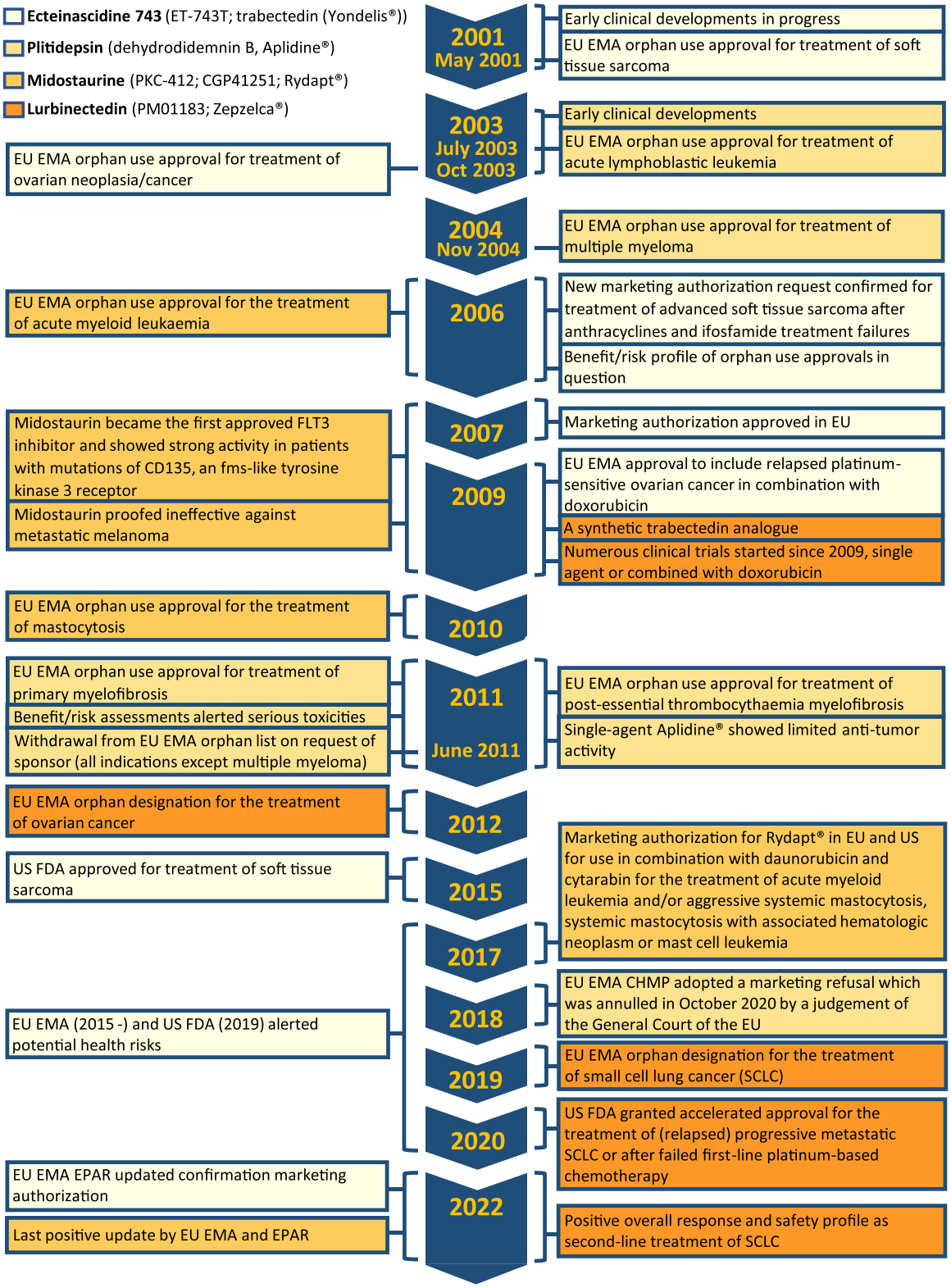

Fig. 1. Milestones for different pharmaceuticals (Ecteinascidine, Plitidepsin, Midostaurine and Lurbinectedin) for a period of 2001–2022.

target goal is often the reason why these marine natural compounds or their analogs are often being tested in combination with other (chemo) therapeutic drugs. Eastman (2017) argued for a change in preclinical testing with different biological endpoints that actually measure viability i.e. live vs. dead cells instead of growth inhibition. Fewer cells do not actually mean that they have lost viability (Eastman, 2017). The ultimate toxicity profile of newly discovered drugs is high specificity and efficacy against target cells with an acceptable safety profile in normal cells. Furthermore, the microorganism-derived MNPs have often analogs that structurally resemble compounds produced by terrestrial microorganisms. The question may rise in those cases if the research on MNPs is not too far-reaching or redundant.

To date 2022, Only one natural and two synthetic drugs derived from ascidians and/or their symbionts have received final approval from regulatory authorities: trabectedin (Yondelis©), the trabectedin derivative Lurbinectedin (Zepsyre©/PM01183) and the staurosporine synthetic derivative midostaurine (Rydapt©/PKC-412/CGP41251/N-benzoylstaurosporine). The natural compound trabectedin is now also being produced synthetically. An overview of the development and milestones for these components are illustrated in Fig 1. Finally, 8 of other semisynthetic or synthetic derivatives of the ascidian-derived staurosporine (lestaurtinib, edotecarin, enzastaurin, becatecarin, UCN-01, CEP-2563, CEP-1347 and sautosporin) have been used in preclinical and clinical trials and/or have been withdrawn for reasons of lack of efficacy, not superior to existing agents or strategic.

Author contributions

KC took the lead in devising the concept and draft preparation of the review **Emerging pharmaceutical therapies of ascidian-derived natural products and derivatives**. KC, BDS and BDW share first authorship while all authors contributed to the progress of the review and agreed to the final version for submission. The ultimate goal of this teamwork is the preparation of a research project on the topic.

Declaration of Competing Interest

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

Data availability

No data was used for the research described in the article.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2023.104254.

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