Natural products from aquatic eukaryotic microorganisms for cancer therapy: perspectives on anti-tumour properties of ciliate bioactive molecules

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Abstract

Several modern drugs, including those for cancer therapy, have been isolated from natural sources, are based on natural products and its derivatives, or mime natural products. Some of them are in clinical use, others in clinical trials. The success of natural products in drug discovery is related to their biochemical characteristics and to the technologic methods used to study their feature. Natural compounds may acts as chemo-preventive agents and as factors that increase therapeutic efficacy of existing drugs, thus overcoming cancer cell drug resistance that is the main factor determining the failure in conventional chemotherapy.

Water environment, because of its physical and chemical conditions, shows an extraordinary collection of natural biological substances with an extensive structural and functional diversity. The isolation of bioactive molecules has been reported from a great variety of aquatic organisms; however, the therapeutic application of molecules from eukaryotic microorganisms remains inadequately investigated and underexploited on a systematic basis.

Herein we describe the biological activities in mammalian cells of selected substances isolated from ciliates, free-living protozoa common almost everywhere there is water, focusing on their anti-tumour actions and their possible therapeutic activity. In particular, we unveil the cellular and molecular machine mediating the effects of cell type-specific signalling protein pheromone *Er*-1 and secondary metabolites, *i.e.* euplotin C and climacostol, in cancer cells. To support the feasibility of climacostol-based approaches, we also present novel findings and report additional mechanisms of action using both *in vitro* and *in vivo* models of mouse melanomas, with the scope of highlighting new frontiers that can be explored also in a therapeutic perspective.

The high skeletal chemical difference of ciliate compounds, their sustainability and availability, also through the use of new organic synthesis/modifications processes, and the results obtained so far in biological studies provide a rationale to consider some of them a potential resource for the design of new anti-cancer drugs.

Keywords: (6) climacostol, natural products, anti-tumour molecules, ciliated protozoa, chemotherapy, tumour growth

1. Introduction

Natural products from diverse natural sources including plants, animals and microorganisms (also of marine origin) have been the basis of treatment of human diseases since antiquity [1]. To date, natural products and their derivatives are the source for a significant fraction of modern drugs [2] and they command a substantial market share, comprising compounds approved in the last 30 years and used in various medical therapeutic areas [3].

Molecules from nature offer a huge structural diversity even vaster than standard combinatorial chemistry, thus they provide major opportunities for finding novel compounds. In the area of drug discovery they are fundamental as targets for production by biotechnological approaches and a source of lead molecules of novel chemical structures. The success of natural products in drug discovery is thus related to their biochemical characteristics and to the technological methods used to study their feature [2, 4-6]. Cytological profiling, for instance, can be utilised for the targeted isolation of compounds with specific modes of action [7, 8]. Also, functional chromatography has been proposed as natural product purification [9] and today, the access to genome sequencing of unicellular and multicellular organisms provides a new approach to natural product discovery and action [2, 10, 11]. By combining technologies such as mass spectrometry and sensitive Nuclear Magnetic Resonance techniques it has become possible to identify and quantify active compounds in natural sources that are of crucial interest for drug discovery [12, 13].

To date, approximately 80% of anticancer drugs approved by the Food and Drug Administration (FDA) belongs to natural sources (naturally occurring drugs or their derivatives) [1, 14-19]. These drugs are characterised by different mechanisms of action, including primarily cell death via apoptosis and/or necrosis, autophagy, interaction with microtubules, inhibition of topoisomerases I or II, alkylation of DNA, and interference with tumour signal transduction [1, 14, 16, 20-22]. Natural compounds may acts as chemo-preventive agents and as factors that increase therapeutic

efficacy of existing drugs, thus overcoming cancer cell drug resistance that is the main factor determining the failure in conventional chemotherapy [1, 14, 16, 19, 20, 23-31].

2. Biologically active compounds from the protozoan ciliates

Water environment because of its physical and chemical conditions shows an extraordinary collection of biological substances with an extensive structural and functional diversity. Aquatic organisms, living in high competitive environment, have evolved biochemical and physiological mechanisms that include the production of unique and potent peptides and metabolites belonging to different chemical classes as terpenoids, alkaloids, polyketides, shikimic acid derivatives, sugars, steroids and a multitude of mixed biogenesis metabolites [17, 32-35]. The discovery of new molecules from the sea poses the problem of supply of biological material from which the same molecules are isolated. Thanks to the technological innovations today it has become possible to identify new molecules from small amounts of material; however, the need remains to obtain adequate amounts of product in order to explore the biological properties. In order to protect biodiversity and ecosystem functions, it is unthinkable to proceed to massive collection of biological material, thus chemical synthesis processes become the crucial task [36]. The isolation of bioactive molecules has been reported from a great variety of aquatic organisms, however, the therapeutic application of molecules from eukaryotic microorganisms remains inadequately investigated and underexploited on a systematic basis. Given this context, this paper proposes to explore eukaryotic microbes (protists), essentially ciliates, as source of natural products.

Ciliophora, commonly called ciliates, is a group of protists within the superphylum Alveolata [37] that are characterised by the presence on their cell surface of hair-like organelles called cilia, and are equipped with two kind of nuclei, a diploid micronucleus and a polyploid macronucleus. Free-living ciliates are common almost everywhere there is water, where they play a key role in microbial food web. There are approximately 8,000 species of ciliates and many of them are able to

synthesise cell type-specific signalling protein pheromones presiding cell-cell communication, and secondary metabolites with important phylogenetic, functional and ecological properties, principally represented by terpenoid and polyketide molecules [38]. Diffusible proteins are designated as 'pheromones' for their activity in modulating intra-specific self- and non-self-recognition phenomena. Pheromones are responsible for the switching between the reproductive (mitotic growth) and mating (sexual) stages of ciliate life cycles [39, 40]. In contrast, secondary metabolites are either stored in the cell cytoplasm or inside extrusomes before being used as highly potent inhibitors (toxins) of physiological processes in the prey, predators or competitors of the organisms that produce them. For instance, *Euplotes raikovi* uses a combination of terpenoids (preraikovenal-raikovenal) as strategy of defence. Raikovenal alone, indeed, is functionally less active than other terpenoids since it showed no killing activity against other ciliates [38, 41]. *Euplotes vannus* and *Euplotes rariseta* morpho-species are also characterised by a great diversity of terpenoid production [38]. It was also shown that a ciliate from antarctic waters, *Euplotes focardii*, produces focardins (focardin and epoxyfocardin) with a defensive role against predacious ciliates [38, 42]. Secondary metabolites built on the hypericin skeleton such as stentorin [43] and blepharismin [44], produced respectively by *Stentor coeruleus* and *Blepharisma japonicum*, have antibacterial and antiviral activity. Stentorin and blepharismin have also a toxic effect against other protozoa, such as Paramecium, when are exposed to visible light [45]. In addition, among the ciliate toxins that not belong to the aforementioned classes of compounds, we should remember: the keronopsins produced by *Pseudokeronopsis rubra* [46], that are able to crippling or killing the predators; the keronopsamides produced by *Pseudokeronopsis riccii* [38], for which the biological activity is to date unknown; the mixture of free fatty acids produced by *Coleps hirtus* [47] to paralyse the prey; the mono-prenyl hydroquinone and the erytrholactones produced by *Spirostomum ambiguum* [48] and *Pseudokeronopsis erytrhina* [49], respectively, as chemical weapons against predators.

Particularly important for the purposes of this paper is the remarkable bioactivity that some molecules produced by ciliates have revealed on human and animal cell lines. In this Perspective paper we provide a concise and unifying picture of the research in the field by authors reviewing reported data on the ciliate pheromone *Er*-1, that may act as an immune-modulatory factor with anti-cancer properties, and on the anti-tumour actions of selected compounds, *i.e* the toxins euplotin C and climacostol. To support the feasibility of climacostol-based approaches, we also present novel preliminary findings and report additional mechanisms of action using both *in vitro* and *in vivo* melanoma models. When appropriate, methodological information is given in the figure captions. The goal in this context is to present new data to further define some of the cellular events/molecular players influenced by climacostol in tumour cells; these suggest new frontiers that can reveal also new therapeutic perspectives.

3. Pheromones

Pheromones have been detected in the culture supernatant of various ciliate species. However, they have been effectively isolated and their structures determined to varying degrees of complexity only in five species, *i.e. Blepharisma japonicum*, *Euplotes raikovi*, *Euplotes octocarinatus*, *Euplotes nobilii*, *and Euplotes crassus* [39, 40]. All the *Euplotes* pheromones are water-soluble proteins that vary in extension between 38 and 109 amino acids. In the marine ciliate *Euplotes raikovi*, in particular, pheromones (denominated *Er*-1, *Er*-2, and so forth) contain 37-51 amino-acid residues, of which only six cysteines forming three intra-chain disulfide bonds are highly conserved [50, 51]. Functionally, these peptides may act both via paracrine-like (or heterotypic) interactions thus inducing mating of cells and through an autocrine binding, to promote the vegetative (mitotic) proliferation of the same cells from which they are released [39, 40, 50, 51].

3.1. Pheromone Er-1: immune-enhancing effects and anti-cancer properties

On the basis of their ability to elicit varied and context-dependent cell responses, pheromones have been proposed to represent potential prototypical cell growth factors. In line with this view, it has been previously demonstrated that the epidermal growth factor and the cytokine interleukin-2 (IL-2) function as effective competitors of pheromone/pheromone-receptor binding reactions [52]. The immune-stimulatory cytokine IL-2 is a growth factor for many leukocytes, such as T cells and natural killer cells [53, 54]. IL-2 signals through the IL-2 receptor (IL-2R) which couples to multiple pathways, including factor signal transducer and activator of transcription 5 (STAT5) and mitogen-activated protein kinases (MAPKs) [53, 54]. Noteworthy, *Euplotes raikovi* pheromone *Er*-1 was shown to bind with different affinities to the α and β chains of IL-2R expressed by the mouse T lymphocyte cells CTLL-2, which depend completely on IL-2 signalling for proliferation and survival [55]. We have recently documented that pheromone *Er*-1 increases DNA synthesis, proliferation and viability of human lymphoid Jurkat T-cells, a cell line expressing functional IL-2R and representing an useful model for immunological studies [56]. *Er*-1 was active at nanomolar concentrations, indicating a high sensitivity of human T-cells to the pheromone, probably due to an efficient activation of the intracellular cascade primed by *Er*-1 binding to cell membranes. Compelling evidence was also provided that the pheromone *Er*-1 acts through the activation of ERK1/2 MAPKs and mimicking IL-2 actions at IL-2R [56]. Further support to *Er*-1/IL-2R coupling comes from preliminary data indicating that STAT5 levels increased in Jurkat cells treated with *Er*-1; no additive effects were achieved in the presence of combination of *Er*-1 and IL-2 (Cervia D. and coll., data not shown). In CTLL-2 cells IL-2 signalling was shown to be modulated by other natural compounds modifying the levels of IL-2 and high affinity IL-2R, as well as interfering with IL-2R transduction mechanisms [57].

From a functional point of view, *Er*-1 increased the Jurkat cell production of different T helper type 1 or type 2 cytokines, including interferon-γ, tumour necrosis factor-α, IL-1β, IL-2, and IL-13 [56].

Of interest, this immuno-enhancing effect was observed to cause an *in vitro* inhibition of human glioma U-373 cells growth. Indeed, *Er*-1 was unable to influence directly the growth of glioma cells (thus supporting the cell-type specificity of *Er*-1 effects). By contrast, using a cell-based assay in which U-373 cells were cultured in conditioned medium from *Er*-1-treated Jurkat cells, we demonstrated that *Er*-1 induced Jurkat cells to synthesise and release factors that, in turn, inhibit the glioma U-373 cell cycle progression, without causing apoptosis [56].

Stimulating the immune system of cancer patients by the administration of cytokines from the IL-2 family is one of the most intriguing areas of current cancer immune-therapy research. In 1992 IL-2 was the first FDA-approved cytokine for cancer therapy and was helpful in proving that immune activation may be an effective manner of eradicating cancer [54]. However, its clinical application remains restricted due to several disadvantages, including adverse reactions and severe toxicities [54]. The use of novel IL-2-like cytokines with reduced toxicity but maintaining or even increasing the ability to activate T cells and natural killer cells efficiently for potent anti-tumour immune responses is a promising alternative approach to replace conventional IL-2 therapy [54, 58]. In this context, the pheromone *Er*-1 property to immuno-enhance T-cell activity through IL-2R binding deserves attention for possible application in pharmacological perspective.

4. Euplotins

The sesquiterpenoids euplotin A, B and C and their biogenic precursor preuplotin are a class of lipophilic species-specific terpenoid secondary metabolites isolated from the marine ciliate *Euplotes crassus* [38]. They all consist of a tricyclic dioxa-cyclopenta[c,d]indene scaffold, and the presence of 5 defined stereocentres in these non-aromatic polycyclic compounds is a fundamental parameter in the search for a correlation between molecular entities and variety of interesting biological activities. Of notice, the cytotoxic potency of euplotins can be modified by the structure of the

geranyl side chain [59]. Euplotins have attracted initial interest for their key ecological role in the adaptive strategies of *Euplotes* species for habitat colonisation.

4.1. Euplotin C

The acetylated compound euplotin C, which contains only an alkenyl chain linked to the tricyclic moiety without other functional groups [60, 61], provides an effective mechanism for damping potential competitors, thus supporting the entrance into new niches. Indeed, euplotin C rapidly kills non-producer *Euplotes* strains whereas at sub lethal levels it alters cell cycle and shape, and inhibits cell motility [38, 61-65]. At molecular level, euplotin C rapidly increases the intracellular concentration of both Ca^{2+} and Na^{+} in *Euplotes vannus* (an *Euplotes* morpho-species that does not produce euplotin C and shares with *Euplotes crassus* the same interstitial habitat), as well as its membrane electrical properties (*i.e.* increase of the amplitude of graded action potentials) [64]. Of interest, euplotin C decreases mitochondrial function, induces profound changes of the mitochondrial ultrastructure, and activates a caspase-dependent type of apoptosis in *Euplotes vannus* without affecting the generation of reactive oxygen species (ROS) or inducing a detectable permeabilisation/rupture of cell membranes [64, 65]. These events drive the dismantling of the ciliary structures/microtubules, the development of aberrant cytoplasmic vacuoles, and the quick decrease of phagocytic activity [64, 65]. Accordingly, euplotin C also enhances lysosomal pH and reduces lysosomal membrane stability of *Euplotes vannus* [64]. The most important and general outcome of these studies is that euplotin C triggers a marked disruption of homeostatic mechanisms, which plays a determinant role in cell-environment interactions.

4.2. Effects of Euplotin C against a wide range of organisms

In relation to its hydrophobic and lipophilic nature, euplotin C has been later found to exert powerful cytotoxic and pro-apoptotic effects not only on other ciliates. For instance, an inhibitory

effect of euplotin C has been observed on food vacuole formation and fluid phase endocytosis of *Paramecium primaurelia*, a process that might be mediated by an altered function of cell membranes as well as by modification of the microtubule network [66, 67]. Noteworthy, the cytotoxic actions of euplotin C has been then broadened to non-marine, free-living and parasitic protozoa, as for instance the pathogenic *Leishmania major* and *Leishmania infantum*, opportunistic yeast *Candida albicans* and several prokaryotic (opportunistic or pathogenic) bacterial strains, such as *Streptococcus* and *Burkholderia* spp. [68], thus suggesting that this substance may be a possible lead compound for chemotherapeutics.

4.3. Cytotoxic and pro-apoptotic effects of Euplotin C in tumour cells

Consistent with the fact that euplotin C is cytotoxic against a variety of organisms, the cellular and molecular basis of its effects were investigated in mammalian cells expressing properties common to both neurones and neurosecretory cells, as for instance AtT-20 and PC12 cells, derived from a corticotropic tumour of the mouse anterior pituitary and a pheochromocytoma of the rat adrenal medulla, respectively [69, 70]. Euplotin C was shown to be a powerful, dose-dependent cytotoxic agent with a moderate (low micromolar) potency and very high efficacy. Interestingly, it displays a very low, or none at all, cytotoxicity for non-tumour cells such as mouse macrophages [68], suggesting a somewhat tissue selective effect in mammals. The key determinants of euplotin C signalling in tumour cells [69, 70] involve a rapid opening of ryanodine receptors leading to depletion of endoplasmic reticulum Ca^{2+} stores and a marked increase of intracellular concentration of Ca^{2+} [70]. Euplotin C also induces a concomitant generation of ROS that sustains, at least in part, Ca^{2+} overload. These are early events participating in cell death by apoptosis and are paralleled by endoplasmic reticulum stress and mitochondrial dysfunction. In particular, the apoptotic machinery coupled to euplotin C is characterised by the formation of Bax-dependent protein-permeable channels in mitochondrial membranes thus inducing the dissipation of the mitochondrial membrane

potential and allowing the release of cytochrome c from the mitochondria to the cytosol. In accordance, euplotin C triggers an increase of Bax/Bcl-2 ratio. These events depend on ROS generation without the involvement of p53. Caspase-3 is then activated and induces the morphological changes typical of apoptosis, *i.e.* cell shrinkage, chromatin condensation, nucleosomal degradation and fragmentation. Also, the endoplasmic reticulum stress-related caspase-12 may participate to the apoptotic process and likely it is involved when the mitochondrial pathway is inhibited. Finally, euplotin C was also shown to activate the Ca^{2+}-dependent protease calpains downstream of ROS generation, although, on the contrary, seem to exert protective effects [69].

Taken together these data indicate that cell death pathway mediated by euplotin C displays many common features in different cells which is consistent with the flexibility/redundancy of euplotin C apoptotic programme and suggests the existence of multiple points of signalling integration.

5. Climacostol

Climacostol (5-(Z)-non-2-enyl-benzene-1,3-diol) is a natural toxin physiologically produced by the freshwater ciliated protozoan *Climacostomum virens* **(Fig. 1A)** for chemical defence against unicellular and multi-cellular predators [71-75]. Climacostol consists of a phenolic structure and a long aliphatic hydrocarbon chain attached to the ring structure, and belongs to resorcinolic lipids, a group of natural amphiphilic molecules detected in prokaryotes and eukaryotes. A hypothetical pathway for the biosynthesis of long-chain resorcinolic lipids could work also in the biosynthesis of climacostol that would be synthesised from the C16-polyketide, with a cyclisation and a decarboxylation [74]. Recently, resorcinolic lipids have drawn attention because of their anti-parasitic, anti-microbial, anti-tumour, and genotoxic activities [76, 77]. In this respect, climacostol exerts a potent antimicrobial activity in a specific manner against a panel of bacterial and fungal pathogens, including *Staphylococcus aureus*, *Escherichia coli, Candida albicans* and

Pseudomonas aeruginosa [48]. Further, climacostol displayed potent cytotoxic activity on free-living freshwater ciliates [78].

5.1. Cytotoxic and pro-apoptotic effects in tumour cells

Climacostol, which is now available by an innovative diastereoselective chemical synthesis [79], was shown to inhibit the growth of several human and rodent tumour cells while being devoid of effects on endothelial cells; a comparative analysis also demonstrated that climacostol inhibited viability of tumour cells with higher potency when compared to non-tumour cells [76, 79, 80]. Moreover, a potent dose-dependent (EC_{50} - the concentration producing half the maximum effect = 6.23 µg/ml), and effective cytotoxic and anti-proliferative role of climacostol was discovered in B16-F10 cells, an high metastatic subline of the mouse B16 melanoma cell line [80]. These findings are intriguing; to better discuss their possible implications we present here *in vitro* experiments on melanoma cell viability analysed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, in which we measured the effects of increasing doses of climacostol at 24 h in low metastatic B16-F1 cells [81-83]. As shown in **Fig. 1B**, climacostol caused a drastic decrease of cell viability with an EC_{50} of 12.5 µg/ml, the double than that obtained in the high metastatic B16-F10 cells. This lend further support to a preferential toxicity of climacostol towards cells with high tumourigenic/colonisation/invasion potential.

At molecular level, climacostol rapidly forms adducts with DNA (either histone-protected or naked DNA) leading to DNA damage and thus cell death [80, 84]. Induction of DNA damage has been demonstrated being effective in treating cancer, and many currently employed anti-tumour drugs, as for instance platinum agents, act through this mechanism [85-87]. In this line, cisplatin and climacostol did not display any additive effects on melanoma viability [80].

Using human leukaemia and squamous carcinoma and mouse melanoma cells the pro-apoptotic effects of climacostol were demonstrated at both molecular and morphological level [76, 80].

Mechanistic insights from these works include the evidence that climacostol induces the intrinsic apoptotic pathway characterised by the central role of the mitochondria dysfunction: dissipation of the mitochondrial membrane potential, rapid increase of ROS, translocation of Bax to the mitochondria, release of cytochrome c from the mitochondria, and activation of caspase 9-dependent cleavage of caspase 3 with enzymatic degradation of DNA. Of interest, the up-regulation of p53 and its targets Noxa and Puma was found to be responsible of the apoptotic programme of climacostol in melanomas [80]. Similarly, different anti-tumour agents, including cisplatin, lead to melanoma cell death with changes in the expression of p53 and its signals [85, 86]. It is interesting to note that climacostol is able to induce morphological changes in rat liver mitochondria through the inhibition of respiratory chain complex I with subsequent production of ROS [88]. Moreover, climacostol was shown to determine a non-enzymatic type of DNA damage in the presence of Cu^{2+} ions via its metal-reducing activity (that produces ROS) [84]. The intracellular Cu^{2+} levels are usually higher in cancer cells than in normal cells: this may further enhance the non-enzymatic DNA cleavage induced by climacostol and reinforce the enzymatic one. These mechanisms may in part explain the preferential cytotoxicity of climacostol towards tumour cells.

5.2. Inhibition of melanoma progression

The anti-cancer activity of climacostol *in vivo* was recently described in a melanoma allograft model, the B16-F10 cells injected subcutaneously in mice [80]. As schematically depicted in **Fig. 1C**, when tumours were established, the experimental procedure consisted in intra-tumour injections (local administration) of climacostol at 600 µg/ml or vehicle (control) every 3-4 days for 3 weeks. Data analysis revealed a persistent inhibition of tumour growth rate when melanomas were treated with climacostol. In addition, it significantly improved the survival of transplanted mice, decreased tumour weight, and induced a remarkable reduction of viable cells inside the tumour [80]. Using the same experimental paradigm, we here define better the effects of the local administration

of climacostol in *in vivo* mouse melanoma allografts by means of immunostaining experiments. Tumours treated with climacostol showed a significant reduction in the phosphorylation state of STAT3 at Ser727 site when compared to control group (**Figure 2A**). In melanoma cells, Ser727 phosphorylation of STAT3 has key positive role in cell survival activity [89]. The cell staining for ki67, a well-established marker of melanoma proliferation [90], was found significantly decreased as well in climacostol-treated melanoma allografts (**Figure 2B**). Taken together these results further support the beneficial role of climacostol intra-tumour delivery during melanoma growth; of interest, this also supports the notion that climacostol effects involve a reduction in the number of viable and proliferating cells.

We have previously demonstrated that B16-F10 melanoma allografts injected intra-tumour with climacostol expressed levels of cleaved caspase-3 higher than controls and displayed a high amount of clustered apoptotic cells with an enhanced expression of p53, Noxa and Puma [80]. The immunostaining experiments of **Figure 2C** now show that the expression of survivin, a prognostic factor in melanoma, is significantly reduced in B16-F10 melanoma allografts after local administration of climacostol. This is in agreement with our previous observations since survivin expression may be induced upon melanocyte transformation or in response to disruption of p53 signalling and is an essential component of melanoma development and metastasis targeting cell division, apoptosis, and motility [91, 92]. Taken together, the published data and the new evidence here reported indicate that climacostol triggers the death process of tumour cells both *in vitro* and *in vivo* as a result of DNA damage and apoptosis; the signalling events responsible for the climacostol-induced pro-apoptotic effects rely on the up-regulation of p53 network that, in turn, activates the intrinsic programed cell death pathway (mitochondrial dysfunction, ROS, Bax/cytochrome c, caspase-9/3).

Normally, chemotherapeutic drugs are administered systemically to reach the cancerous cells. However, intra-tumour administration, for solid tumours, is a potential approach for delivering high

doses of drugs directly at the tumour site while minimising systemic exposure. Indeed, conventional chemotherapy is severely compromised because of its systemic toxicity. Melanoma is a highly malignant tumour able to form cutaneous, subcutaneous and nodal metastases; it is one of those tumours in which intra-tumour administration of drugs has been proposed and tested as a clinically viable intervention. In particular, several strategies of regional delivery have been implemented to improve the drug effects on melanoma *in vivo* and reduce the adverse effects [93, 94], as for instance intra-tumour administration of cisplatin [95]. For these reasons, our results represent a first proof-of-concept that local delivery of climacostol has therapeutic activity in *in vivo* tumours, although the possibility to use climacostol *via* the systemic route should not be excluded. In this respect, the anti-cancer therapeutic potential of climacostol by systemic administration is still unknown and deserves to be investigated.

New targeted therapies and immunotherapies which have provided significant advances towards control of tumour growth and metastases formation have been recently approved [87, 96-98]. Climacostol, while not substituting for these therapies may be developed as a co-therapy to increase anti-tumour potential of these new drugs. Indeed the possibility exists that melanoma cells become resistant to anti-cancer compounds and circumvent their effects. In this respect, the quest for novel combination therapies which may improve the treatments currently in use is of relevance in clinical perspective [87, 96-98].

5.3. Implications in melanoma vasculature framework

Survivin knock-down in B16-F10 cells has recently been shown to diminish the number of blood vessels in tumour allografts [99] and there is considerable interest from a clinical point of view on the potential role of angiogenesis in the development and progression of malignant melanomas [100]. To evaluate tumour-associated angiogenesis, sections from B16-F10 allografts were double stained with CD31 antibody (as endothelial cell marker) and cleaved caspase-3 (as apoptotic cell

marker), and analysed by immunohistochemistry. As shown in **Fig. 3A**, differences were observed between the different tumours with regard to vessel abundance and distribution. In particular, tumours locally treated with climacostol (600 µg/ml every 3-4 days for 3 weeks) were characterised by low vessel density when compared to vehicle (control)-treated melanomas, with the apoptotic areas inside the tumours being largely avascular. These new data are consistent with the notion that climacostol may induce a decrease in microvessel sprouting that contributes to inhibition of melanoma growth.

We also investigated possible vascular growth factors involved in climacostol effects. Among them, platelet-derived growth factor (PDGF) has been shown to be secreted by melanoma cells both *in vitro* and *in vivo*, including B16 cells [101, 102]. It may function as an autocrine growth factor, as well as an angiogenetic factor in melanoma development, although contrasting results have been also reported [103]. Noteworthy, the levels of PDGF receptor α (PDGF-Rα), which binds to PDGF-A, correlated with the metastatic potential of the melanoma cells [104, 105]. It is also interesting the fact that activated PDGF-Rα was shown to suppress the levels of p53 [106]. As shown in Real-Time PCR experiments of **Figure 3B**, *in vitro* treatment of B16-F10 cells with 30 µg/ml climacostol significantly decreased over time the mRNA expression of PDGF-Rα versus vehicle-treated control cells. The expression of PDGF-A was also down-regulated by 6 h administration of climacostol. One of the downstream events relevant to PDGF signalling is the activation of the MAP kinases ERK1/2. Indeed, previous studies demonstrated that chemotherapeutic drugs reduce both PDGF-Rα expression and ERK1/2 phosphorylation, in B16 mouse melanomas; this was accompanied by decreased tumour growth and prevention of vascular integrity [107]. We analysed whether climacostol affected this pathway by western blot experiments measuring the activation of the MAP kinases ERK1/2. B16-F10 cells treated *in vitro* for 24 h with 30 µg/ml climacostol exhibited a significant increase of phosphorylated ERK1/2 levels (**Fig. 3C**). Sustained pro-apoptotic stimuli in B16 cells are coupled to ERK1/2 activation [81]. The effects of

climacostol may thus be explained based on the controversial role of ERK1/2 when activated by anti-tumour agents, including cisplatin [85, 86, 108, 109]. Indeed, the activation of the MAPK pathway, as it is observed in most melanomas, mediates a strong anti-apoptotic response and limits the efficacy, by enhancing chemoresistance, of chronic administrations of DNA damaging agents. As a consequence, combinations of MAPK pathway inhibitors and other drugs (including those targeting tumour vasculature) arise as a rationale treatment strategy and are already tested in clinical trials [109, 110]. Interestingly, as shown **Fig. 3D**, 30 μg/ml climacostol at 6 h significantly decreased phosphorylated ERK1/2 levels in B16-F10 cells, thus indicating a time-dependent dual role of the toxin on MAPK modulation. In light of these observation it is tempting to speculate that climacostol acts downregulating PDGF-A/PDGF-Rα signalling, likely involving ERK1/2 activation, thus affecting melanoma angiogenesis. This possibility is intriguing and deserves to be further investigated.

6. Conclusion

Nature is an excellent source of potential chemotherapeutic agents and lead compounds that have provided the basis and insight for the semi-synthesis or total synthesis of several effective new drugs. Natural compounds could be clinically used on their own or in combination with other natural products or standard antineoplastic drugs. The possibility also exists of synergistic approaches for combining natural compounds with various forms of immunotherapy to promote cancer cell death and an effective anti-tumour immune response [54, 111, 112]. Many agents from water organisms, mostly from marine sources, have been studied at preclinical levels and entered clinical trials in cancer; to date some of them have been approved for use in humans [1, 17, 18]. Ara-C (Cytarabine, Cytosar-U®) and Trabectedin (ET743, Yondelis®) are examples of anticancer drugs from the sea, isolated respectively from the sponge *Cryptotheca crypta* and from the tunicate *Ecteinacidia turbinate*. Cytarabine is used to treat different forms of leukaemia and lymphoma

while Trabectedin was approved for the treatment of unresectable and metastatic liposarcoma or leiomyosarcoma. In addition, Eribulin mesylate (Halaven®), a synthetic derivative based on the structure of halichondrin B derived from the sponge *Halichondria* sp., is used for treating metastatic breast cancer. The story of dolastatins also deserves interest. Indeed, monomethylauristatin E (MMAE, vedotin) is a synthetic derivative of dolastatin 10, originally isolated from the sea hare *Dolabella auricularia* but in fact produced by the cyanobacteria *Symploca hydnoides* and *Lyngbya majuscula*, which are part of the sea hare's diet. After the development of a technology to couple MMAE analogues to monoclonal antibodies, Brentuximab vedotin was successfully synthesised (Adcetris®) and in 2011 was approved by FDA for the treatment of patients with Hodgkin's lymphoma or systemic anaplastic large cell lymphoma.

The quality of lead compounds arising from natural sources is high and often bio-friendly, due to their coevolution with the targets in biological systems. Although the application of natural compounds in the treatment of cancer has resulted in increased therapeutic efficacy, currently, a great deal of effort is still aimed at discovering novel molecules. One prerequisite to natural product discovery that remains paramount is the range and novelty of molecular diversity. Natural products possess enormous structural and chemical diversity that is unsurpassed by any synthetic libraries. However, some challenges of animal-derived natural products in drug discovery and development include very low yields, limited supply, complex structures posing enormous difficulty for structural modifications, and complex structures precluding practical synthesis. In this respect the chemical synthesis is still the best way to solve supply problems, and the pharmaceutical industry in particular relies on chemical methods for the production of some medicines derived from natural products [113].

Microbial natural products have several intrinsic properties favouring their consideration in drug discovery and development. For instance, the advantage of using eukaryotic microbes, essentially ciliates, as source of new drugs relies on the sustainability of the natural resource practically with

no environmental impact. In fact, worldwide there is water, it is very frequent that *Euplotes* species can be easily collected without affecting biodiversity. Although a vast and representative number of species is already in collection only a small number of different ciliate species is cultivated in the laboratories, implying that the vast biodiversity of eukaryotic microbial natural products remains underappreciated. Single-celled eukaryotes can be well adapted to captivity, cheap to cultivate under laboratory conditions, perpetuated even into large-scale cultures at reduced costs and with no legal restrictions, and simple to manage for the production of candidate drugs with practically no waste.

From the structural chemist's point of view the most intriguing aspect of ciliate compounds is represented by the high skeletal chemical difference found in this group of organisms [38]. It should be noted that the anti-tumour activity of euplotin C and climacostol is strongly related to their structural feature [114]. The activity differences between new molecular structures of various ciliated derivatives can be explained by diastereoslective interaction with DNA [115]. This relationship between biological activity and stereochemical structure highlights the importance of developing highly stereoselective organic synthesis processes. These structure-activity relationships play an important part in finding new active euplotin C and climacostol derivatives, which exhibit the optimum in potency, most selectively, and least toxicity. This involves the optimisation of the synthetic route for organic compounds that demonstrates of how the biological activity is correlated with synthetic modifications of the structure of interest.

In summary, the vast, untapped, ecological biodiversity of ciliates and their molecules holds great promise for the discovery of novel natural products and deserve to be exploited by drug pharmacology. This could help to create more effective and better-tolerated anti-cancer drugs to be incorporated into the therapeutic armoury.

Conflict of interest

The authors declare no conflict of interest.

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References

1. J. Khazir, D.L. Riley, L.A. Pilcher, P. De-Maayer, B.A. Mir, Anticancer agents from diverse natural sources, Nat Prod Commun 9 (2014) 1655-1669.
2. Y. Luo, R.E. Cobb, H. Zhao, Recent advances in natural product discovery, Curr Opin Biotechnol 30 (2014) 230-237.
3. D.J. Newman, G.M. Cragg, Natural products as sources of new drugs over the 30 years from 1981 to 2010, J Nat Prod 75 (2012) 311-335.
4. M. Kibble, N. Saarinen, J. Tang, K. Wennerberg, S. Makela, T. Aittokallio, Network pharmacology applications to map the unexplored target space and therapeutic potential of natural products, Nat Prod Rep 32 (2015) 1249-1266.
5. A.L. Harvey, R. Edrada-Ebel, R.J. Quinn, The re-emergence of natural products for drug discovery in the genomics era, Nat Rev Drug Discov 14 (2015) 111-129.
6. L.W. Sumner, Z. Lei, B.J. Nikolau, K. Saito, Modern plant metabolomics: advanced natural product gene discoveries, improved technologies, and future prospects, Nat Prod Rep 32 (2015) 212-229.
7. J.L. Ochoa, W.M. Bray, R.S. Lokey, R.G. Linington, Phenotype-Guided Natural Products Discovery Using Cytological Profiling, J Nat Prod 78 (2015) 2242-2248.
8. J. Chang, H.J. Kwon, Discovery of novel drug targets and their functions using phenotypic screening of natural products, J Ind Microbiol Biotechnol 43 (2016) 221-231.
9. E.C. Lau, D.J. Mason, N. Eichhorst, P. Engelder, C. Mesa, E.M. Kithsiri Wijeratne, G.M. Gunaherath, et al., Functional chromatographic technique for natural product isolation, Organic & Biomolecular Chemistry 13 (2015) 2255-2259.
10. K.L. Kurita, E. Glassey, R.G. Linington, Integration of high-content screening and untargeted metabolomics for comprehensive functional annotation of natural product libraries, Proc Natl Acad Sci U S A 112 (2015) 11999-12004.
11. P.R. Jensen, K.L. Chavarria, W. Fenical, B.S. Moore, N. Ziemert, Challenges and triumphs to genomics-based natural product discovery, J Ind Microbiol Biotechnol 41 (2014) 203-209.
12. D. Kumar, Nuclear magnetic resonance (NMR) spectroscopy: Metabolic profiling of medicinal plants and their products, Crit Rev Anal Chem 0 (2015) 1-13.
13. C.S. Clendinen, G.S. Stupp, R. Ajredini, B. Lee-McMullen, C. Beecher, A.S. Edison, An overview of methods using (13)C for improved compound identification in metabolomics and natural products, Front Plant Sci 6 (2015) 611.
14. S. Nobili, D. Lippi, E. Witort, M. Donnini, L. Bausi, E. Mini, S. Capaccioli, Natural compounds for cancer treatment and prevention, Pharmacol Res 59 (2009) 365-378.
15. T.N. Chinembiri, L.H. du Plessis, M. Gerber, J.H. Hamman, J. du Plessis, Review of natural compounds for potential skin cancer treatment, Molecules 19 (2014) 11679-11721.
16. M. Trendowski, Recent Advances in the Development of Antineoplastic Agents Derived from Natural Products, Drugs 75 (2015) 1993-2016.
17. F. Javed, M.I. Qadir, K.H. Janbaz, M. Ali, Novel drugs from marine microorganisms, Crit Rev Microbiol 37 (2011) 245-249.
18. D.J. Newman, G.M. Cragg, Marine-sourced anti-cancer and cancer pain control agents in clinical and late preclinical development, Mar Drugs 12 (2014) 255-278.
19. N. Sithranga Boopathy, K. Kathiresan, Anticancer drugs from marine flora: an overview, J Oncol 2010 (2010) 214186.
20. R. Beesoo, V. Neergheen-Bhujun, R. Bhagooli, T. Bahorun, Apoptosis inducing lead compounds isolated from marine organisms of potential relevance in cancer treatment, Mutat Res 768 (2014) 84-97.
21. A. Ranjan, N.M. Fofaria, S.H. Kim, S.K. Srivastava, Modulation of signal transduction pathways by natural compounds in cancer, Chin J Nat Med 13 (2015) 730-742.

22. Y. Safdari, M. Khalili, M.A. Ebrahimzadeh, Y. Yazdani, S. Farajnia, Natural inhibitors of PI3K/AKT signaling in breast cancer: emphasis on newly-discovered molecular mechanisms of action, Pharmacol Res 93 (2015) 1-10.
23. C.B. Magne Nde, S. Zingue, E. Winter, T.B. Creczynski-Pasa, T. Michel, X. Fernandez, D. Njamen, et al., Flavonoids, breast cancer chemopreventive and/or chemotherapeutic agents, Curr Med Chem 22 (2015) 3434-3446.
24. M. Schnekenburger, M. Dicato, M. Diederich, Plant-derived epigenetic modulators for cancer treatment and prevention, Biotechnol Adv 32 (2014) 1123-1132.
25. V.S. Thakur, G. Deb, M.A. Babcook, S. Gupta, Plant phytochemicals as epigenetic modulators: role in cancer chemoprevention, AAPS J 16 (2014) 151-163.
26. P. Wang, B. Wang, S. Chung, Y. Wu, S.M. Henning, J.V. Vadgama, Increased chemopreventive effect by combining arctigenin, green tea polyphenol and curcumin in prostate and breast cancer cells, Rsc Advances 4 (2014) 35242-35250.
27. P. Wang, H.L. Yang, Y.J. Yang, L. Wang, S.C. Lee, Overcome Cancer Cell Drug Resistance Using Natural Products, Evid Based Complement Alternat Med 2015 (2015) 767136.
28. P. Baharuddin, N. Satar, K.S. Fakiruddin, N. Zakaria, M.N. Lim, N.M. Yusoff, Z. Zakaria, et al., Curcumin improves the efficacy of cisplatin by targeting cancer stem-like cells through p21 and cyclin D1-mediated tumour cell inhibition in non-small cell lung cancer cell lines, Oncol Rep 35 (2016) 13-25.
29. R. Khalife, S. El-Hayek, O. Tarras, M.H. Hodroj, S. Rizk, Antiproliferative and proapoptotic effects of topotecan in combination with thymoquinone on acute myelogenous leukemia, Clin Lymphoma Myeloma Leuk 14 Suppl (2014) S46-55.
30. J. Uzoigwe, E.R. Sauter, Cancer prevention and treatment using combination therapy with plant- and animal-derived compounds, Expert Rev Clin Pharmacol 5 (2012) 701-709.
31. V.O. Zambelli, K.F. Pasqualoto, G. Picolo, A.M. Chudzinski-Tavassi, Y. Cury, Harnessing the knowledge of animal toxins to generate drugs, Pharmacol Res in press (2016)
32. B. Haefner, Drugs from the deep: marine natural products as drug candidates, Drug Discov Today 8 (2003) 536-544.
33. T.F. Molinski, D.S. Dalisay, S.L. Lievens, J.P. Saludes, Drug development from marine natural products, Nat Rev Drug Discov 8 (2009) 69-85.
34. A. Penesyan, S. Kjelleberg, S. Egan, Development of novel drugs from marine surface associated microorganisms, Mar Drugs 8 (2010) 438-459.
35. L. Zhang, R. An, J. Wang, N. Sun, S. Zhang, J. Hu, J. Kuai, Exploring novel bioactive compounds from marine microbes, Curr Opin Microbiol 8 (2005) 276-281.
36. K. Li, Y.W. Chung-Davidson, U. Bussy, W. Li, Recent advances and applications of experimental technologies in marine natural product research, Mar Drugs 13 (2015) 2694-2713.
37. M.A. Ruggiero, D.P. Gordon, T.M. Orrell, N. Bailly, T. Bourgoin, R.C. Brusca, T. Cavalier-Smith, et al., A higher level classification of all living organisms, PLoS One 10 (2015) e0119248.
38. G. Guella, D. Skropeta, G. Di Giuseppe, F. Dini, Structures, biological activities and phylogenetic relationships of terpenoids from marine ciliates of the genus *Euplotes*, Mar Drugs 8 (2010) 2080-2116.
39. P. Luporini, A. Vallesi, C. Alimenti, C. Ortenzi, The cell type-specific signal proteins (pheromones) of protozoan ciliates, Curr Pharm Des 12 (2006) 3015-3024.
40. P. Luporini, C. Alimenti, A. Vallesi, Ciliate pheromone structures and activity: a review, Italian Journal of Zoology 82 (2015) 3-14.
41. G. Guella, F. Dini, F. Pietra, From epiraikovenal, an instrumental niche-exploitation sesquiterpenoid of some strains of the marine ciliated protist Euplotes raikovi, to an unusual intramolecular tele-dienone-olefin [2+2] photocycloaddition, Helv Chim Acta 78 (1995) 1747-1754.
42. A. Valbonesi, P. Luporini, Description of 2 New Species of Euplotes and Euplotes-Rariseta from Antarctica, Polar Biology 11 (1990) 47-53.

43. C.S. Lobban, S.J. Hallam, P. Mukherjee, J.W. Petrich, Photophysics and multifunctionality of hypericin-like pigments in heterotrich ciliates: a phylogenetic perspective, Photochem Photobiol 83 (2007) 1074-1094.
44. B. Pant, Y. Kato, T. Kumagai, T. Matsuoka, M. Sugiyama, Blepharismin produced by a protozoan Blepharisma functions as an antibiotic effective against methicillin-resistant Staphylococcus aureus, FEMS Microbiol Lett 155 (1997) 67-71.
45. Y. Kato, T. Matsuoka, Photodynamic action of the pigment in ciliated protozoan Blepharisma, Journal of Protozoology Research 5 (1995) 136-140.
46. G. Hofle, S. Pohlan, G. Uhlig, K. Kabbe, D. Schumacher, Keronopsin-a and Keronopsin-B, Chemical Defense Substances of the Marine Ciliate Pseudokeronopsis-Rubra (Protozoa) - Identification by Ex-Vivo Hplc, Angewandte Chemie-International Edition in English 33 (1994) 1495-1497.
47. F. Buonanno, A. Anesi, G. Guella, S. Kumar, D. Bharti, A. La Terza, L. Quassinti, et al., Chemical offense by means of toxicysts in the freshwater ciliate, Coleps hirtus, J Eukaryot Microbiol 61 (2014) 293-304.
48. D. Petrelli, F. Buonanno, L.A. Vitali, C. Ortenzi, Antimicrobial activity of the protozoan toxin climacostol and its derivatives, Biologia 67 (2012) 525-529.
49. A. Anesi, F. Buonanno, G. di Giuseppe, C. Ortenzi, G. Guella, Metabolites from the Euryhaline Ciliate Pseudokeronopsis erythrina, Eur J Org Chem 2016 (2016) 1330-1336.
50. A. Vallesi, G. Giuli, R.A. Bradshaw, P. Luporini, Autocrine mitogenic activity of pheromones produced by the protozoan ciliate *Euplotes raikovi*, Nature 376 (1995) 522-524.
51. A. Vallesi, P. Ballarini, B. Di Pretoro, C. Alimenti, C. Miceli, P. Luporini, Autocrine, mitogenic pheromone receptor loop of the ciliate Euplotes raikovi: pheromone-induced receptor internalization, Eukaryot Cell 4 (2005) 1221-1227.
52. C. Ortenzi, C. Miceli, R.A. Bradshaw, P. Luporini, Identification and initial characterization of an autocrine pheromone receptor in the protozoan ciliate *Euplotes raikovi*, J Cell Biol 111 (1990) 607-614.
53. T.R. Malek, I. Castro, Interleukin-2 receptor signaling: at the interface between tolerance and immunity, Immunity 33 (2010) 153-165.
54. G.C. Sim, L. Radvanyi, The IL-2 cytokine family in cancer immunotherapy, Cytokine Growth Factor Rev 25 (2014) 377-390.
55. A. Vallesi, G. Giuli, P. Ghiara, G. Scapigliati, P. Luporini, Structure-function relationships of pheromones of the ciliate *Euplotes raikovi* with mammalian growth factors: cross-reactivity between E*r*-1 and interleukin-2 systems, Exp Cell Res 241 (1998) 253-259.
56. D. Cervia, E. Catalani, M.C. Belardinelli, C. Perrotta, S. Picchietti, C. Alimenti, G. Casini, et al., The protein pheromone Er-1 of the ciliate Euplotes raikovi stimulates human T-cell activity: involvement of interleukin-2 system, Exp Cell Res 319 (2013) 56-67.
57. N.A. Forward, D.M. Conrad, M.R. Power Coombs, C.D. Doucette, S.J. Furlong, T.J. Lin, D.W. Hoskin, Curcumin blocks interleukin (IL)-2 signaling in T-lymphocytes by inhibiting IL-2 synthesis, CD25 expression, and IL-2 receptor signaling, Biochem Biophys Res Commun 407 (2011) 801-806.
58. A.M. Levin, D.L. Bates, A.M. Ring, C. Krieg, J.T. Lin, L. Su, I. Moraga, et al., Exploiting a natural conformational switch to engineer an interleukin-2 'superkine', Nature 484 (2012) 529-533.
59. Y.T. Ge, S. Kondo, S. Katsumura, K. Nakatani, S. Isoe, Absolute-Configuration of Novel Marine Diterpenoid Udoteatrial Hydrate Synthesis and Cytotoxicities of Ent-Udoteatrial Hydrate and Its Analogs, Tetrahedron 49 (1993) 10555-10576.
60. G. Guella, E. Callone, I. Mancini, G. Uccello-Barretta, F. Balzano, F. Dini, Chemical and structural properties of the inclusion complex of euplotin C with heptakis(2,6-di-O-methyl)-b-cyclodextrin through NMR spectroscopy, electrospray mass spectrometry and molecular mechanics investigations, Eur J Org Chem 2004 (2004) 1308-1317.
61. G. Guella, F. Dini, F. Pietra, Hydrolytic breakdown of the euplotins, highly strained, adaptive, hemiacetal esters of the marine ciliate Euplotes crassus: a mimic of degradative pathways in nature and a trick for the assignment of the absolute configuration, Helv Chim Acta 79 (1996) 710-717.

62. F. Dini, G. Guella, I. Giubbilini, I. Mancini, F. Pietra, Control of interspecific relationships in marine ciliate protists by most evolved natural products, Naturwissenschaften 80 (1993) 84-86.
63. G. Guella, F. Dini, A. Tomei, F. Pietra, Preuplotin, a putative biogenetic precursor of the euplotins, bioactive sesquiterpenoids of the marine ciliated protist Euplotes crassus, J Chem Soc Perkin Trans 1 (1994) 161-166.
64. F. Trielli, D. Cervia, G. Di Giuseppe, C. Ristori, T. Kruppel, B. Burlando, G. Guella, et al., Action Mechanisms of the Secondary Metabolite Euplotin C: Signaling and Functional Role in Euplotes, J Euk Microbiol 55 (2008) 365-373.
65. D. Cervia, G. Di Giuseppe, C. Ristori, D. Martini, G. Gambellini, P. Bagnoli, F. Dini, The secondary metabolite euplotin C induces apoptosis-like death in the marine ciliated protist *Euplotes vannus*, J Euk Microbiol 56 (2009) 263-269.
66. P. Ramoino, C. Usai, S. Maccione, F. Beltrame, A. Diaspro, M. Fato, G. Guella, et al., Effect of the bioactive metabolite euplotin C on phagocytosis and fluid-phase endocytosis in the single-celled eukaryote Paramecium, Aquat Toxicol 85 (2007) 67-75.
67. P. Ramoino, F. Dini, P. Bianchini, A. Diaspro, G. Guella, C. Usai, Biophysical effects of the natural product euplotin C on the Paramecium membrane, J Comp Physiol A Neuroethol Sens Neural Behav Physiol 195 (2009) 1061-1069.
68. D. Savoia, C. Avanzini, T. Allice, E. Callone, G. Guella, F. Dini, Antimicrobial activity of euplotin C, the sesquiterpene taxonomic marker from the marine ciliate *Euplotes crassus*, Antimicrob Agents Chemother 48 (2004) 3828-3833.
69. D. Cervia, M. Garcia-Gil, E. Simonetti, G. Di Giuseppe, G. Guella, P. Bagnoli, F. Dini, Molecular mechanisms of euplotin C-induced apoptosis: involvement of mitochondrial dysfunction, oxidative stress and proteases, Apoptosis 12 (2007) 1349-1363.
70. D. Cervia, D. Martini, M. Garcia-Gil, G. Di Giuseppe, G. Guella, F. Dini, P. Bagnoli, Cytotoxic effects and apoptotic signalling mechanisms of the sesquiterpenoid euplotin C, a secondary metabolite of the marine ciliate *Euplotes crassus*, in tumour cells, Apoptosis 11 (2006) 829-843.
71. F. Buonanno, T. Harumoto, C. Ortenzi, The Defensive Function of Trichocysts in Paramecium tetraurelia Against Metazoan Predators Compared with the Chemical Defense of Two Species of Toxin-containing Ciliates, Zool. Sci. 30 (2013) 255-261.
72. A. Miyake, F. Buonanno, P. Saltalamacchia, M.E. Masaki, H. Iio, Chemical defence by means of extrusive cortical granules in the heterotrich ciliate Climacostomum virens, Eur J Protistol 39 (2003) 25-36.
73. M.E. Masaki, T. Harumoto, M.N. Terazima, A. Miyake, Y. Usuki, H. Iio, Climacostol, a defense toxin of the heterotrich ciliate Climacostomum virens against predators, Tetrahedron Lett. 40 (1999) 8227-8229.
74. M.E. Masaki, S. Hiro, Y. Usuki, T. Harumoto, M.N. Terazima, F. Buonanno, A. Miyake, et al., Climacostol, a defense toxin of Climacostomum virens (protozoa, ciliata), and its congeners, Tetrahedron 60 (2004) 7041-7048.
75. F. Buonanno, Variations in the efficiency of ciliate extrusomal toxins against a common ciliate predator, the catenulid Stenostomum sphagnetorum, Ital. J. Zool. 72 (2005) 293-295.
76. F. Buonanno, L. Quassinti, M. Bramucci, C. Amantini, R. Lucciarini, G. Santoni, H. Iio, et al., The protozoan toxin climacostol inhibits growth and induces apoptosis of human tumor cell lines, Chem Biol Interact 176 (2008) 151-164.
77. A. Kozubek, J.H. Tyman, Resorcinolic Lipids, the Natural Non-isoprenoid Phenolic Amphiphiles and Their Biological Activity, Chem Rev 99 (1999) 1-26.
78. F. Buonanno, C. Ortenzi, The protozoan toxin climacostol and its derivatives: Cytotoxicity studies on 10 species of free-living ciliates, Biologia 65 (2010) 675-680.
79. D. Fiorini, S. Giuli, E. Marcantoni, L. Quassinti, M. Bramucci, C. Amantini, G. Santoni, et al., A Straightforward Diastereoselective Synthesis and Evaluation of Climacostol, A Natural Product with Anticancer Activities, Synthesis 9 (2010) 1550-1556.

80. C. Perrotta, F. Buonanno, S. Zecchini, A. Giavazzi, F. Proietti Serafini, E. Catalani, L. Guerra, et al., Climacostol reduces tumour progression in a mouse model of melanoma via the p53-dependent intrinsic apoptotic programme, Sci Rep 6 (2016) 27281.
81. L. Bizzozero, D. Cazzato, D. Cervia, E. Assi, F. Simbari, F. Pagni, C. De Palma, et al., Acid sphingomyelinase determines melanoma progression and metastatic behaviour via the microphtalmia-associated transcription factor signalling pathway, Cell Death Differ 21 (2014) 507-520.
82. E. Assi, D. Cervia, L. Bizzozero, A. Capobianco, S. Pambianco, F. Morisi, C. De Palma, et al., Modulation of Acid Sphingomyelinase in Melanoma Reprogrammes the Tumour Immune Microenvironment, Mediators Inflamm 2015 (2015) 370482.
83. D. Cervia, E. Assi, C. De Palma, M. Giovarelli, L. Bizzozero, S. Pambianco, I. Di Renzo, et al., Essential role for acid sphingomyelinase-inhibited autophagy in melanoma response to cisplatin, Oncotarget 7 (2016) 24995-25009.
84. L. Quassinti, F. Ortenzi, E. Marcantoni, M. Ricciutelli, G. Lupidi, C. Ortenzi, F. Buonanno, et al., DNA binding and oxidative DNA damage induced by climacostol-copper(II) complexes: implications for anticancer properties, Chem Biol Interact 206 (2013) 109-116.
85. Z.H. Siddik, Cisplatin: mode of cytotoxic action and molecular basis of resistance, Oncogene 22 (2003) 7265-7279.
86. A. Basu, S. Krishnamurthy, Cellular responses to Cisplatin-induced DNA damage, J Nucleic Acids 2010 (2010) 201367.
87. V.A. Nikolaou, A.J. Stratigos, K.T. Flaherty, H. Tsao, Melanoma: new insights and new therapies, J Invest Dermatol 132 (2012) 854-863.
88. Y. Muto, Y. Tanabe, K. Kawai, Y. Okano, H. Iio, Climacostol inhibits Tetrahymena motility and mitochondrial respiration, Central European Journal of Biology 6 (2011) 99-104.
89. M. Sakaguchi, M. Oka, T. Iwasaki, Y. Fukami, C. Nishigori, Role and regulation of STAT3 phosphorylation at Ser727 in melanocytes and melanoma cells, J Invest Dermatol 132 (2012) 1877-1885.
90. S.J. Ohsie, G.P. Sarantopoulos, A.J. Cochran, S.W. Binder, Immunohistochemical characteristics of melanoma, J Cutan Pathol 35 (2008) 433-444.
91. J.A. McKenzie, D. Grossman, Role of the apoptotic and mitotic regulator survivin in melanoma, Anticancer Res 32 (2012) 397-404.
92. X. Chen, N. Duan, C. Zhang, W. Zhang, Survivin and Tumorigenesis: Molecular Mechanisms and Therapeutic Strategies, J Cancer 7 (2016) 314-323.
93. S. Sloot, O.M. Rashid, J.S. Zager, Intralesional therapy for metastatic melanoma, Expert Opin Pharmacother 15 (2014) 2629-2639.
94. S.S. Agarwala, Intralesional therapy for advanced melanoma: promise and limitation, Curr Opin Oncol 27 (2015) 151-156.
95. T.L. Hwang, W.R. Lee, S.C. Hua, J.Y. Fang, Cisplatin encapsulated in phosphatidylethanolamine liposomes enhances the in vitro cytotoxicity and in vivo intratumor drug accumulation against melanomas, J Dermatol Sci 46 (2007) 11-20.
96. L. Finn, S.N. Markovic, R.W. Joseph, Therapy for metastatic melanoma: the past, present, and future, BMC Med 10 (2012) 23.
97. B. Homet, A. Ribas, New drug targets in metastatic melanoma, J Pathol 232 (2014) 134-141.
98. O. Michielin, C. Hoeller, Gaining momentum: New options and opportunities for the treatment of advanced melanoma, Cancer Treat Rev 41 (2015) 660-670.
99. J.G. Fernandez, D.A. Rodriguez, M. Valenzuela, C. Calderon, U. Urzua, D. Munroe, C. Rosas, et al., Survivin expression promotes VEGF-induced tumor angiogenesis via PI3K/Akt enhanced beta-catenin/Tcf-Lef dependent transcription, Mol Cancer 13 (2014) 209.
100. G. Jour, D. Ivan, P.P. Aung, Angiogenesis in melanoma: an update with a focus on current targeted therapies, J Clin Pathol 69 (2016) 472-483.

101. A. Willenberg, A. Saalbach, J.C. Simon, U. Anderegg, Melanoma cells control HA synthesis in peritumoral fibroblasts via PDGF-AA and PDGF-CC: impact on melanoma cell proliferation, J Invest Dermatol 132 (2012) 385-393.
102. M. Furuhashi, T. Sjoblom, A. Abramsson, J. Ellingsen, P. Micke, H. Li, E. Bergsten-Folestad, et al., Platelet-derived growth factor production by B16 melanoma cells leads to increased pericyte abundance in tumors and an associated increase in tumor growth rate, Cancer Res 64 (2004) 2725-2733.
103. D. Faraone, M.S. Aguzzi, G. Toietta, A.M. Facchiano, F. Facchiano, A. Magenta, F. Martelli, et al., Platelet-derived growth factor-receptor alpha strongly inhibits melanoma growth in vitro and in vivo, Neoplasia 11 (2009) 732-742.
104. E.C. McGary, A. Onn, L. Mills, A. Heimberger, O. Eton, G.W. Thomas, M. Shtivelband, et al., Imatinib mesylate inhibits platelet-derived growth factor receptor phosphorylation of melanoma cells but does not affect tumorigenicity in vivo, J Invest Dermatol 122 (2004) 400-405.
105. R.L. Barnhill, M. Xiao, D. Graves, H.N. Antoniades, Expression of platelet-derived growth factor (PDGF)-A, PDGF-B and the PDGF-alpha receptor, but not the PDGF-beta receptor, in human malignant melanoma in vivo, Br J Dermatol 135 (1996) 898-904.
106. H. Lei, G. Velez, A. Kazlauskas, Pathological signaling via platelet-derived growth factor receptor {alpha} involves chronic activation of Akt and suppression of p53, Mol Cell Biol 31 (2011) 1788-1799.
107. A. Pirraco, P. Coelho, A. Rocha, R. Costa, L. Vasques, R. Soares, Imatinib targets PDGF signaling in melanoma and host smooth muscle neighboring cells, J Cell Biochem 111 (2010) 433-441.
108. A. Mirmohammadsadegh, R. Mota, A. Gustrau, M. Hassan, S. Nambiar, A. Marini, H. Bojar, et al., ERK1/2 is highly phosphorylated in melanoma metastases and protects melanoma cells from cisplatin-mediated apoptosis, J Invest Dermatol 127 (2007) 2207-2215.
109. R.J. Sullivan, K. Flaherty, MAP kinase signaling and inhibition in melanoma, Oncogene 32 (2013) 2373-2379.
110. A. Marzuka, L. Huang, N. Theodosakis, M. Bosenberg, Melanoma Treatments: Advances and Mechanisms, J Cell Physiol 230 (2015) 2626-2633.
111. G. Graziani, L. Tentori, P. Navarra, Ipilimumab: a novel immunostimulatory monoclonal antibody for the treatment of cancer, Pharmacol Res 65 (2012) 9-22.
112. T. Kitamura, J.W. Pollard, Therapeutic potential of chemokine signal inhibition for metastatic breast cancer, Pharmacol Res 100 (2015) 266-270.
113. B. David, J.L. Wolfender, D.A. Dias, The pharmaceutical industry and natural products: historical status and new trends, Phytochem Rev 14 (2015) 299-315.
114. F.A.M. Silva, F. Borges, C. Guimaraes, J.L.F.C. Lima, C. Matos, S. Reis, Phenolic acids and derivatives: Studies on the relationship among structure, radical scavenging activity, and physicochemical parameters, Journal of Agricultural and Food Chemistry 48 (2000) 2122-2126.
115. H. Caner, E. Groner, L. Levy, I. Agranat, Trends in the development of chiral drugs, Drug Discov Today 9 (2004) 105-110.
116. D. Cervia, S. Fiorini, B. Pavan, C. Biondi, P. Bagnoli, Somatostatin (SRIF) modulates distinct signaling pathways in rat pituitary tumor cells; negative coupling of SRIF receptor subtypes 1 and 2 to arachidonic acid release, Naunyn Schmiedebergs Arch Pharmacol 365 (2002) 200-209.
117. C. Armani, E. Catalani, A. Balbarini, P. Bagnoli, D. Cervia, Expression, pharmacology, and functional role of somatostatin receptor subtypes 1 and 2 in human macrophages, J Leukoc Biol 81 (2007) 845-855.
118. C. Perrotta, M. Buldorini, E. Assi, D. Cazzato, C. De Palma, E. Clementi, D. Cervia, The thyroid hormone triiodothyronine controls macrophage maturation and functions: protective role during inflammation, Am J Pathol 184 (2014) 230-247.
119. G. Di Giuseppe, D. Cervia, A. Vallesi, Divergences in the Response to Ultraviolet Radiation Between Polar and Non-Polar Ciliated Protozoa : UV Radiation Effects in Euplotes, Microb Ecol 63 (2011) 334-338.

120. D. Cervia, D. Fehlmann, D. Hoyer, Native somatostatin sst2 and sst5 receptors functionally coupled to Gi/o-protein, but not to the serum response element in AtT-20 mouse tumour corticotrophs, Naunyn Schmiedebergs Arch Pharmacol 367 (2003) 578-587.
121. C. Nunn, D. Cervia, D. Langenegger, L. Tenaillon, R. Bouhelal, D. Hoyer, Comparison of functional profiles at human recombinant somatostatin sst2 receptor: simultaneous determination of intracellular Ca2+ and luciferase expression in CHO-K1 cells, Br J Pharmacol 142 (2004) 150-160.
122. D. Cervia, D. Langenegger, E. Schuepbach, M. Cammalleri, P. Schoeffter, H.A. Schmid, P. Bagnoli, et al., Binding and functional properties of the novel somatostatin analogue KE 108 at native mouse somatostatin receptors, Neuropharmacology 48 (2005) 881-893.
123. C. De Palma, R. Di Paola, C. Perrotta, E. Mazzon, D. Cattaneo, E. Trabucchi, S. Cuzzocrea, et al., Ibuprofen-arginine generates nitric oxide and has enhanced anti-inflammatory effects, Pharmacol Res 60 (2009) 221-228.
124. E. Catalani, D. Cervia, D. Martini, P. Bagnoli, E. Simonetti, A.M. Timperio, G. Casini, Changes in neuronal response to ischemia in retinas with genetic alterations of somatostatin receptor expression, Eur J Neurosci 25 (2007) 1447-1459.
125. D. Cervia, E. Catalani, M. Dal Monte, G. Casini, Vascular endothelial growth factor in the ischemic retina and its regulation by somatostatin, J Neurochem 120 (2012) 818-829.
126. A. D'Alessandro, D. Cervia, E. Catalani, F. Gevi, L. Zolla, G. Casini, Protective effects of the neuropeptides PACAP, substance P and the somatostatin analogue octreotide in retinal ischemia: a metabolomic analysis, Mol Biosyst 10 (2014) 1290-1304.
127. D. Cazzato, E. Assi, C. Moscheni, S. Brunelli, C. De Palma, D. Cervia, C. Perrotta, et al., Nitric oxide drives embryonic myogenesis in chicken through the upregulation of myogenic differentiation factors, Exp Cell Res 320 (2014) 269-280.
128. S. Alboni, D. Cervia, B. Ross, C. Montanari, A.S. Gonzalez, M. Sanchez-Alavez, M.C. Marcondes, et al., Mapping of the full length and the truncated interleukin-18 receptor alpha in the mouse brain, J Neuroimmunol 214 (2009) 43-54.
129. C. De Palma, F. Morisi, S. Pambianco, E. Assi, T. Touvier, S. Russo, C. Perrotta, et al., Deficient nitric oxide signalling impairs skeletal muscle growth and performance: involvement of mitochondrial dysregulation, Skelet Muscle 4 (2014) 22.
130. B. Pavan, S. Fiorini, M. Dal Monte, L. Lunghi, C. Biondi, P. Bagnoli, D. Cervia, Somatostatin coupling to adenylyl cyclase activity in the mouse retina, Naunyn Schmiedebergs Arch Pharmacol 370 (2004) 91-98.

Figure legends

Fig. 1. A, Photomicrographs of *Climacostomum virens* and the chemical structure of climacostol. Chemically synthesised climacostol ($C_{15}H_{22}O_2$) was obtained and prepared as previously described [79, 80]. B16-F1 and B16-F10 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 10% heat-inactivated foetal bovine serum, glutamine (200 mM), penicillin/streptomycin (100 U/ml) (Euroclone, Pero, Italy), and grown at 37°C in a humidified atmosphere containing 5% CO_2 [80-83, 116]. B, Dose-response effects of climacostol on B16-F1 cell viability. Using published protocols [56, 69, 70, 80, 81, 117-119], cell viability was evaluated by MTT analysis (Sigma-Aldrich, St. Louis, MO, USA). MTT absorbance in *in vitro* B16-F1 cells treated with increasing concentrations of climacostol for 24 h was quantified spectrophotometrically using a Glomax Multi Detection System microplate reader (Promega, Milano, Italy). EC_{50} was determined by non-linear regression curve analysis of the concentration-effect responses [120-122]. The GraphPad Prism software package (GraphPad Software, San Diego, CA, USA) was used. Data are expressed by setting the absorbance of the reduced MTT in the absence of climacostol as 100%. The data points represent the results (means ± SEM) obtained from 4 independent experiments. C**,** Schematic picture depicting *in vivo* treatments of mice bearing B16-F10 melanoma allografts. Female C57BL/6 mice (8-10 weeks old) were purchased from Charles River Laboratories (Calco, Italy), housed in a regulated environment and provided with food and water ad libitum [80-83]. All studies were conducted in accordance with the Italian law on animal care N° 116/1992 and the European Communities Council Directive EEC/609/86. All efforts were made to reduce both animal suffering and the number of animals used. Using published protocols [80-83], mice received subcutaneous injections of B16-F10 in the lower-right flank. When the syngeneic implantation was established (usually 10 days after tumour cells inoculation) and the tumour was palpable (volume range between 15-30 mm^3), mice with almost similar size of tumours were randomly assigned to one of the two experimental groups. In particular, transplanted mice received 100 µl intra-tumour

injections of climacostol (600 μg/ml, equivalent to ca. 3.5 mg/kg dose, respectively) or vehicle every 3-4 days for 3 weeks.

Fig. 2. Climacostol decreased the expression of pSTAT3, ki67, and survivin in B16-F10 melanoma allografts. Mice treated with vehicle (control) or climacostol were sacrificed at day 16 for immunostaining experiments. Briefly, *in vivo* resected B16-F10 tumours were immersion-fixed in 4% paraformaldehyde, as already described. [80, 83, 123]. Tumour sections were then cut at 10 μm and incubated overnight with the rabbit polyclonal anti-phosphoSTAT3 (phospho S727), anti-ki67, and anti-survivin primary antibodies (Abcam, Cambridge, UK). For fluorescence detection, sections were stained with the appropriate Alexa Fluor secondary antibody (Life Technologies, Monza, Italy) and cover-slipped with a phosphate buffer-glycerin mixture containing DAPI (Sigma-Aldrich, St. Louis, MO, USA). Incubation in secondary antibody alone was performed as negative control. Images were acquired by a Zeiss Axioskop 2 plus microscope equipped with the Axiocam MRC photocamera and the Axiovision software (Carl Zeiss, Oberkochen, Germany). Using published protocols [124-128], each image was converted to grayscale and normalised to background using an image editing software (Adobe Photoshop CS3; Adobe Systems, Mountain View, CA, USA). Mean gray levels were then measured in the immune-stained cells area. A, B, C, Images of immunofluorescence staining for pSTAT3 (A), ki67 (B), and survivin (C). DAPI (blue) was used for nuclei detection. Scale bar: 20 μm. The images are representative of 3 different tumours per experimental group. Right panels: quantitative analysis of immunostaining for pSTAT3, ki67, and survivin. Each histogram represent the results (means ± SEM) obtained from at least 2 representative images of 3 different tumours per experimental group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ *vs* control (unpaired Student's *t*-test; GraphPad Prism, GraphPad Software, San Diego, CA, USA).

Fig. 3. A, Climacostol decreased tumour-associated angiogenesis in B16-F10 melanoma allografts. Tumour sections from *in vivo* resected B16-F10 melanomas at day 16 were immune-stained with the rat polyclonal anti-CD31 (as endothelial cell marker) (BD Pharmigen, San Diego, CA, USA) and rabbit polyclonal anti-cleaved caspase 3 (as apoptotic cell marker) (Cell Signaling Technology, Danvers, MA, USA) primary antibodies, as previously detailed (see the legend of Fig. 2). Images depicted immunofluorescence staining for CD31 (red) and cleaved caspase-3 (punctate green) in vehicle (control) or climacostol-treated melanomas. DAPI (blue) was used for nuclei detection. Scale bar: 20 μm. The images are representative of 3 different tumours per experimental group. B, Climacostol decreased PDGF system in B16-F10 cells. Real-Time PCR experiments were performed in *in vitro* B16-F10 cells treated with vehicle (control) or 30 μg/ml climacostol at 6, 10 and 24 h. mRNA expression was analysed as previously described [80, 81, 117, 118, 127, 129] using the SsoAdvanced Universal SYBR Green Supermix and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). A set of primer pairs was designed to hybridise to unique regions of the appropriate gene sequence (**Table 1**). Fold change was determined relative to the control after normalizing to Rpl32 (internal standard) through the use of the formula $2^{-\Delta\Delta CT}$. Data are expressed as the fold change over the control (set as 1 - dashed line). Each histogram represents the results (means ± SEM) of the mRNA levels of PDGF-A and PDGF-Rα as obtained from 4-8 independent experiments. $*p < 0.05$ and $**p < 0.01$ *vs* the respective control (one-way ANOVA followed by Bonferroni's multiple comparison post-test; GraphPad Prism, GraphPad Software, San Diego, CA, USA). C, D, Dual role of climacostol on MAPK activity in B16-F10 cells. Western blot experiments were performed in *in vitro* B16-F10 cells treated with vehicle (control) or 30 μg/ml climacostol at 24 h (C) or 6 h (D). Protein expression was analysed as previously described [80, 81, 130] using the rabbit polyclonal anti-phospho-p44/p42 MAPK (pERK1/2) primary antibody (Cell Signaling Technology, Danvers, MA, USA). To monitor for potential artefacts in loading and transfer among samples in different lanes, the blots were routinely treated with the Restore Western

Blot Stripping Buffer (ThermoFisher Scientific, Waltham, MA, USA) and re-probed with the rabbit polyclonal anti-p44/42 MAPK (total ERK1/2) primary antibody (Cell Signaling Technology, Danvers, MA, USA), that recognises the protein independently of its phosphorylation state. Right panels: densitometric analysis (means ± SEM) of ERK1/2 activity (n = 3). Data are expressed as the fold change over the normalised control. *$p < 0.001$ and **$p < 0.0001$ *vs* control (unpaired Student's *t*-test; GraphPad Prism, GraphPad Software, San Diego, CA, USA).