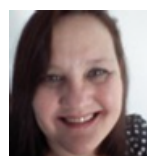# Natural cytotoxic diterpenoids, a potential source of drug leads for melanoma therapy.

Jose M. Prieto[*,a,b], and Dâmaris Silveira[b]

1. [a] *Department of Pharmaceutical and Biological Chemistry, UCL School of Pharmacy, London, UK;*
   [b] *Department of Pharmacy, Faculty of Health Sciences, Universidade de Brasília, Brasília, Brazil.*

Abstract: Diterpenes present complex structure and due to their unique carbon skeleton and interesting biological activities, have been the focus of continuous studies for the development of new anticancer agents. Phorbol esters have been known for their activity against skin malignancies since ancient times. Taxol was first studied in melanoma cells, and recently ingenol mebutate has been approved for the chemoprevention of melanoma in actinic keratosis patients. Therefore, there is scope for research on this class of compounds. We here aim to review the relevant original research reporting on isolated diterpenes with cytotoxic and/or antitumoral activities upon melanoma cells. By collating and discussing the implications of past and current developments on diterpenes, we hope to steer further interest on this field and facilitate the drug discovery activities of the scientific community towards finding potential alternatives to current melanoma chemotherapy.

Keywords: Melanoma, Diterpenes, Cytotoxicity, Cell Migration, Cell Invasion.

1 INTRODUCTION

Terpenoids are a large class of compounds presenting both structurally and stereochemically very diverse skeletons. Typical structures are originated from the C_5 isoprene units and are classified as hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterpenes (C_{25}), triterpenes (C_{30}), and tetraterpenes (C_{40}). They have been -together with alkaloids- one of the most successful sources for anticancer drugs. This review focuses on the diterpenes, which arise from geranylgeranyl diphosphate (GGPP) and comprise more than 18,000 different natural compounds presenting 126 different carbon skeletons [1]. Such array of structures and functionalities provides a pool of interesting molecules for therapeutic purpose.

The screening program run by the US National Cancer Institute in the 70s and 80s reached its climax with the discovery of taxol from *Taxus brevifolia* Nutt. (Taxaceae) which took the clinical world by storm and put natural products back as a valid source of medicines. This compound was isolated in 1971 by Wall and co-workers from Pacific Yew extracts guided by their powerful cytotoxic activity against a melanoma cell line. Eventually, taxol -now known as paclitaxel- was approved in the USA by the FDA in 1993, not for the treatment of melanoma but mainly ovarian and breast cancers. Since then, melanoma treatment keeps revolving on the use of synthetic chemotherapy drugs such as dacarbazine and temozolamine, alone or in combination with either interleukin-2 (IL-2) or monoclonal antibodies (vemurafenib, ipilimumab, dabrafenib, and trametinib). These therapies would need to be improved for long-term efficacy and decreased toxicity [2].

The relationship between diterpene-rich plants and skin tumours has been exploited-since ancient times. The spurges (a group of *Euphorbia* species) have been known since ancient times for their topical pro-inflammatory activity externally and cathartic effects when taken inwardly. Their characteristic milky sap is loaded with diterpenes. Its careful application onto warts removes them very effectively, but it is not devoid of local adverse effects, usually acute painful oedema. Chronic application of naturally occurring diterpenes in spurges, notably phorbol esters, is known to both induce cancerous changes on normal cells and differentiation in cancer cells.

The recent approval of the diterpenes derivative ingenol mebutate (Picato®) for the treatment of actinic keratosis -a skin condition which leads to melanoma in a very high proportion of patients- has renewed the interest on diterpenes as a source of anticancer drugs with particular activities on skin malignancies.

We here aim to review relevant original research reporting on isolated diterpenes with cytotoxic and/or antitumor activities upon melanoma cells. By collating and discussing the implications of past and current developments on diterpenes, we hope to steer further interest on this field and facilitate the drug discovery activities of the scientific community towards finding new avenues to current melanoma chemotherapy. Pubmed and Scopus have been searched for 'MELANOMA' and 'DITERPEN' to allow for diterpenes and diterpenoids to be retrieved.

All relevant papers to the review, regardless date of publication were used.

2 MELANOMA: PHYSIOPATHOLOGICAL AND MOLECULAR ASPECTS

Skin cancer is classified into non-melanoma skin cancer (NMSC) and melanoma. Melanoma originates from pigment-producing cells called melanocytes which produce melanin, a substance that is responsible for skin colour. While NMSC is the most common form, melanoma is so aggressive that causes approximately 80% of all deaths from skin cancer [3,4]. Melanoma pathogenesis goes through two major stages: the first is melanocytic transformation (melanogenesis) and the second is the spread of melanoma to other organs (metastasis).

Although patterns of UV exposure and personal characteristics greatly influence melanogenesis, this eventually occurs as a result of the accumulation of genetic changes. The most important oncogenes responsible for melanoma malignancy are: BRAF [5], NRAS [6], and mutant NF1 which are found in up to 50%, 20% and 14% of melanoma patients, respectively [7]. This led to a genomic classification consisting of four subtypes based on the pattern of the most prevalent significantly mutated genes: mutant BRAF, mutant RAS, mutant neurofibromin 1 (NF1), and Triple-WT (wild-type) [8]. We may soon be adding other molecular oncogenes to the list such as the tandem cyclin-dependent kinase 4 (CDK4) / cyclin D1 (CCND1): melanomas with wild-type BRAF or N-RAS frequently had increases in the number of copies of these genes [7].

BRAF and NRAS mutations seem to be mutually exclusive in the majority of melanomas, but mutant NRAS can drive a dual pathway signalling if the tumour suppressor PTEN is depleted. Then both the MAPK and the PI3K-AKT signalling pathways are activated enough to support the survival of the cancerous cell (see Fig 1) [9].

The identification of these targets facilitated the development of drugs with specific inhibitory activity upon BRAF and NRAS. Patients enjoy longer expectancy of life with such treatments, but resistance emerges very fast [10]. It looks like the above oncotargets cooperate with each other some combination therapies may be a better approach. In drug-resistant melanomas, the mutant BRAF seems to cooperate with NF1 loss to continue driving melanoma development through the abrogation of oncogene-induced senescence [11]. Furthermore, recent evidence points towards increased expression of the Microphthalmia-associated transcription factor (MITF) to contribute to resistance to BRAF pathway inhibition [12]. Although coming last in the percentage of cases within melanoma patients with NF1-mutated subtype seems to have a higher mutational burden and strongest UV mutation signature than any of the previous ones [13]. NF1 mutant melanomas –characterised by a high level of CRAF expression and a differential MAPK activation- [14]

Another strategy is enhancing the natural cell defences by upregulating key tumour suppressors in melanoma such as p53, phosphatase and tensin homolog (PTEN), and p14-ARF. There is compelling evidence that the physiological regulation of p53 function depends on MDM2 - itself the product of a p53- inducible gene. The two molecules are linked to each other through an 'autoregulatory negative feedback loop' maintaining low cellular p53 levels in the absence of stress. DNA damage induces rapid MDM2 phosphorylation, which is thought to contribute to the p53 activation mechanism, in order to keep MDM2 localised in the nucleolus so that it cannot degrade p53 a 15-kDa protein called p14ARF [15]. Its effect blocks the G1 and G2 phases in the cell cycle and inhibits the growth of abnormal cells by indirectly activating p53. Therefore p14ARF could be considered as important as p53 as a key tumour suppressor in melanoma [16]. It is affected by several proteins but also small xenobiotics/molecules such as the flavonoid apigenin, thus proving that natural small molecules may be viable co-adjuvants in the battle against cancer [17].

A further pathway enhancing cell growth and proliferation without the involvement of the MAPK pathway is the reduction of retinoblastoma (RB) protein family activity [18]. This pathway is a less common one which results in the activation of the cell cycle regulators -and melanoma onto targets as previously explained- Cyclin D1 or CDK4 and initiate clonal expansion. However, it links with the senescence pathway which is central in melanoma. One of the key protein effectors here is p16-CDKN2A acting together with telomere length control. This protein inhibits the activity of cyclin D / CDK4 complex and blocks the hyperphosphorylation of RB to keep E2F transcription factors bound to RB.

Conversely, inactivation of p16-CDKN2A by mutation deletion or promoter silencing causes phosphorylation of RB protein which leads to the release of E2F allowing cell cycle progression. Clearly, this signalling system is the key regulator of melanocyte senescence, and its disruption enables cells to avoid senescence and causes uncontrolled cell growth as well as the aggressiveness of transformed melanocytes [19–22].

Resistance to apoptosis is required for the development of melanoma which is associated with mutations of p53, a major tumour suppressor gene.

P53, p14-CDKN2A and Bcl-2 proteins are the major proteins that are involved in this pathway [22]. As such apoptosis serves as a prophylactic mechanism to cancer development,g cell death, the characteristic mechanisms of programmed cell death include chromatin condensation, membrane blebbing, and cell fragmentation, where the fragments are phagocytised by the neighbouring inflammatory cells [22]. These cellular changes occur because of the activity of caspase, an aspartate-specific cysteine protease, which is an essential element in promoting cell death. The caspase cascade system is required for initiation, transduction and amplification of apoptotic signals. It is regulated by several proteins and messengers such as Bcl-2 and Ca^{+2} and activated either through extracellular signals (extrinsic pathway) or intracellular signal (intrinsic pathway). The Bcl-2 family of proteins regulates apoptosis by controlling mitochondrial permeability. Failing that, Cytochrome C leaks into the mitochondrion and activates the caspases which cleave specific cellular substrates to effect cellular death [23].

Metastasis is the second stage in melanoma progression spread of cells from the primary tumour to distant locations where they form a secondary tumour. Tumour metastasis is responsible for 90% of cancer deaths [24]. In metastasis, several signal-transduction pathways are involved, enabling cancer cells to proliferate, remodel their neighbouring environment, invade and migrate to distant locations, and differentiate. Under normal skin condition, melanocytes remain under the control of keratinocytes and only proliferate after keratinocyte stimulation. However, deregulated tight junctions between melanocytes and their neighbouring stromal cells allow melanocytes to escape the tight control by adjacent keratinocytes [19–22].
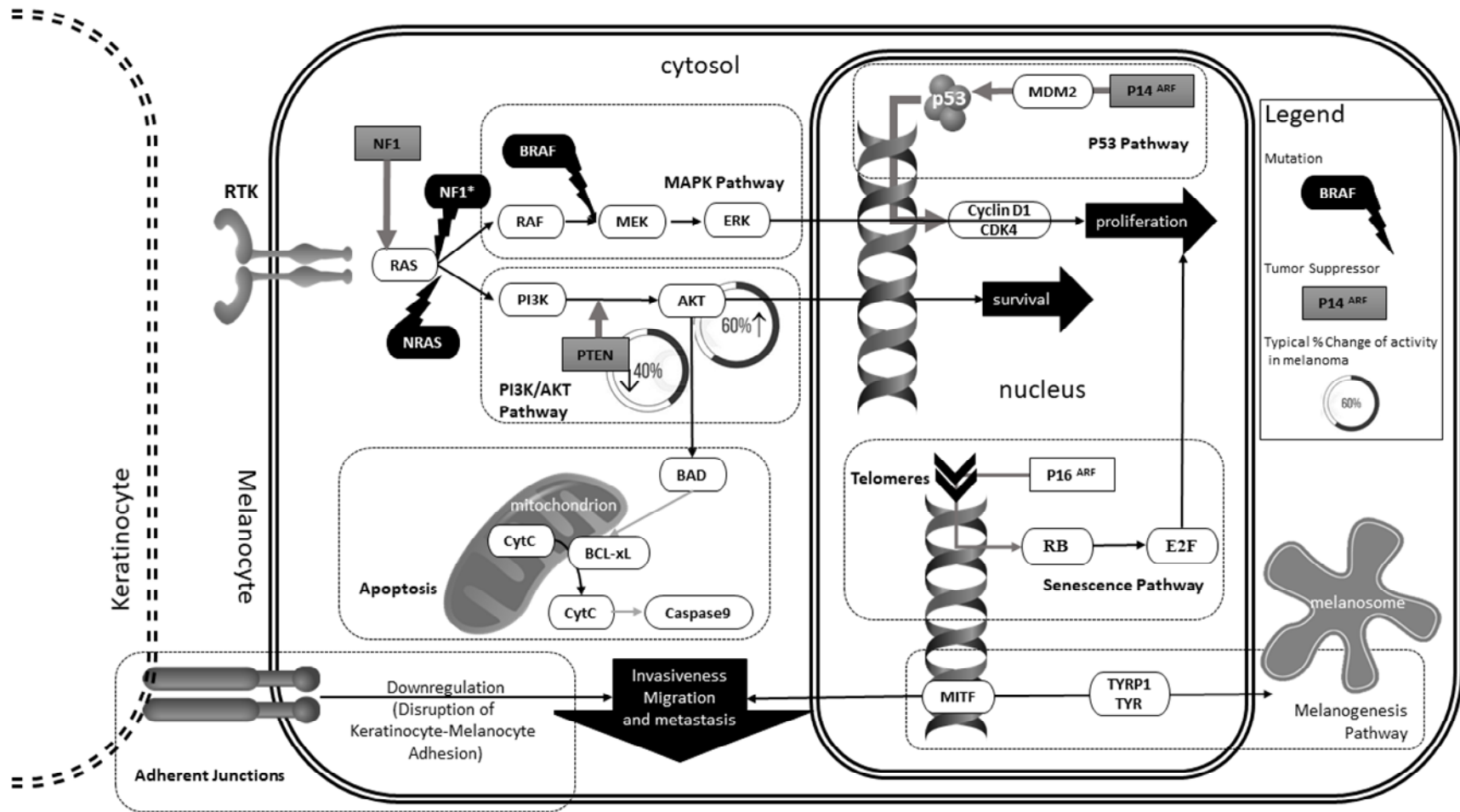
Fig 1. Scheme of the main pathways, mutations / oncogenes and key tumor suppressors in melanoma.

3 DITERPENES FROM PLANTS

3.1 Phytane

Phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol) (**01**) is one of the simplest diterpenes. It is a reduced form of geranylgeraniol, which forms the lipophilic side chain of chrorophylls, therefore is produced by almost all photosynthetic organisms including plants, marine animals such as corals and bacteria [25]. It boasts an enormous variety of biological activities, ranging from antimicrobial and cytotoxic actions to immune-modulating properties [26]. It stimulates ROS production in B16F10 murine melanoma cells. This radical burst reduces melanogenesis *via* phosphorylation of the extracellular signal-regulated protein kinase (ERK) which in turn induces the proteasomal degradation of MITF [27].

Geranylgeranylacetone (**02**) (also known as all *E*-teprenone) is a pharmaceutical drug used for the treatment of gastric ulcers in Japan and other South-Asian countries. It was shown that it also induces apoptosis in human melanoma cells *via* the intrinsic pathway [28]. Although teprenone is not a naturally occurring diterpene, can be synthesised from natural compounds such as acyclic sesquiterpenes and polyprenylacetic acids [29,30].

3.2 Labdanes

Forskolin (**03**) is a labdane diterpene that is produced by the Indian Coleus plant (*Plectranthus barbatus* Andrews; Lamiaceae). It can influence the regulation of adenylate cyclase in murine melanoma tumour cell clones in a similar manner than melanocyte-stimulating hormone (MSH): both produce a significantly higher accumulation of intracellular cyclic adenosine 3', 5' monophosphate (cAMP) in strongly metastatic clones than in weakly metastatic B16 tumour cell clones. cAMP-dependent molecular processes are required for the expression of B16 melanoma experimental metastatic potential [31-33], but forskolin seems not to act as a hormone [34]. Clock genes are suppressed in B16 cells and tumours, but forskolin restores circadian rhythmicity and subsequently cell cycle gene expression resulting in fewer cells in S phase and more in G1 phase. Accordingly, both *in vitro* proliferation and *in vivo* tumour growth slow after forskolin treatment [35].

Triptolide (**04**), from *Tripterygium wilfordii* Hook. f. (Celastraceae) shows promising activities both *in vitro* and *in vivo*. It induces cell arrest at S phase in A375.S2 human melanoma cells *via* the inhibition of cyclin E and CDC25A. Triptolide triggers mitochondrial-dependent apoptosis with caspase [36]. It also impairs DNA damage repair in A375.S2 [37]. Additionally, it inhibits migration and invasion in B16F10 cells by a Nuclear Factor kappaB (NF-κB) dependent pathway [38]. Triptolide decreases circulating T cells and plasma levels of IL-10, TGF-β, and VEGF in C57BL/6 mice bearing B16-F10 [39].

Andrographolide (**05**), from *Andrographis paniculata* (Burm.f.) Nees (Acanthaceae) exerted direct cytotoxicity on melanoma cells by cell-cycle arrest at G0/G1 phase through induction of cell-cycle inhibitory protein p27 and decreased expression of cyclin-dependent kinase 4 (CDK4). Moreover, andrographolide is able to increase the cytotoxic activity of lymphocytes against cancer cells by increased proliferation of lymphocytes and production of interleukin-2, enhanced synthesis of the tumour necrosis factor-alpha, and increased CD marker expression. These *in vitro* activities were confirmed in two *in vivo* syngenic xenograft models using B16F0 melanoma cells [40]. The activity of andrographolide has stimulated researchers to design semisynthetic derivatives which may be more easily protected in patents such as 3,14,19-tripropionylandrographolide (SRS06) (**06**). This compound targets NF-κB both by reducing its gene expression and reducing its DNA binding activity to p65 [41]. Moreover, it also suppresses melanin synthesis through the Akt/GSK3β/β-catenin signal pathway [42] and inhibits melanoma tumour growth by inactivating the TLR4/NF-κB signalling pathway [43].
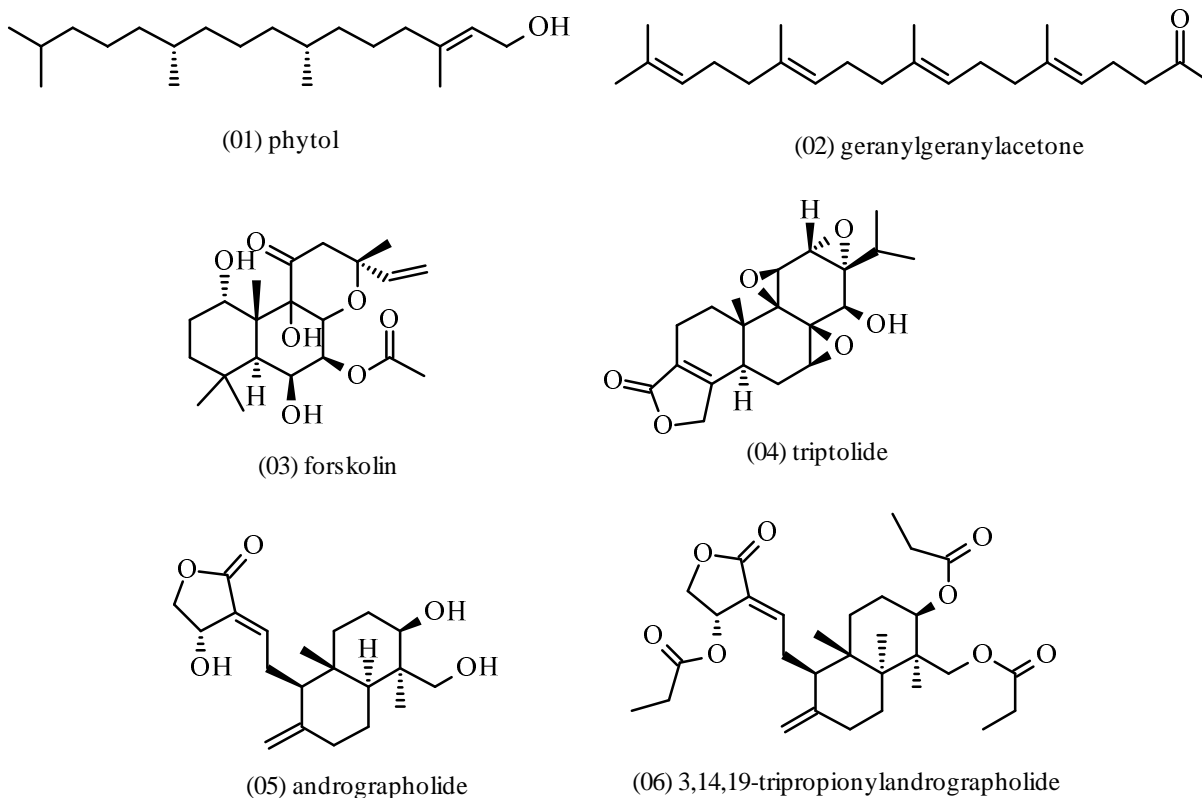
(01) phytol

(02) geranylgeranylacetone

(03) forskolin

(04) triptolide

(05) andrographolide

(06) 3,14,19-tripropionylandrographolide

Fig 2. Phytane and Labdane-type diterpenes with activity on melanoma cells

3.3   Pimaranes

The tubers of *Icacina* species (Icacinaceae). are a rich source of diterpenoids with (9βH)-17-norpimarane and (9bH)-pimarane structures, the latest being a very rare structural class. 17-hydroxyicacinol (**07**), icacinol (**08**), and humirianthol (**09**), as well as two (9bH)-17-norpimaranes, humirianthenolide C (**10**) and icacenone (**11**), and the icacinlactones A–G (**12**-**18**) exhibited cytotoxic activity against MDA-MB-435 in the low micromolar range with humirianthenolide C being the most potent of all, [44–46].

3.4   Scopadulanes

The genus *Calceolaria* (Calceolariaceae), native of South America, have yielded several new diterpenes with the scopadulane skeleton, such as thyrsiflorin A (**19**). Its semisynthetic derivative, demalonyl thyrsiflorin A (**20**) is cytotoxic to melanoma A375 cells at 6.25 μM by inducing an apoptotic response accompanied by the reduction of Heat Shock Protein 70 (Hsp70) expression and reactive oxygen species (ROS) production. It inhibits the caspase cascade at increased concentrations (25-50 μM) an effect still accompanied by ROS increase, probably switching its mechanisms from apoptosis to necrosis [47].
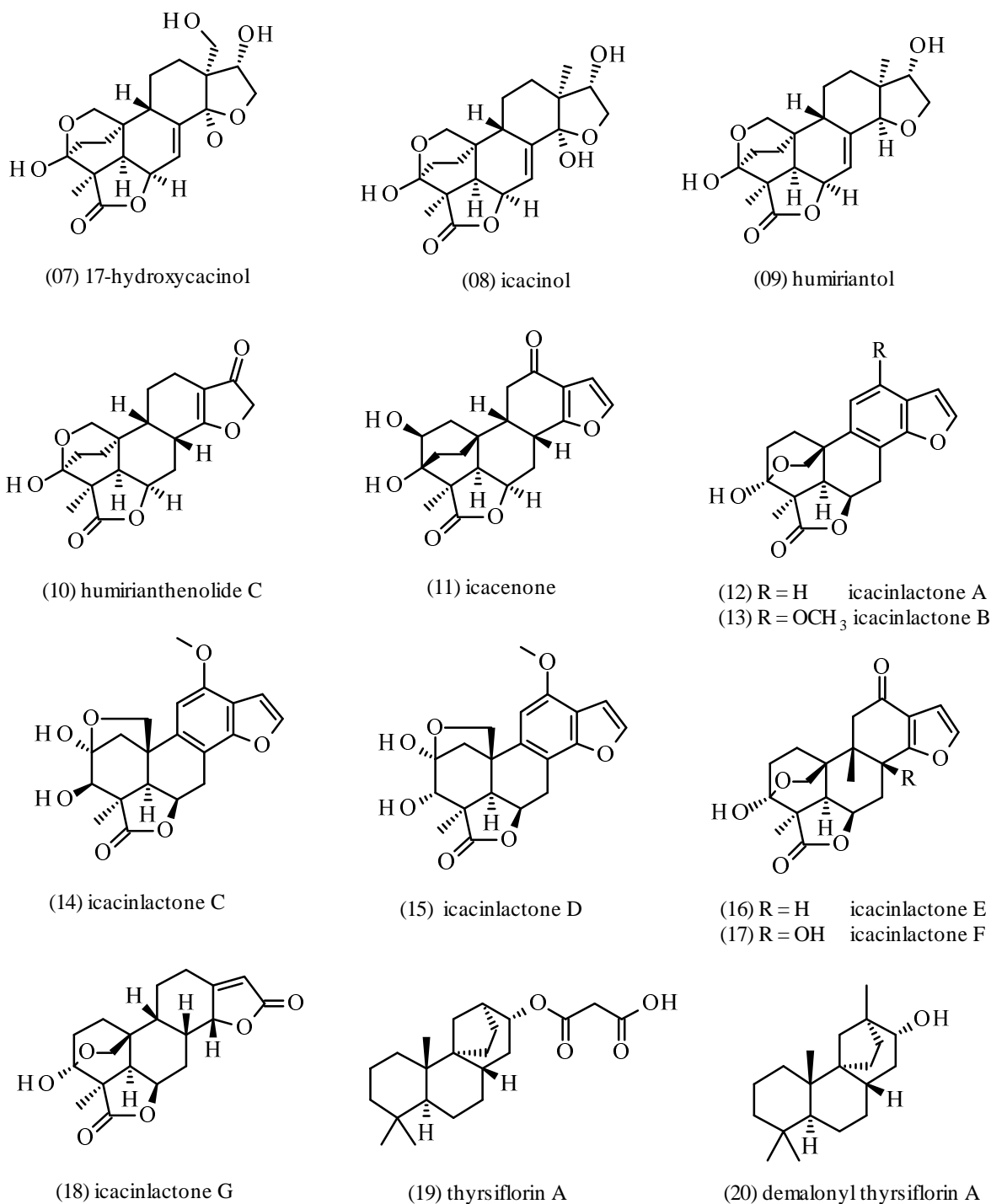
(07) 17-hydroxycacinol

(08) icacinol

(09) humiriantol

(10) humirianthenolide C

(11) icacenone

(12) R = H   icacinlactone A
(13) R = OCH$_3$ icacinlactone B

(14) icacinlactone C

(15) icacinlactone D

(16) R = H   icacinlactone E
(17) R = OH   icacinlactone F

(18) icacinlactone G

(19) thyrsiflorin A

(20) demalonyl thyrsiflorin A

Fig 3. Pimarane-type and Scopadulane-type diterpenes with activity on melanoma cells

3.5   Abietane

Carnosol (**21**), a well-known constituent of *Rosmarinus officinalis* L. (Lamiaceae) extracts, is a phenolic diterpene with antioxidant and anticarcinogen properties in melanoma cells [48,49].

Other researchers have also found that a carnosol-rich extract from *Callicarpa longissima* (Hemsl.) Merr. (Lamiaceae) inhibits melanogenesis in B16F10 melanoma cells [50]. It inhibits B16/F10 cell migration and invasion *in vitro* by differentially

inhibiting MMP-9 mRNA levels over those of MMP-2. Moreover, carnosol was able to inhibit the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, AKT, p38, JNK and prevented the activation of the transcription factors NF-κB and c-Jun [48]. Furthermore, carnosol is both a potent genoprotective agent against radiation in normal skin cells and a sensitising agent, increasing the cellular death in the melanoma cell line B16F10 [51].

Carnosic acid (**22**) is another natural abietane diterpene found in rosemary and common sage in quantities up to 1.5 - 2.5% respectively. It may act as a transcriptional inducer of the NQO1 gene, and therefore suppresses the cytotoxic effect of inhibitors of this enzyme such as rhododendrol [52]. On the one hand, it suppresses the phosphorylation of Src, FAK, and AKT as well as the secretion of MMP-9, tissue inhibitor of metalloproteinase 1 (TIMP-1), urokinase plasminogen activator (uPA), and vascular cell adhesion molecule (VCAM)-1. On the other hand, increased the secretion of TIMP-2 at 10 μM. Remarkably, it both suppresses the mesenchymal markers such as N-cadherin and induces epithelial markers such as E-cadherin thus potentially reverting the epithelial-mesenchymal transition thus hampering B16F10 cell migration [53].

Parvifloron D (**23**), a natural diterpene isolated from *Plectranthus ecklonii* Benth (Lamiaceae) presents broad but not selective cytotoxicity towards many human tumour cells, being able to induce cell death on human A375 and mouse B16V5 cell lines among others [54].

Although sharing commonalities with other abietane diterpenes, 11,12,16-trihydroxy-2-oxo-5-methyl-10-demethyl-abieta-1[10],6,8,11,13-pentene (**24**) isolated from *Premna serratifolia* L. (Lamiaceae) appears to be a novel compound based on a new diterpene skeleton with cytotoxic activity on B16 cells, presenting a promising $IC_{50} = 5$ μM [55].

Several diterpenes containing an abietane skeleton were isolated from roots of *Peltodon longipes* A. St. Hill. Ex Benth (Lamiaceae) and evaluated about cytotoxicity against the human melanoma cancer cell line MV-3. Among them, the compounds 7a-acetoxyroyleanone (**25**) and horminone (**26**) presented the highest activities with IC_{50} values of 7.4 and 16.7 μM, respectively [56].

3.6 Halimanes

Compounds containing halimane skeleton can be found in different plant species from several families, as well as in marine organisms and microorganism. Biogenetically speaking they are a small group of diterpenes closely related to both labdane and clerodane diterpenoids [1]. One such compound, (5R,8R,9S,13R)-halim-1,10-ene-15,16-diol (**27**), was isolated from *Vellozia kolbekii* Alves (Velloziaceae) and showed cytotoxicity against three human cancer cell lines, including MDA-MB-435 by a mechanism of action involving cell cycle arrest at the S/G2M phases [57].

3.7 Ingenol

The diterpene ingenol (**28**) was first isolated in 1968 by Opferkuch & Hecker. These authors were searching for the "*Euphorbia* factors" (skin irritant and tumour-promoting principles) present in *Euphorbia ingens* E. Mey. (Euphorbiaceae). Phorbol 12-myristate 13-acetate (PMA) is the reference in this category. It is a naturally occurring protein kinase C activator diterpene in many *Croton* species, currently classified as a Category 2 carcinogenic chemical hazard. However, they also isolated some "non-irritant" esters of ingenane-type polyfunctional diterpene alcohols devoid of "reasonable tumorigenic activity in mouse (*i.e.* about 1/10 less irritant than PMA). These authors could elucidate the structure through X-ray crystallography in 1970 and described it as endowed with a unique macrocyclic core. The development of this chemopreventative drug has its origins in the use of *Euphorbia peplus* L. (Euphorbiaceae) in Australian folk medicine for the treatment of actinic keratoses and skin cancer. Actinic keratosis is a precancerous condition which if untreated, usually leads to a melanoma [58].

Pharmacological work carried out during the 1990s showed that ingenol is endowed with both antitumour and *in vitro* anti-HIV activities. Mechanistic studies of its effects on actinic keratosis unveiled a dual mechanism of action including rapid necrosis of the affected skin cells and a specific neutrophil-mediated, antibody-dependent cellular cytotoxicity. Destruction of actinic keratosis lesions is accomplished in 2 or 3 days only, and the subsequent immune-mediated response prevents the development of any residual dysplastic epidermal cells. Direct isolation of ingenol mebutate from *E. peplus* is relatively inefficient (*c.a.* 1 mg of ingenol mebutate per Kg of plant) but still commercially viable so it was chosen as the herbal drug for pharmaceutical production. Partial or total synthesis has been extensively explored as a means to obtain both the active principle and other derivatives with the improved therapeutic profile. In 2012 the diterpene ingenol mebutate (**29**) (commercialised as Picato®) was finally approved for the topical treatment of actinic keratosis. The first post-marketing studies on the safety and tolerability of Picato® in the treatment of actinic keratosis seem to be very favourable with mild to moderate adverse effects (*i.e.* erythema, flaking/scaling and crusting) and resolve quickly. Further studies to apply it to the treatment of

superficial basal cell carcinomas with a 0.05% gel have been successful [59], so it looks like ingenol mebutate is poised to be a drug of choice for the treatment of superficial melanoma in the years to come.

The tetrahydroingenol diterpene 7,13-diacetyl-5-angeloyl-20-nicotinyl-3-propionyl-1,2,6,7-tetrahydroingenol (DANPT) (**30**), isolated from *Euphorbia erythradenia* Boiss. (Euphorbiaceae), is cytotoxic against A375 and HMCB cells (IC$_{50}$ < 20 µM). It induced G2/M arrest associated with down-regulation of cyclin B and Cdk-1 and subsequent up-regulation of p53 and p21. Its apoptosic activity occurs with an increased Bax/Bcl-2 ratio, activation of caspase-3, ROS production and loss of mitochondrial membrane potential (ΔYm) [60].

3.8 Ent-Kauranes

The epimer of kaurenoic acid (EKA) (**31**) isolated from the medicinal plant *Croton antisyphiliticus* Mart. (Euphorbiaceae) inhibited mitochondrial viability and induced apoptosis in B16 with an IC$_{50}$ value of 60 µg/mL but was not specific when compared with 3T3 control cells.

Kaurenic acid (**32**), isolated from *Espeletia semiglobulata* Cuatrec (Asteraceae), presented potent cytotoxicity against B16-F1 melanoma cells, with an IC$_{50}$ value of 0.8 µM, two orders of magnitude lower than taxol (IC$_{50}$ = 19 µM). Moreover, kaurenic acid was able to, decrease tumour development in C57BL/6 male mice transplanted with B16-F1 cells, with an inhibition rate of 70 and 80 % at 1.0 and 20.0 mg/kg, respectively, with taxol showing a rate of 54%, at 15 mg/kg [61].

The diterpenoid oridonin (**33**), isolated from *Isodon rubescens* (Hemsl.) H. Hara (Lamiaceae) (previously known as *Rabdosia rubescens*) induced apoptotic effects to human melanoma A375-S2 cells (IC$_{50}$ = 15 µM) by induction of caspases-3 and -8, increased expression of Bax without affecting Bcl-2, as well as the release of cytochrome C and downstream activation of caspase-9. It shown weak cytotoxicity against PBMC thus pointing out to a good therapeutic window. By contrast, ponicidin (**34**), which is present in the same plant and is structurally very close, was not as active against these melanoma cells and markedly inhibited the growth of PBMC under the same conditions. However, treatment with increased concentrations of oridonin (137 µM) for 12 h, exerted cytotoxicity by necrosis as measured by an LDH activity-based assay. These results may be interpreted in the context of convergent apoptotic and necrotic pathways in cancer cells [62].

Further work established that oridonin induced A375-S2 cell apoptosis by activating p53 and ERK pathways in parallel, which explains the apoptotic cell death [63]. Recently its activity upon uveal cells of melanoma origin was also confirmed. The authors found a significantly increased expression of the proapoptotic Bcl-2 family protein Bim and this mechanism was confirmed by knockdown by small interfering RNA. Additionally, oridonin suppressed Fatty Acid Synthase (FAS) expression in uveal melanoma cells, also confirmed by enforced FAS expression by insulin partially rescuing the cells from oridonin-induced apoptosis, showing that inhibition of FAS also contributed to oridonin-mediated apoptosis [64].

3.9 Daphnane

The daphnane-type diterpene daphneresiniferins A (**35**) and B (**36**), yuanhuacine (**37**), yuanhuadine (**38**), yuanhuahine (**39**), genkwadaphnin (**40**), genkwanine A (**41**), genkwanine F (**42**), and genkwanine H (**43**), were isolated from the methanol extract of the flower buds of *Daphne genkwa* Siebold & Zucc. (Thymelaeaceae). All compounds were assayed for both their cytotoxicity and inhibitory effects of the melanogenesis in α-MSH-activated B16 melanoma cells, with IC$_{50}$ values in the range 0.06-9.0 µM, while arbutin and Kojic acid, used as controls presented IC$_{50}$ values of 140 and 39 µM, respectively. On the other hand, only daphneresiniferin B showed significant cytotoxicity on B16 melanoma cells, with an IC$_{50}$ value of 6.6 µM [65].

Gnidimacrin (**44**), isolated from roots of *Stellera chamaesjasme* L (Thymelaeaceae), a plant used in Traditional Chinese Medicine (TCM), was active *in vivo* on B16 bearing mice at doses of 0.06 µg/kg/day [66]. Hirsein A (4**5**) and B (**46**), and gnidilatin (4**7**) from *Thymelaea hirsuta* (L.) Endl. were capable of lowering melanin content in B16 cells without reduction in cell viability. Hirsein A, at 0.1 µM, reducing the melanin content by 37%, while hirsein B reduced the melanin content by 26 %. The process involves the downregulation of the MITF gene, an important oncotarget in melanoma [67–69].
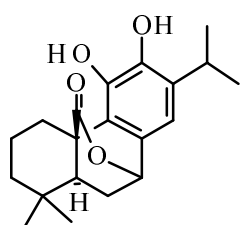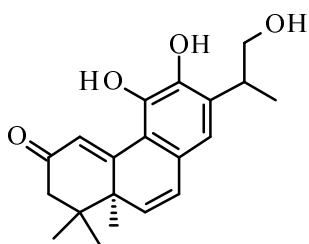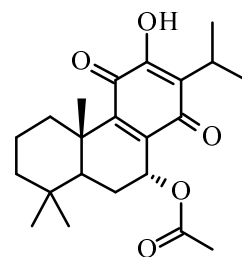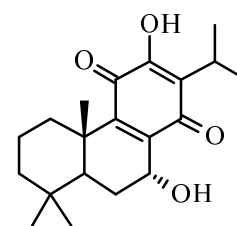
(21) carnosol
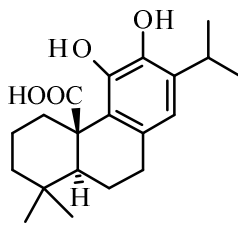
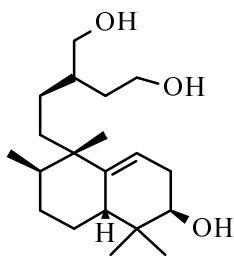(22) carnosic acid
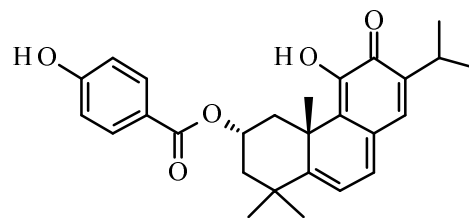
(23) parvifloron D

(24) 11,12,16-trihydroxy-2-oxo-5-methyl-10-demethyl-
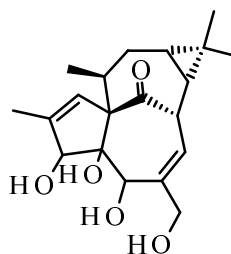abieta-1[10],6,8,11,13-pentene

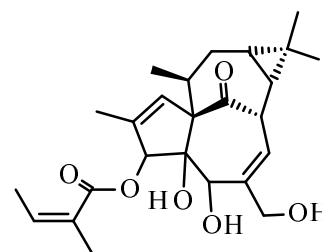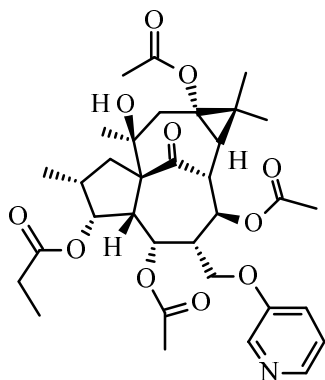(25) 7a-acetoxyroyleanone

(26) horminone

(27) (5R,8R,9S,13R)-halim-1,10-ene-15,16-diol

(28) ingenol

(29) ingenol mebutato

(30) 7,13-diacetyl-5-angeloyl-20nicotinyl-3-propionyl-
1,2,6,7-tetrahydroingenol (DANPT)

Fig 4. Abietane-type, Halimane-type and Ingenol diterpenes with activity on melanoma cells
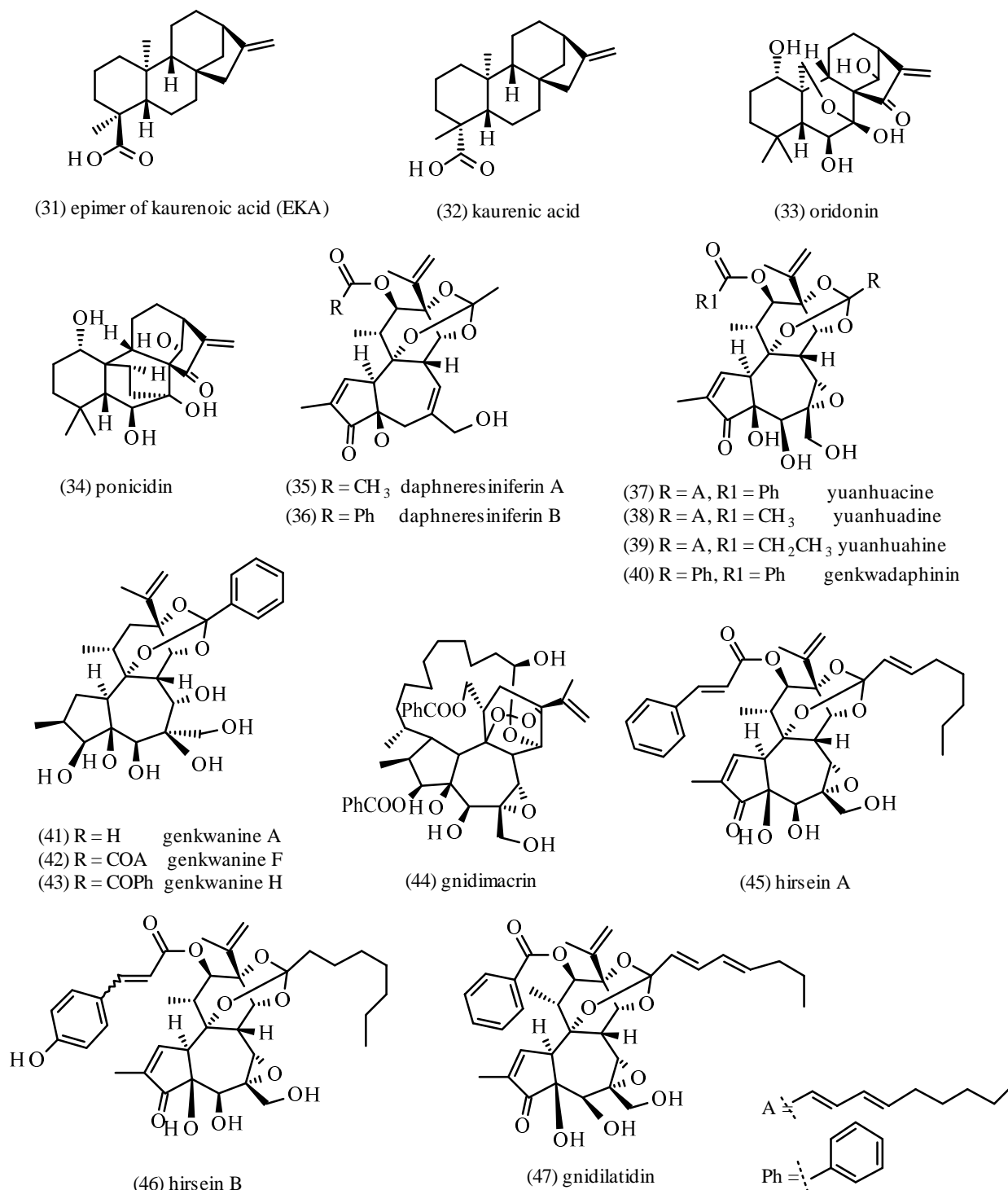
(31) epimer of kaurenoic acid (EKA)

(32) kaurenic acid

(33) oridonin

(34) ponicidin

(35) R = CH$_3$ daphneresiniferin A
(36) R = Ph   daphneresiniferin B

(37) R = A, R1 = Ph   yuanhuacine
(38) R = A, R1 = CH$_3$ yuanhuadine
(39) R = A, R1 = CH$_2$CH$_3$ yuanhuahine
(40) R = Ph, R1 = Ph   genkwadaphinin

(41) R = H   genkwanine A
(42) R = COA   genkwanine F
(43) R = COPh genkwanine H

(44) gnidimacrin

(45) hirsein A

(46) hirsein B

(47) gnidilatidin

A =

Ph =

Fig 5. Kaurane-type and Daphnane-type diterpenes with activity on melanoma cells

3.10 Neoclerodane

Four neoclerodane-type diterpenes, isolated from *Ajuga decumbens* Thunb. (Lamiaceae), namely, ajugacumbin K (**48**), ajugacumbin L (**49**), ajugacumbin M (**50**), and ajugacumbin N (**51**) were evaluated about cytotoxicity on B16 cell line. All compounds presented cytotoxicity on B16, with an IC$_{50}$ value ranging from 24.2 to 44.5 μM [70].

3.10 Others

Hypoestoxide (**52**), a verticillane diterpene found in *Hypoestes rosea* P. Beauv. (Acanthaceae), presented potent nonsteroidal anti-inflammatory effects, and exhibited highly powerful activity against B16 melanoma growth in C57BL/6 mice. At a low maximal effective dose of 5 mg/kg, hypoestoxide was able to induce significant *in vivo* antitumor activity that was better than or comparable with most of the standard chemotherapeutic antiangiogenic agents

cortisone acetate, vincristine, bleomycin, adriamycin, 5-fluorouracil, cyclophosphamide, and etoposide, except vincristine [71].
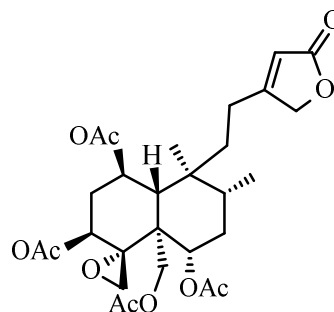
The compound vouacapane-6α,7β,14β,19-tetraol (**53**), isolated from *Pterodon pubescens* Benth. seeds presented *in vitro* cytotoxicity on SK MEL 37 melanoma cells in a dose-dependent way, with IC_{50} values of 32 μM, similar to doxorubicin (IC_{50} = 35 μM) under identical assay conditions [72].
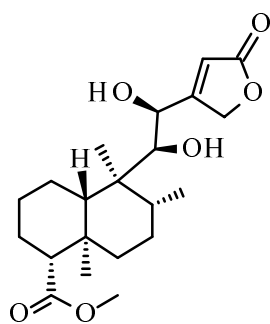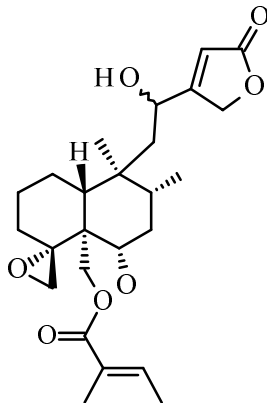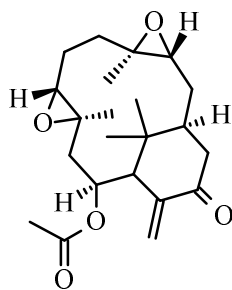

(48) ajugacumbin K
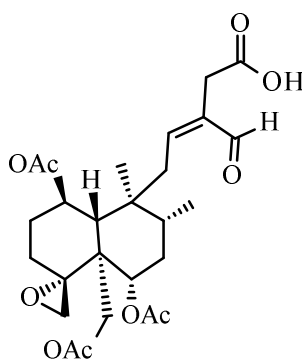
(49) ajugacumbin L

(50) ajugacumbin M

(51) ajugacumbin N

(52) hipoestoxide (HE)
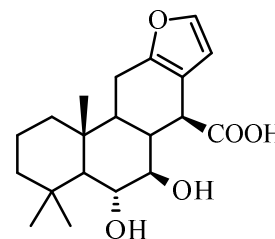
(53) vouacapane-6a,7b,14b,19-tetraol

Fig 6. Miscellaneous plant-derived diterpenes with activity on melanoma cells

4 DITERPENES FROM MARINE ORGANISMS

4.1 Cembranolides

Cembrane-type diterpenoids can be found in terrestrial and marine organisms, e.g. soft corals [73,74] and plants [75,76].

Sinularin (**46**), isolated from the soft coral *Sinularia flexibilis* Quoy and Gaimard (Alcyoniidae)

inhibited A2058 melanoma cell proliferation (IC_{50} = 5 μg/mL), migration, and G2/M phase arrest. The apoptotic effect was evidenced by proteomic data and Western blot displaying the levels of several associated proteins including annexin A1, voltage‐dependent anion‐selective channel protein 1 and prohibitin (upregulated), heat shock protein 60, heat shock protein beta-1, and peroxiredoxin-2 (downregulated) in A2058 melanoma cells exposed to

sinularin. It increased the expression of p53, cleaved-caspase-3, cleaved-caspase-8, cleaved-caspase-9, p21, and Bax, and decreased expression of Bcl-2 [77].

Sarcophytol A (**47**) and sarcophine (**48**), from the soft coral *Sarcophyton glaucum* Quoy and Gaimard (Alcyoniidae), are naturally occurring cembranolides with potent chemopreventive activity [78]. Their low yield in nature sparked a long-term interest in the synthesis of analogues with similar properties [78–84].

4.2 Norcembranoids

From *Sinularia gardneri* (Pratt) (Alcyoniidae) was obtained, besides other compounds, a diterpene with norcembranoid skeleton, singardin (**49**), that presented cytotoxicity against MEL-28, in the concentration of 5 μg/mL [85].

4.3 Biscembranoids

Sarcophytolide M (**50**) was active in the low micromolar range to SK-Mel2, followed by sarcophytolide N (**51**), sarcophytolide I (**52**), lobophytone J (**53**) sarcophytolide L (**54**) lobophytone O (**55**), lobophytone U (**56**), methyl totuoate A (**57**) methyl tortuoate B (**58**) and methyl sartortuoate (**59**) all isolated from the Vietnamese soft coral *Sarcophyton pauciplicatum* (Alcyoniidae) [86].

(46) sinularin   (47) sarcophytol   (48) sarcophine   (49) singardin

(50) sarcophytolide M   (51) sarcophytolide N   (52) R = a-OCH3   sarcophytolide I
                                                              (57) R = b-OH   methyltotuoate A

(53) lobophytone J   (54) R = a-OH sarcophytolide L   (55) lobophytone O
                            (56) R = b-OH lobophytone U

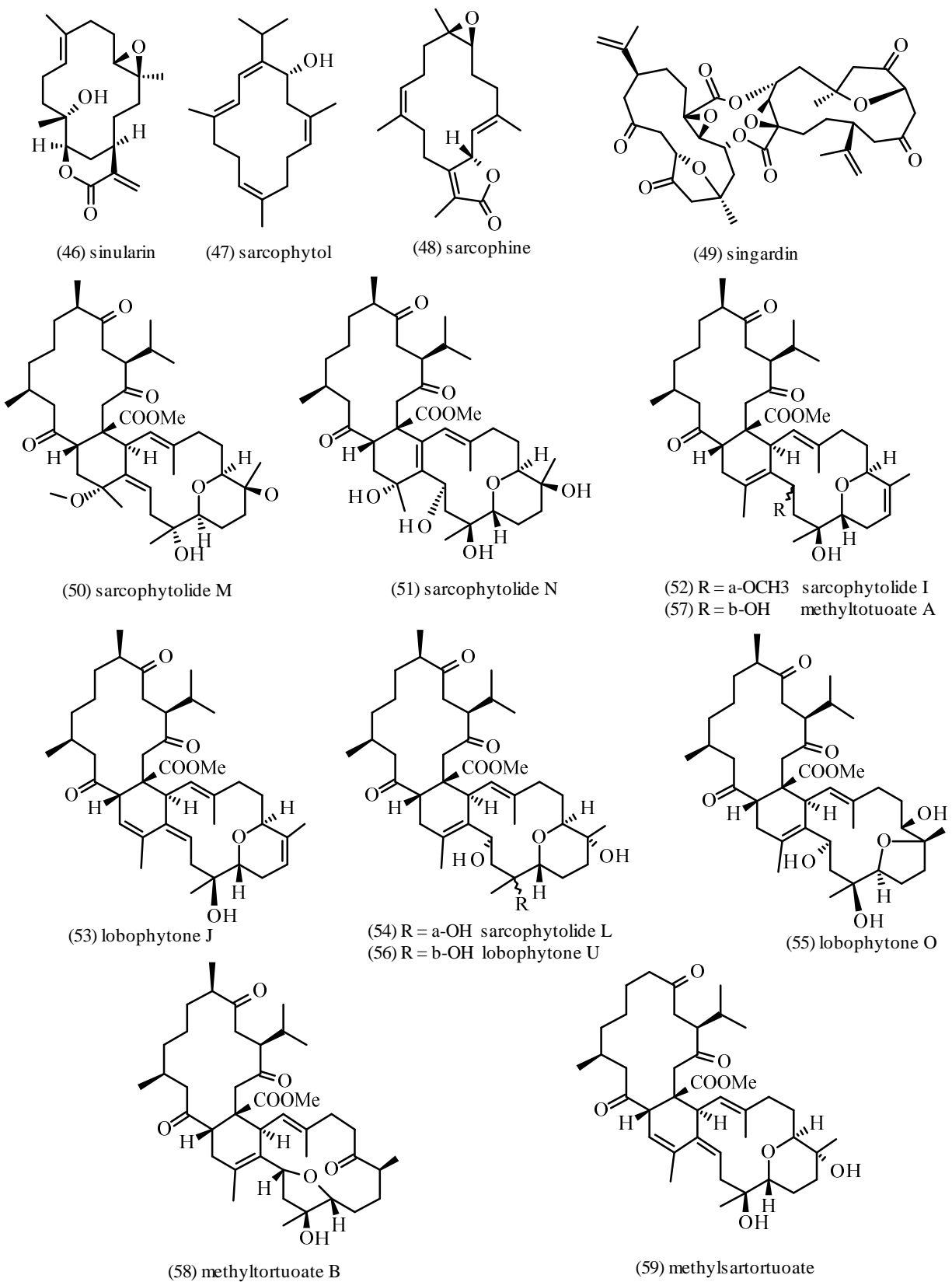(58) methyltortuoate B   (59) methylsartortuoate

Fig 7. Cembrane-type diterpenes from marine organisms with activity on melanoma cells.

4.4 Furanoditerpenes

The activity of the marine furanoditerpenes, spongiatriol (**60**) and epispongiatriol (**61**), was evaluated in MM96E and MM96L - two sublines of human melanoma cells derived from the same metastatic lesion. The two strains were very similar although MM96E presented higher tyrosinase activity and lower expression of alkaline phosphatase than MM96L. Spongiatriol caused dendritic morphology in a proportion of MM96L cells whilst epispongiatriol was able to induce a marked G2/M arrest in MM96E cells. Treatment with cytostatic doses of both diterpenes for 72 h inhibited B8G3 expression and tyrosinase activity with no significant effect on the expression of tyrosinase antigen, with MM96L cells more affected than MM96E. It is believed that these subtle differences in human melanoma phenotypes, is correlated with the expression of different neurotransmitter receptors [87].

4.5 Enhygromic Acids

Enhygromic acid (**62**), isolated from a marine myxobacterium, *Enhygromyxa* sp. exhibited cytotoxicity against B16 melanoma cells [88].

4.6 Spatanes

Spatane diterpenoids isolated from the brown marine algae *Stoechospermum marginatum* (C. Agardh) Küntzing, such as 5(R),19-diacetoxy-15,18(R and S)-dihydrospata-13,16(E)-diene (**63**), induce apoptosis in B16F10 melanoma cells more selectively than in THP1, U937, COLO205, and HL60 cells. The generation of ROS induces unfavourable changes in the Bax/Bcl-2 ratio, eventually disrupting the mitochondrial $\Delta\Psi$m. Cytochrome C leaks to the cytoplasm and activates the caspase-mediated apoptotic pathway. Also, there is a deregulation of the PI3K/AKT signalling pathway. Cell arrest occurs at the S-phase arrest in the cell cycle. This diterpene effectively inhibited tumour growth in C57BL/6 mice bearing B16F10 melanoma without apparent toxic effects [89].

4.7 Lissoclimides

Chlorinated lissoclimide-type diterpenes can be isolated from the mollusc species *Pleurobranchus* (Pleurobranchidae). These diterpenes are presumably metabolites of a *Lissoclinum* species of sea squirts (family Didemnidae) on which the molluscs use to feed. Chlorolissoclimide (**64**) and dichlorolissoclimide (**65**) were found to be potent cytotoxins in the National Cancer Institute's screening panel of 60 tumour cell lines and showed some selectivity for melanomas.

4.8 Briaranes

Diterpenes with briarane skeleton can be found in soft corals, nudibranchs, and sponges [90]. Tubiporein (**67**), isolated from soft coral *Tubipora* sp, was able to inhibit the growth of B-16 mouse melanoma cells at the $IC_{50} = 2$ µg/mL [91].

5 DITERPENES FROM FUNGI

Sphaeropsidin A (**66**) is a fungal bioactive secondary metabolite, with a rearranged primarane skeleton with Na+/K+/2Cl− cotransporter and/or anion exchanger inhibitor activities. It can overcome apoptosis as well as multidrug resistance in SKMEL-28 and mouse B16F10 by inducing a marked and rapid cellular shrinkage related to the loss of intracellular Cl^- and the decreased HCO_3^- concentration in the culture supernatant [92].

(60) epispongiatriol

(61) spongiatriol

(62) enhygromic acid

(63) 5(R),19-diacetoxy-15,18(R and S)-dihydrospata-13,16(E)-diene

(64) chlorolissoclimide

(65) dichlorolissoclimide
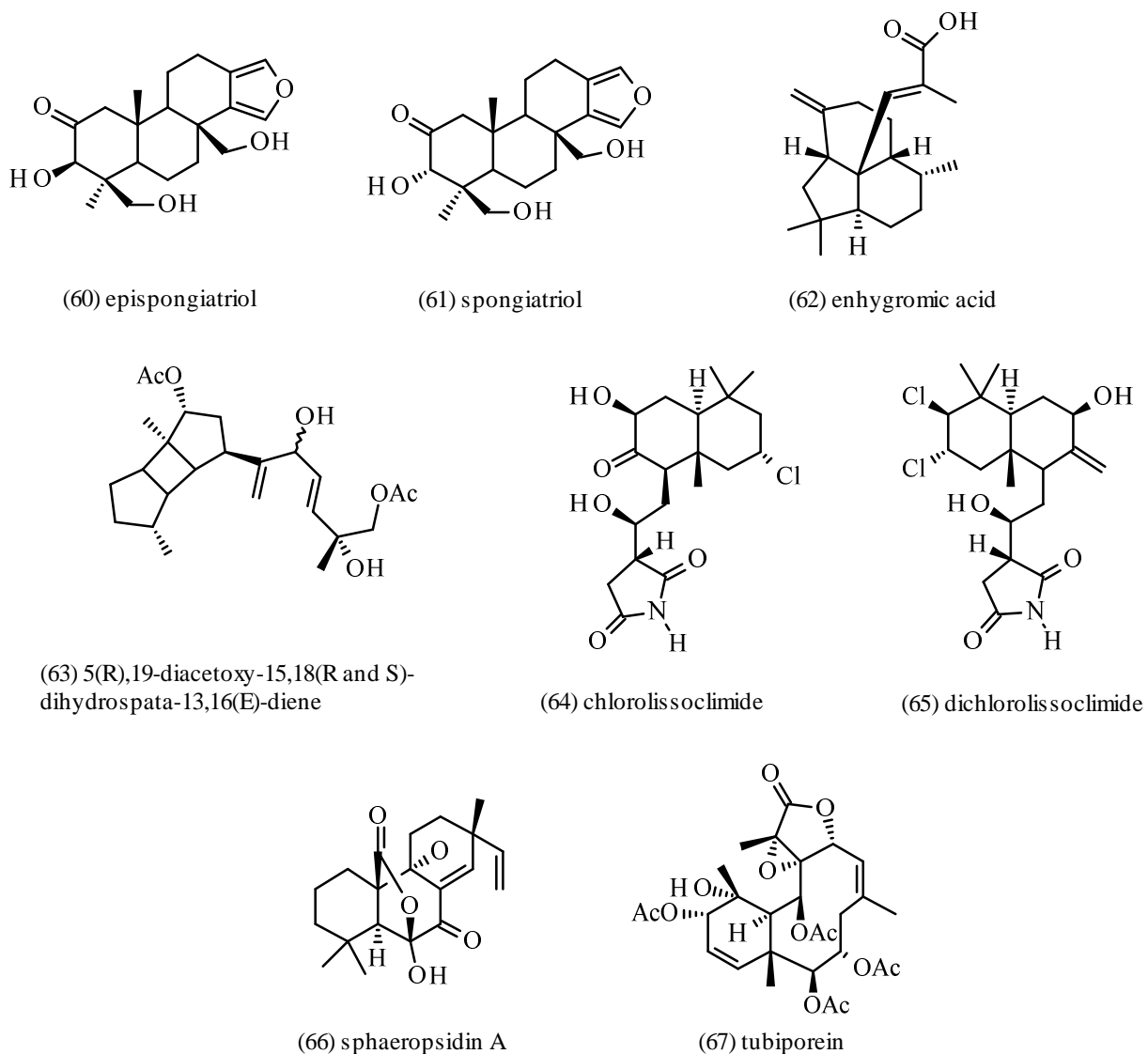

(66) sphaeropsidin A

(67) tubiporein

Fig 8. Other miscellaneous diterpenes from marine organisms and fungi with activity on melanoma cells.

6   COMBINATION THERAPIES

The management of patients with metastatic malignant melanoma remains difficult. Conventional chemotherapy has been disappointingly ineffective. Early work in going beyond cytotoxic monotherapy saw taxanes combined with DTIC, temozolomide, cisplatin, carboplatin and tamoxifen. The results are suggesting that they are at least as effective as various other combination regimens [93].

The combination of recombinant human fibroblast interferon (INF-δ) and mezerein (**68**) (itself a PKC activator) results in a synergistic antiproliferative effect on human melanoma cells accompanied by an increase in melanin synthesis [94]. Mezerein was first described as a major toxic principle of *Daphne mezereum* L. (Thymelaeaceae) [95].

One molecular target for this activity is human gamma interferon (IFN-γ) binding and Class I HLA and melanoma-associated antigen (MAA) expression in the HO-1 human melanoma cell line. Studies using HO-1 demonstrated the superiority of the combination Mezerein + IFN-δ. This synergistic combination also increased the expression of HLA Class I antigens but decreased the expression of the high molecular weight-melanoma associated antigen (HMW-MAA) [94].

In vitro combinations of sphaeropsidin A (Sph A) (**66**) with either cisplatin or temozolomide, showed that combining 4 μM Sph A with 75 μM cisplatin for 72 h had a synergistic cytotoxic effect independent of BRAF or NRAS mutations [96].

Tanshinone (**69**) isolated from *Radix Astragali* acts synergically with carboplatin to inhibit B16 cell growth after excessive UV radiation by inducing apoptosis [97].

A combination therapy that uses both triptolide (**04**) and VNP20009 (an antitumour factor from *Salmonella*) significantly improved the tumour colonisation of VNP20009 by reducing both the number of infiltrated neutrophils and the expression of VEGF synergistically, thus retarding the growth of the melanoma [98].
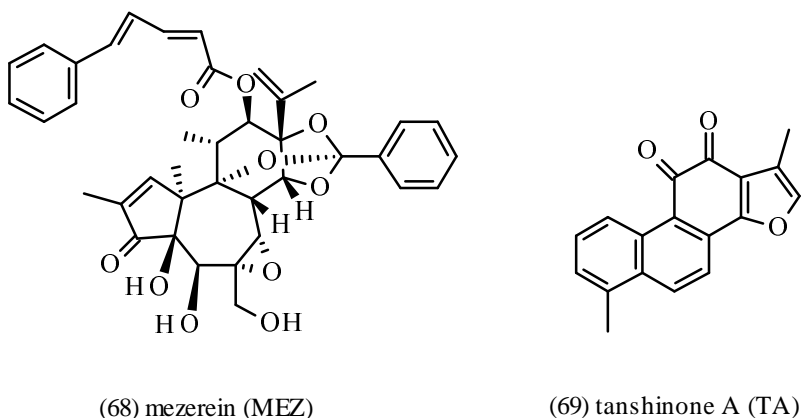

(68) mezerein (MEZ)   (69) tanshinone A (TA)

Fig 9. Examples of diterpenes can act synergistically with drugs used in melanoma therapy

7 FUTURE DIRECTIONS

The commercialisation of Paclitaxel (Taxol®) and docetaxel (Taxotere®) did not discourage researchers to continue designing and synthesising new taxane analogues, and some 9-beta-dihydrobaccatin-9,10-acetals exhibited potent antitumor effects against B16 melanoma BL6 *in vivo* by both iv and p.o. administration [99]. Similarly, the synthesis of derivatives from the above reviewed natural diterpenes is a logical 'next step' which is actively pursued by a number of groups working on the ingenol skeleton [100,101], leelamine [102], andrographolide [103], the cembranolides from the soft coral *Sarcophyton glaucum* [78–84]. This approach depends on a reliable source for the core skeleton, and diterpenes are notoriously minor compounds in most cases. Cell culture in bioreactors may afford a more reliable and sustainable supply such in the case of the bioactive abietane diterpenes from *Salvia sclarea* (Lamiaceae) which synthesis *in vitro* can be by elicited transcriptional reprogramming of their hairy roots [106]. Total synthesis is always complicated and usually the last resort but nevertheless possible and always with the advantage of giving a total control on the quality, purity and supply of the drug. Earlier developments tapped into close precursors in high quantities such as communic acid (**70**) –up to 50% w/w in the cones of some conifers- to synthesise podolactone-type diterpenes. In this manner, Barrero and co-workers obtained some bioactive derivatives with one-digit micromolar to submicromolar range IC$_{50}$s (LL-Z1271α, **71**) [107,108].

However, modern combinatorial metabolic engineering of microbial or plant hosts, using the enzymes of the biosynthetic pathways, offers an alternative environmentally benign access to structurally complex diterpenoids with specific and novel stereochemistry, which defines their therapeutic value. Using such approach, the group of Prof Hamberger achieved the stereoselective biosynthesis of over 50 diterpene skeletons, including natural variants and new enantiomeric or diastereomeric counterparts and demonstrated its scalable biotechnological production in engineered strains of *Saccharomyces cerevisiae* [109].
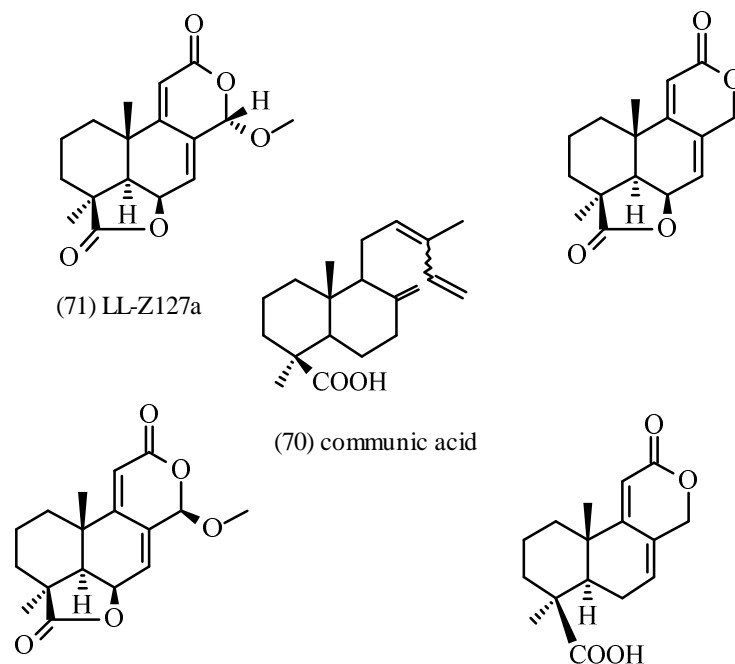
(71) LL-Z127a

(70) communic acid

Fig. 10. Podolactone-type diterpenes derived from communic acids

8   CONCLUDING REMARKS

Diterpenoids can still be a source of lead compounds for future treatments either preventative or curative in melanoma. Given the enormous diversity of this class of compounds, and that much of them are still to be discovered in marine organisms or fungi, the availability of data seems scarce. Moreover, not all reports on diterpenoids show activity against melanoma, for example, some jatrophane diterpenoid esters [110] and even– a powerful and well-established DNA polymerase inhibitor - and a series of semisynthetic derivatives were not of significant activity on melanoma cells [111]. There is a need to understand the structure-activity relationship when more expedite procedures for the synthesis of these molecules become mainstream as reviewed in the previous section.

Still what we already know shows great potential. The most studied compounds are the labdanes which have given several potential blockbusters: forskolin, triptolide carnosic acid, carnosol and andrographolide have both potentials for industrial sourcing and a well-known favourable safety profiles. It is andrographolide which is well ahead of the race with several developers putting forward patented derivatives for serious clinical development. The long time necessary for the development and approval of anticancer compounds makes any forecast difficult, but the case of ingenol is an enormous encouragement for researchers in the field. There is still room for natural products to beat purely synthetic drugs providing new mechanisms of action and better treatments. They are just waiting for us out there.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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