When cholesterol meets histamine, it gives rise to dendrogenin A: a tumour suppressor metabolite1

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When cholesterol meets histamine, it gives rise to dendrogenin A: a tumour suppressor metabolite[1]

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Abstract

Dendrogenin A (DDA) is the first steroidal alkaloid (SA) to be identified in human tissues to date and arises from the stereoselective enzymatic conjugation of 5,6α-epoxycholesterol (5,6α-EC) with histamine (HA). DDA induces the re-differentiation of cancer cells *in vitro* and *in vivo* and prevents breast cancer (BC) and melanoma development in mice, evidencing its protective role against oncogenesis. In addition, DDA production is lower in BCs compared with normal tissues, suggesting a deregulation of its biosynthesis during carcinogenesis. The discovery of DDA reveals the existence of a new metabolic pathway in mammals which lies at the crossroads of cholesterol and HA metabolism and which leads to the production of this metabolic tumour suppressor.

Introduction

Cholesterol is a crucial component of cell membranes. It plays an important role in the organization of the lipid bilayers and is essential for membrane biogenesis and cell proliferation. In addition to these roles, cholesterol is subject to an active metabolism that leads to the production of different structural classes of sterol lipids, such as steroid hormones, bile acids, sterol conjugates and oxysterols. It was initially thought that bile acids, oxysterols and sterol conjugates represented various ways of storing or eliminating cholesterol to preserve cholesterol homoeostasis, however more recent genetic and functional genomic studies now strongly suggest that they play physiological functions [1,2]. This has led to the identification of sterol metabolites that display important functions in the immune system [3–5], in the central nervous system [6,7] and in pathologies such as cancer [8,9].

Cancers are among the leading causes of death in the world and many aspects of their diagnoses and treatment are global unmet medical needs. Great progress has been made in our understanding of these diseases, which has in turn significantly improved their management, particularly through the development of targeted therapies and the emergence of personalized medicine. One of the first targeted therapies was the hormonotherapy of breast cancers (BCs) expressing the oestrogen receptor with the drug tamoxifen (Tam; Figure 1A). V. Craig Jordan first demonstrated that Tam blocks the mitogenic effects of 17β-oestradiol and protects rodents against the development of oestrogen receptor-positive BCs ($ER^{(+)}$ BC) [10]. This led to the clinical development of Tam for the treatment and chemoprevention of $ER^{(+)}$ BC [11]. It was later found that Tam displays a more complex pharmacology than expected [12,13] and an additional molecular target called the antioestrogen-binding site (AEBS) was identified [14]. This was shown to contribute to the anticancer pharmacology of Tam thanks to the development of selective AEBS ligands with no affinity for the ER such as PBPE and DPPE (Figure 1A) [12,14–32]. These observations paved the way for the identification of new parameters in cholesterol biosynthesis and the metabolism of cholesterol-5,6-epoxides that are involved in the control of sensitivity and resistance to Tam [18]. One of the most exciting outcomes of these studies was the discovery of dendrogenin A (DDA; Figure 1B), a cholesterol metabolite with tumour suppressing properties whose production is impaired during oncogenesis [8,9,33]. The discovery of DDA has opened up new promising opportunities for cancer treatments and new routes to understand the aetiology of cancers.

Dendrogenins: products of the conjugation of 5,6α-epoxycholesterol and amines with pharmacological properties

Pharmacogenomic studies were used to identify the AEBS as a hetero-oligomeric complex made up of two subunits

Key words: cancer, cholesterol, cholesterol-5,6-epoxide hydrolase, dendrogenin, steroidal alkaloid, tamoxifen.

Abbreviations: AEBS, microsomal antioestrogen-binding site; BC, breast cancer; 5,6α-EC, 5,6α-epoxycholesterol; 5,6β-EC, 5,6β-epoxycholesterol; ChEH, cholesterol-5,6-epoxide hydrolase; CT, cholestane-3β,5α,6β-triol; D8D7I, EBP, 3β-hydroxysteroid-Δ^8,Δ^7-isomerase; DDA, dendrogenin A; DDAs, dendrogenin A synthase; DDB, dendrogenin B; DHCR7, 3β-hydroxysteroid-Δ^7-reductase; DPPE, tesmilifene; PBPE, 1-[2-[4-(phenylmethyl)phenoxy]ethyl]-Pyrrolidine; SA, steroidal alkaloid; tamoxifen, Tam.

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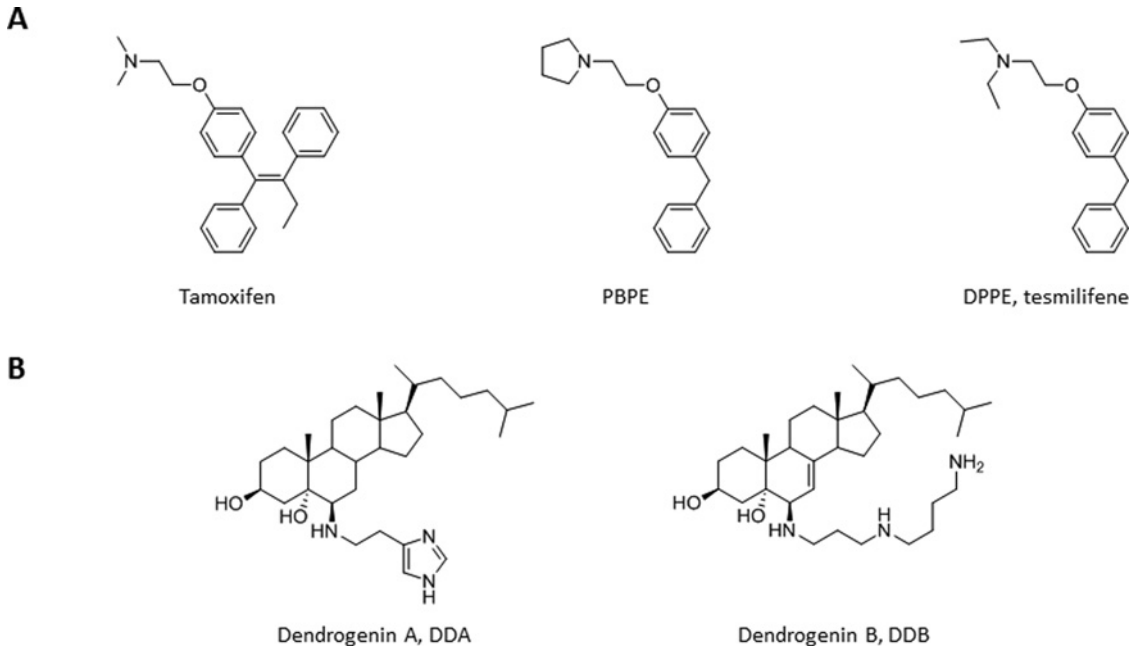
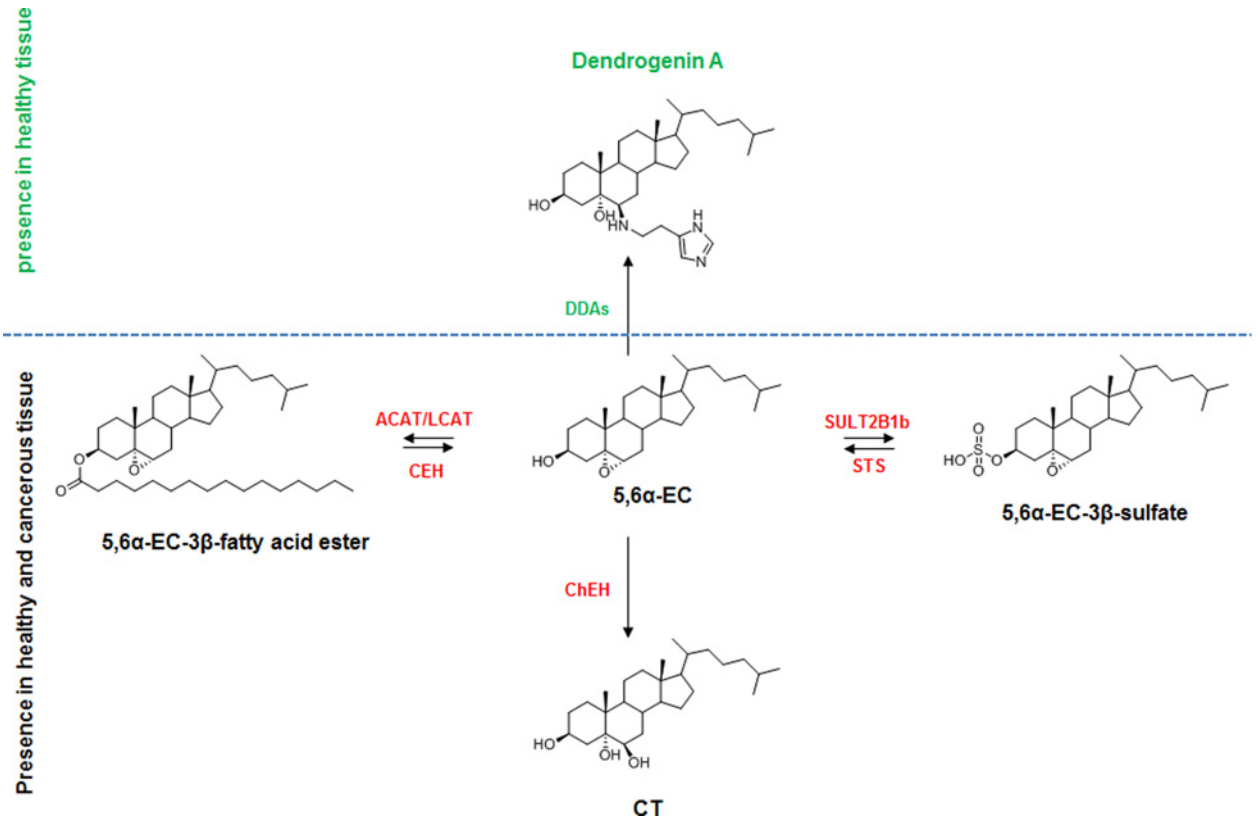
Figure 1 | **Chemical structure of compounds**
(**A**) Tam, PBPE and DPPE; (**B**) DDA and DDB.



involved in cholesterol biosynthesis: the 3β-hydroxysteroid-Δ^8,Δ^7-isomerase (D8D7I, also called EBP) and the 3β-hydroxysteroid-Δ^7-reductase (DHCR7) [14]. It was later found that these subunits catalysed the enzymatic activity of cholesterol-5,6-epoxide hydrolase (ChEH) and that D8D7I was the catalytic subunit of ChEH and DHCR7 the regulatory subunit [20]. ChEH catalyses the hydration of 5,6-epoxycholesterols (5,6-ECs) to give cholestane-3β,5α,6β-triol (CT; Figure 2) [17]. Tam and its analogues are potent inhibitors of ChEH and their treatment leads to the accumulation of 5,6-ECs in cells [16]. 5,6-ECs exist as two diastereoisomers: 5,6α-EC and 5,6β-EC [34]. Interestingly, 5,6α-EC was shown to mediate the BC cell re-differentiation induced by Tam [16]. Substances bearing epoxide are often prone to spontaneous reaction with nucleophiles, however 5,6-EC are exceptions and remain unreactive at ambient temperatures due to the localization of the epoxide ring on the steroid backbone [34,35]. Under catalytic conditions, 5,6α-EC can react stereoselectively with nucleophiles such as ethanolamine or mercaptoethanol to give a single conjugation product [34,35]. The identification of this stereoselective transformation was important in the discovery of DDA as it provided scientists with a new possible metabolic pathway centred on 5,6α-EC transformation [34]. Interestingly, conjugation of 5,6α-EC with histamine (HA) and polyamines has also been reported [36]. For each amine, a single product was obtained [8,36] that resulted from the *trans* diaxial ring opening of the epoxide by the primary amine at C6 (Figure 2). Since D8D7I and DHCR7, the two subunits of the ChEH, had been reported to be able to control mammalian developmental programmes [37], it was postulated that 5,6-EC metabolism could be involved in organogenesis and cell differentiation [8]. To test this hypothesis, the putative metabolites described above were tested *in vitro* to determine if they could induce cell differentiation. Cancer cells were first used because they are dedifferentiated cells and some have been shown to undergo re-differentiation following specific treatment [8,36]. *In vitro* tests showed that, among the different molecules tested, 5α-hydroxy-6β-[2-(1*H*-imidazol-4-yl)-ethylamino]-cholestan-3β-ol (called DDA; Figure 1B) induced tumour cell re-differentiation at nanomolar concentrations in cancer cells of different tissue origins [36]. The effects of DDA on the human BC cell line MCF-7 and the human melanoma cell line SKMEL-28 were then studied. Treatment of MCF-7 cells with DDA triggered the arrest of cells in the G_0–G_1-phase of the cell cycle. DDA modified cell morphology and cells became flattened with increased granulosity. Oil red O staining of cells revealed the accumulation of vesicles containing neutral lipids. Biochemical analysis showed that these neutral lipids were triacylglycerides (TG; Figure 3A). Additionally, DDA induced the expression of milk fat globulin, one of the major proteins found in milk [8,9,36]. Thus, together these data showed that DDA induced MCF-7 cell differentiation and activated the lactation function of the original breast epithelial cells from lactating ducts. However, perhaps the most exciting observation was that DDA appeared to be at least five times more efficient at inducing MCF-7 cell re-differentiation compared with a range of other compounds, including Tam. When tested on SKMEL-28 cells, DDA induced morphological changes, from spindle to dendritic morphology. Ultrastructure analysis of cells by electron

Figure 2 | **Primary metabolism of 5,6α-EC in healthy and cancerous tissues**

DDA was only detected in healthy tissues whereas 5,6α-EC, 5,6α-EC-fatty acid esters, 5,6α-EC-3β-sulfate and CT are detected in both healthy and cancerous tissues. 5,6α-EC can be metabolized into: (1) CT by the ChEH, (2) 5,6α-EC-fatty acid esters by the acylcoA:cholesterol acyltransferases (ACAT) and lecithin-cholesterol acyltransferase (LCAT) and the reverse reaction is catalysed by the cholesteryl ester hydrolases (CEH); (3) 5,6α-EC-3β-sulfate by a sulfotransferase (Sult2B1b) and the reverse reaction is catalysed by a steroid sulfatase (STS), (4) into DDA by a DDAs.



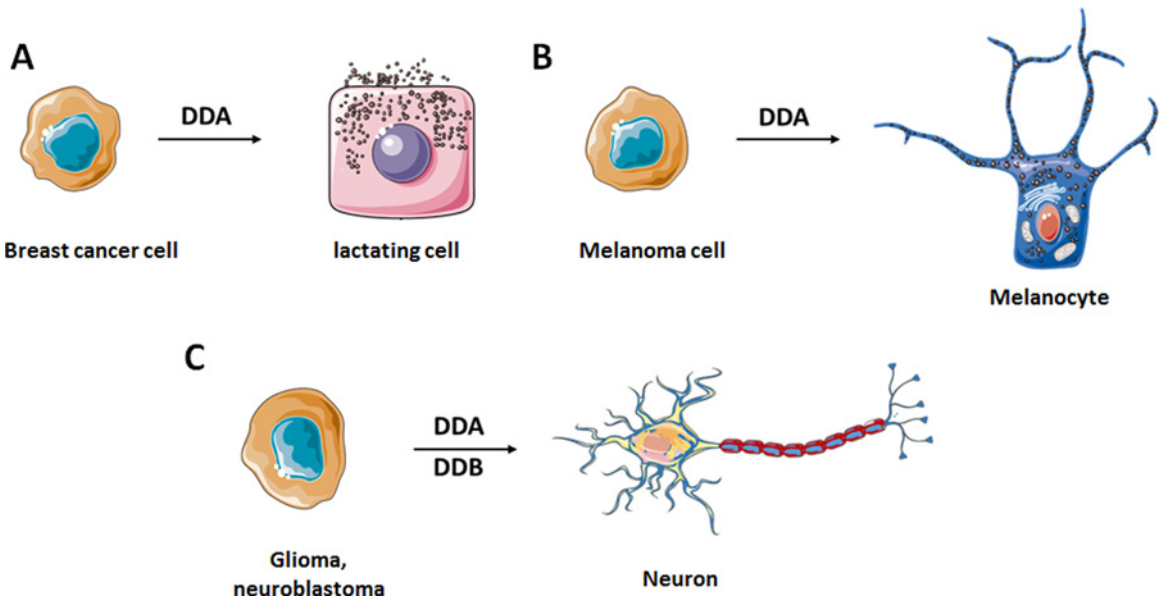
microscopy revealed the presence of melanosomes on the dendritic extensions of cells. Biochemical analysis revealed that DDA re-activated melanogenesis in these cells. Cell cycle analyses also showed that DDA had arrested cells in the G_0–G_1-phase of the cell cycle. Since these are the main characteristics observed in normal melanocytes, this provided evidence that DDA can also stimulate melanoma cell re-differentiation (Figure 3B). When tested on cancer cells of neuronal origin, DDA was found to stimulate neurite outgrowth. Interestingly, DDA also induced the selective differentiation of pluripotent carcinoembryonic P19 cells into neurons. Altogether, these data showed that DDA triggers the re-differentiation of cancer cells of different tissue origins. Substituting HA with spermine or spermidine on these 5,6α-EC derivatives (Figure 3C) also yielded compounds that displayed neuro-differentiation and neurotrophic properties and the most potent compound of this series (called dendrogenin B, DDB; Figure 1B) was found to be not cytotoxic up to 1 mM [36,38,39].

In contrast, it was observed that longer exposures or higher concentrations of DDA triggered cytotoxicity. DDA was found to induce a dose-dependent cytotoxicity in all cell lines tested, with IC_{50} values after a 72 h treatment ranging from 0.4 μM in an intestinal carcinoma cell line (SW620) to 5.5 μM in an acute myeloid leukaemia cell line (KG1) that is highly resistant to chemotherapy Interestingly, both human and mouse cell lines representative of different cancers such as leukaemia (U937, NB4, KG1), colorectal cancer (HCT-8, SW-620), neuroblastoma (SK-N-SH, SH-SY5Y, Neuro2A), glioma (U87), lung carcinoma (A549), melanoma (SKMEL-28, B16F10) and BC (MCF-7, TSA^{H2d}) were all found to be highly sensitive to DDA [36].

DDA has also been studied *in vivo* on tumours implanted into normal immunocompetent mice. In the first set of experiments TSA^{H2d} cells were implanted into the mammary fat pad of BALB/c mice. TSA^{H2d} cells are breast adenocarcinoma cells ($ER^{(+)}$) obtained from BALB/c mice and are syngeneic for this strain of mouse [9]. In a chemopreventive setting, DDA was injected at the same time as the tumour cells were implanted. Mice were treated every day over a 30 day period by subcutaneous peri-tumoral injection of DDA. DDA was found to be very effective

Figure 3 | **Effect of dendrogenins on cancer cells**

DDA triggers breast (**A**) and melanoma cell (**B**) differentiation. DDA and DDB trigger the neuronal differentiation of neuroblastoma (**C**).



at controlling tumour development and, importantly, on improving mouse survival. Dose escalation was limited due to drug solubility at that time, but a strong efficacy of DDA was observed even at extremely low doses (3.7 μg/kg). Similar effects of Tam were seen at 5.6 mg/kg, which corresponded to a 1500-fold greater concentration than that of the injected dose of DDA. Importantly, although Tam controlled tumour development, it did not improve mouse survival, in contrast with DDA. A similar efficacy was obtained for the treatment of established breast tumours implanted 10 days before DDA treatment (tumours detected by manual palpation), showing that DDA also displayed anticancer properties in BC cells [9]. To determine whether DDA could control the development of other types of tumours *in vivo*, DDA was also tested on metastatic melanoma, one of the most aggressive types of cancer in humans. The B16F10 metastatic melanoma cell line was implanted into immunocompetent syngeneic C57/BL6 mice and it was found that DDA was extremely efficient at delaying tumour development and improving mouse survival. Evaluation of this second tumour model showed that DDA was extremely efficient even at low doses, evidencing DDA as a good candidate for melanoma treatment and prevention. Analyses of the phenotypic characteristics of BC and melanoma tumours from DDA-treated animals showed that DDA induced the same differentiation characteristics *in vivo* as those observed *in vitro*. Additionally, the use of immunocompetent mice made it possible to check tumour infiltration by immune cells after DDA treatment. It was found that DDA strongly stimulated T lymphocytes and dendritic cells infiltration, suggesting that the immune system contributes to the anticancer activities of DDA. Additionally, as shown for BC tumours, DDA was found to be very efficient in controlling the growth of established B16F10 tumours [9].

Together these data suggest that DDA could be an interesting anticancer drug candidate to develop for clinical evaluation. Since DDA induces cell differentiation and death in human cancer cell lines of different origins *in vitro*, it will be of interest in future studies to determine the anticancer potency of DDA in pre-clinical models relevant to human cancers.

Dendrogenin A is a mammalian metabolite

The observation that DDA displayed very potent properties at nanomolar concentrations suggested that it could exist as a cholesterol metabolite in mammalian tissues. It was found that synthetic DDA displayed a specific MS/MS fragmentation profile that could distinguish it from its regioisomer in which HA is grafted by its imidazole ring. A deuterated form of DDA was chemically synthesized from commercial deuterated cholesterol and was used for isotope dilution quantification. Analysis of mouse brain extracts then established that DDA existed as a metabolite whose concentration in brain tissue was estimated at 46 ng/g [9]. It was also detected in the liver, blood, spleen and lungs and was found not only in murine but also in human and bovine tissues with a concentration range that fluctuated from 3 nM (human plasma) to 500 nM (mouse liver) [9]. This suggested that DDA was common to mammals and therefore that other mammalian tissues may also contain DDA. However, the presence of DDA in a given tissue does not mean that DDA was produced by that tissue itself. DDA could have been ingested or produced by a specific tissue and

targeted to different tissues through the blood circulation. Further experiments found that DDA was synthesized in mouse brain extracts in the presence of 5,6α-EC and HA in a saturable manner following a Michaelis–Menten kinetic. DDA neosynthesis was strictly dependent on the presence of tissue extract and was abolished by heat and proteinase K treatment of the tissue extract [9]. This established that DDA is produced in mice by an as-yet unidentified enzyme. DDA is therefore the first steroidal alkaloid (SA) identified to date in mammals. Other SA derivatives of cholesterol have been identified in plants (tomatidine) and fishes (squalamine). Interestingly, the functions that have been attributed to these cholesterol derivatives have been in the protection of the organisms against external attacks [40,41].

In human cells, DDA has now been found to be present in normal mammary epithelial cells (8.2 ng/mg protein) and normal melanocytes (5.6 ng/mg protein) grown *in vitro*. However, DDA could not detected in BC cell lines or melanoma cell lines representative of different melanoma and BC subtypes, suggesting a deregulation of DDA metabolism in breast and skin cancers. DDA was also undetectable in tumour cell lines from different types of cancers including leukaemia, glioma, neuroblastoma, colon, intestinal and lung cancers. When analysing DDA content in tumour biopsies from patients with BC compared with normal matched tissues it was found that while DDA was present in normal breast tissue, its concentration drastically decreased in tumours (from ≈140 to ≈30 nM) [9]. The primary metabolism of 5,6α-EC is schematized in Figure 2 showing that DDA was its only metabolite found in healthy tissues whereas others can be produced both in healthy and cancerous tissues and cells [34]. These data established that DDA production is decreased in cancers, suggesting a deregulation of DDA biosynthesis during carcinogenesis, something which deserves further studies.

Natural compounds extracted from plants, microorganisms and marine organisms are the main source of natural chemopreventive and anticancer agents, and are usually identified through pharmacological screens [42]. Here we describe the identification of DDA as the first SA found to date in mammals using a rational approach. A combination of pharmacogenomic and chemical reactivity data was used to synthesize the candidate chemical, to validate its pharmacological properties and to produce specific tools that made it possible to identify DDA as a metabolite that is present in and produced by mammalian tissues. So far, the human metabolome has not been fully explored in order to identify whether pharmocologically active metabolites against degenerative diseases exist. The discovery of DDA has now opened up new and very exciting fields of research and defining its mechanism of action in the induction of cell differentiation and death remains a major project. The identification of the DDA synthase (DDAs) enzyme is also a major challenge since no similar enzymatic conjugation of epoxides with HA or other biogenic amines has been reported to date. Scientists originally thought that 5,6-EC were produced only artefactually since they are known as autoxidation products of cholesterol [34], however the discovery of DDA has established that a metabolic pathway does indeed exist and is involved in the transformation of 5,6α-EC. Interestingly, 5,6α-EC is more than just an autoxidation product of cholesterol since an as-yet-unidentified cytochrome P450 enzyme has been reported to catalyse the stereoselective biosynthesis of 5,6α-EC in bovine adrenals [43]. These data strongly suggest that the $\Delta^{5,6}$ double bond of cholesterol is a template for its stereoselective transformation to produce compounds such as DDA. The tumour suppressive, chemopreventive and neuroprotective properties of DDA make this compound a very promising one for the complementation therapy of degenerative diseases in which its biosynthesis is altered.

Conflict of interest

Dendrogenins have been developed for clinical applications by the company AFFICHEM, of which Marc Poirot and Sandrine Silvente-Poirot are founders.

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