Triterpenoids as new promising anticancer drugs

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Triterpenoids are structurally diverse organic compounds, characterized by a basic backbone modified in multiple ways, allowing the formation of more than 20 000 naturally occurring triterpenoid varieties. Several triterpenoids, including ursolic and oleanolic acid, betulinic acid, celastrol, pristimerin, lupeol, and avicins possess antitumor and anti-inflammatory properties. To improve antitumor activity, some synthetic triterpenoid derivatives have been synthesized, including cyano-3,12-dioxooleana-1,9 (11)-dien-28-oic (CDDO), its methyl ester (CDDO-Me), and imidazolide (CDDO-Im) derivatives. Of these, CDDO, CDDO-Me, and betulinic acid have shown promising antitumor activities and are presently under evaluation in phase I studies. Triterpenoids are highly multifunctional and the antitumor activity of these compounds is measured by their ability to block nuclear factor-κB activation, induce apoptosis, inhibit signal transducer, and activate transcription and angiogenesis. *Anti-Cancer Drugs* 20:880–892 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Recent years have witnessed considerable advances in our understanding of cancer pathogenesis. It has now become evident that cancer development underlies a collection of multiple genetic abnormalities through a multistep, mutagenic process. As a consequence of these multiple genetic abnormalities and of many epigenetic modifications, cancer cells acquire a set of biological properties that include virtually unlimited proliferation potential, independent exogenous growth factors, and increased survival through resistance to antiapoptotic signals. Tumor cells also acquire the ability to establish a privilegiate relationship with the surrounding microenvironment, drawing nourishment from stromal cells, and attracting and promoting the formation of new blood vessels to transport nutrients and oxygen for tumor growth. Finally, tumor cells also acquire the ability to evade immune surveillance, and, ultimately, metastasize to distal organs.

Many of the phenotypic features of cancer cells are related to genetic alterations occurring as a consequence of gene mutations or epigenetic changes. These mutations can be subdivided into gain-of-function mutations, amplifications and/or overexpression of cellular oncogenes and loss-of-function mutations, and deletions and/or epigenetic silencing involving tumor suppressor genes. All these genetic alterations lead to multiple and complex changes at the level of cellular programs involved in the control of cell proliferation, differentiation, apoptosis, migration, and tissue homeostasis.

The complexity of the genetic lesions and the variation of the derivative phenotypic changes of cancer cells indicate that multifunctional drugs capable of affecting tumor cells at various levels must be used for the treatment of these diseases. In this study, we discuss a class of these agents, naturally occurring and synthetic triterpenoids, that are clearly effective in cancer prevention and treatment in various animal models and that are under evaluation as anticancer drugs and for other therapeutic properties. These molecules are typical examples of drugs exerting antitumor activity through effects on various cellular regulatory and metabolic networks.

Chemical structure and main pharmaceutical properties of triterpenoids

Triterpenoids are metabolites of isopentenyl pyrophosphate oligomers and comprise the largest group of plant natural products, with over 20 000 known members. Triterpenoids are synthesized from isopentenyl pyrophosphate through the 30-carbon intermediate squalene, and include protostanes, lanostanes, holostanes, cycloartanes, dammaranes, euphanes, tirucallanes, tetranortriterpenoids, quassinoids, lupanes, oleananes, friedelanes, ursanes, hopanes, isomalabaricanes, and saponins. Triterpenoid carbon frameworks are cyclized by members of the oxidosqualene cyclase family, which has expanded greatly in plants. Oxidosqualene cyclases convert oxidosqualene to one or more cyclic triterpene alcohols with up to six carbocyclic rings [1]. Saponins can be chemically categorized as comprising an aglycone linked to one or more sugar chains. There are two groups of saponins, one containing a steroidal aglycone and the other containing a triterpenoid aglycone. Squalene is considered the common precursor for biosynthesis of both steroid and triterpenoid systems [2]. Saponins are a large family

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of steroid or triterpenoid glycosides, widely distributed in plants and in some marine organisms, in which hydrophilic monosaccharides or oligosaccharides are attached to a hydrophobic sapogenin backbone. They have received considerable attention because of their diverse, promising biological and pharmaceutical properties, including antitumor, antiviral, antifungal, anti-inflammatory, and other activities. They have also recently been shown to show significant effects on plant growth [3].

Oleanolic acid (OA, 3/3-hydroxy-olea-12-en-28oic acid) and its isomer, ursolic acid (38-hydroxy-urs-12-en-28oic acid), are triterpenoid compounds that exist widely in natural plants in the form of free acid or aglycones for triterpenoid saponins [2].

OA and ursolic acid are weakly anti-inflammatory and anti-tumorigenic *in vivo* [4]. Synthetic oleanane triterpenoids are the most potent anti-inflammatory and anti-carcinogenic triterpenoids known. The anti-inflammatory activity of these compounds is measured by their ability to block the de-novo synthesis of inducible nitric oxide synthase (iNOS) in primary macrophages stimulated with interferon-γ (IFNγ) [4]. With regard to its antitumor activity, the cyano-3,12-dioxooleana-1,9 (11)-dien-28-oic (CDDO) is the most studied synthetic oleanane [4]. CDDO and its derivatives were originally developed as inhibitors of nitric oxide production in macrophages and as antiproliferative agents for tumor cells. C-28 methyl ester (CDDO-Me) and C-28 imidazolide (CDDO-Im) derivatives of CDDO have shown an improvement in the antitumor activities for various tumor types compared with CDDO, with IC_{50s} in the nanomolar range. At the same time, very few, if any, side effects were detected at clinically relevant concentrations of CDDO-Me and CDDO-Im. Other C-28 amide derivatives have also been synthesized; new compounds studied here include CDDO-ethyl amide (CDDO-EA), CDDO-diethyl amide (CDDO-DE), CDDO-trifluoroethyl amide (CDDO-TFEA) [5], and CDDO-methyl amide (CDDO-MA) [6]. CDDO-Me is a methyl ester derivative of CDDO, and this compound is used in phase I clinical trials for the treatment of leukemia, solid tumors, and other non-neoplastic diseases. As a group, these synthetic oleanane triterpenoids are potent multifunctional molecules in cell culture assays; depending on the dose, they can suppress inflammation, activate cytoprotective pathways, induce differentiation, inhibit proliferation, and induce apoptosis [4].

During the development of CDDO, very important structure–activity relationships, showing that a 2-cyano-1-en-3-one in ring A and a 9(11)-en-12-one in ring C are essential for the high potency of CDDO, were found. The entire oleanane skeleton might not be necessary for potency. Consequently, a tricyclic compound [tricyclic-bis-enone (TBE) compound] was designed which has the same A, B, and C rings as CDDO. TBE compounds with various functionalities at different positions can be obtained. Such 'diversity-oriented synthesis' could lead to new potential anti-inflammatory and cancer chemopreventive drugs that have high oral potency and high water solubility for ease of administration and formulation, and high biological selectivity for removal of possible side effects, and would be inexpensive for large-scale synthesis. Therefore, the synthesis of optically active TBE compounds is very important for a comparison of the biological potency of both enantiomers [7,8]. Moreover, TBEs are entirely synthetic and are thus not hindered by limited functional groups or by steric obstruction, and these new compounds may have more structural diversity, better stability, and enhanced pharmacokinetic and pharmacodynamic profiles compared with oleanane triterpenoids. The acetylenic tricyclic bis-(cyano enone) TBE-31 [(F)-(4aa,8aa,10ah)-1,2,4a,6,8a,9,10,10a-octahydro-8a-ethynyl-1,1,4a-trimethyl-2, 6-dioxophenanthrene-3, 7-dicarbonitrile], synthesized from cyclohexanone as a starting material, is much more active than CDDO. TBE-31 potently inhibits iNOS, activates the phase 2 response, and induces differentiation and apoptosis of cancer cells. TBE-31 is also orally available and is exceptionally effective at blocking both the formation of aflatoxin-B1–DNA adducts and aflatoxin-B1-induced tumorigenesis *in vivo*; moreover, it has better pharmacokinetic and/or pharmacodynamic properties than many pentacyclic oleanane triterpenoids [9].

Maslinic acid (2-alpha,3-beta-dihydroxyolean-12-en-28-oic acid) is a triterpenoid compound present in plants such as *Olea europaea*, as is OA. It is known for its significant effects as an antioxidant, anti-inflammatory, and antimicrobial, as well as its antiviral activities [10]. Maslinic acid also has potent differentiating and anti-proliferation properties [11,12].

Lupeol, betulin, and betulinic acid are natural pentacyclic triterpenes of the lupane-type saponins that can be found in the external bark of various *Betula* spp., such as *B. papyrifera*, and have recently been investigated for their various pharmacological and medicinal properties [13]. Lupeol (Lup-20(29)-en-3h-ol) was found in fruits, in many vegetables (e.g. olive, mango, and fig), and in several medicinal plants, and possesses strong antioxidant, anti-inflammatory, antiarthritic, antimutagenic, and antimalarial activity in in-vitro and in-vivo systems. It also acts as a potent inhibitor of protein kinases and serine proteases, and inhibits the activity of DNA topoisomerase II, a target for anticancer chemotherapy. Lupeol inhibits nuclear factor (NF)-κB signaling, including phosphorylation of IkBα protein, DNA binding of NF-κB complex, and NF-κB-dependent reporter gene activity. It has also been shown that lupeol induces differentiation and inhibits the cell growth of mouse melanoma and human leukemia cells. Recently, it was shown in a two-stage model of mouse skin carcinogenesis that lupeol exhibits significant antitumor-promoting activity [14].

Betulinic acid is a derivative of betulin with anti-inflammatory, anti-HIV, and antineoplastic activities. On account of its selective antimelanoma activity and its favorable therapeutic index, betulinic acid is currently undergoing clinical trials at the National Cancer Institute. Nevertheless, the medical uses of betulinic acid and its derivatives in the pharmaceutical industry are limited because of their poor hydrosolubility and pharmacokinetic properties (absorption, distribution, metabolism, and elimination). To overcome this major problem, some laboratories have undertaken the synthesis of more water-soluble betulinic acid derivatives. For example, it was reported that the introduction of polar moieties at the C-3 and C-28 positions, such as amino acids and phthalates, enhances, in certain cases, the hydrosolubility and anticancer activity of betulin and betulinic acid [15].

Celastrol, also known as tripterine, a quinone methide triterpenoid, isolated from the Chinese Thunder of God Vine [16], is currently being investigated for its antioxidant and anti-inflammatory properties. Celastrol was shown to inhibit the proliferation of a variety of tumor cells, including those from leukemia [17], gliomas [18], and prostate cancer [19]. Celastrol was also shown to inhibit tumor necrosis factor (TNF)-induced tumor cell invasion [20], in addition to both constitutive and inducible NF-κB activation [21]. Furthermore, celastrol acts as an inhibitor of heat and shock response activation [22], and as a proteasome inhibitor (it inhibits the chymotrypsin-like activity of a purified 20S proteasome and cancer cell 26S proteasome) [19].

Using analogs of celastrol, it was possible to define the chemical determinants of this molecule responsible for its antitumor activity. The celastrol methylester (pristimerin) was equipotent or more potent than celastrol in inducing apoptosis of a tumor cell; dihydrocelastrol, a reduced variant of celastrol that lacks the quinone methide moiety of the parent molecule, is inactive in inducing apoptosis of tumor cells, thus suggesting that the quinone methide moiety in celastrol is crucial for apoptotic activity [23]. Modification of the carboxylic acid moiety in celastrol generated some compounds, Ca16 benzyl ester and CA19 isopropyl ester, exhibiting strong antitumor activity [23]. Using in-vivo melanoma tumor models, it was shown that both the parental compound celastrol and its derivative, CA19, are active in inhibiting primary and metastatic tumor growth, whereas the compound CA16 was less active [23].

Recently, the antitumor properties of the celastrol methylester pristimerin, a natural triterpenoid isolated from *Celastrus* and *Maytenus* spp., have been reported, showing that this compound was more potent than celastrol in inhibiting NF-κB and proteasome activity and in inducing myeloma cell apoptosis *in vitro* and *in vivo* [24].

Avicins are a family of triterpenoid saponins isolated from *Acacia victoriae* (Leguminosae). Avicins contain an acacic acid core with two acylic monoterpene units connected by a quinovose sugar [25] (Fig. 1). Avicin D and G possess two important properties as potential anticancer drugs: (i) they inhibit DNA binding of NF-κB complex and the expression of NF-κB-dependent genes [26] and (ii) they induce apoptosis of tumor cells through mitochondrial perturbation [25].

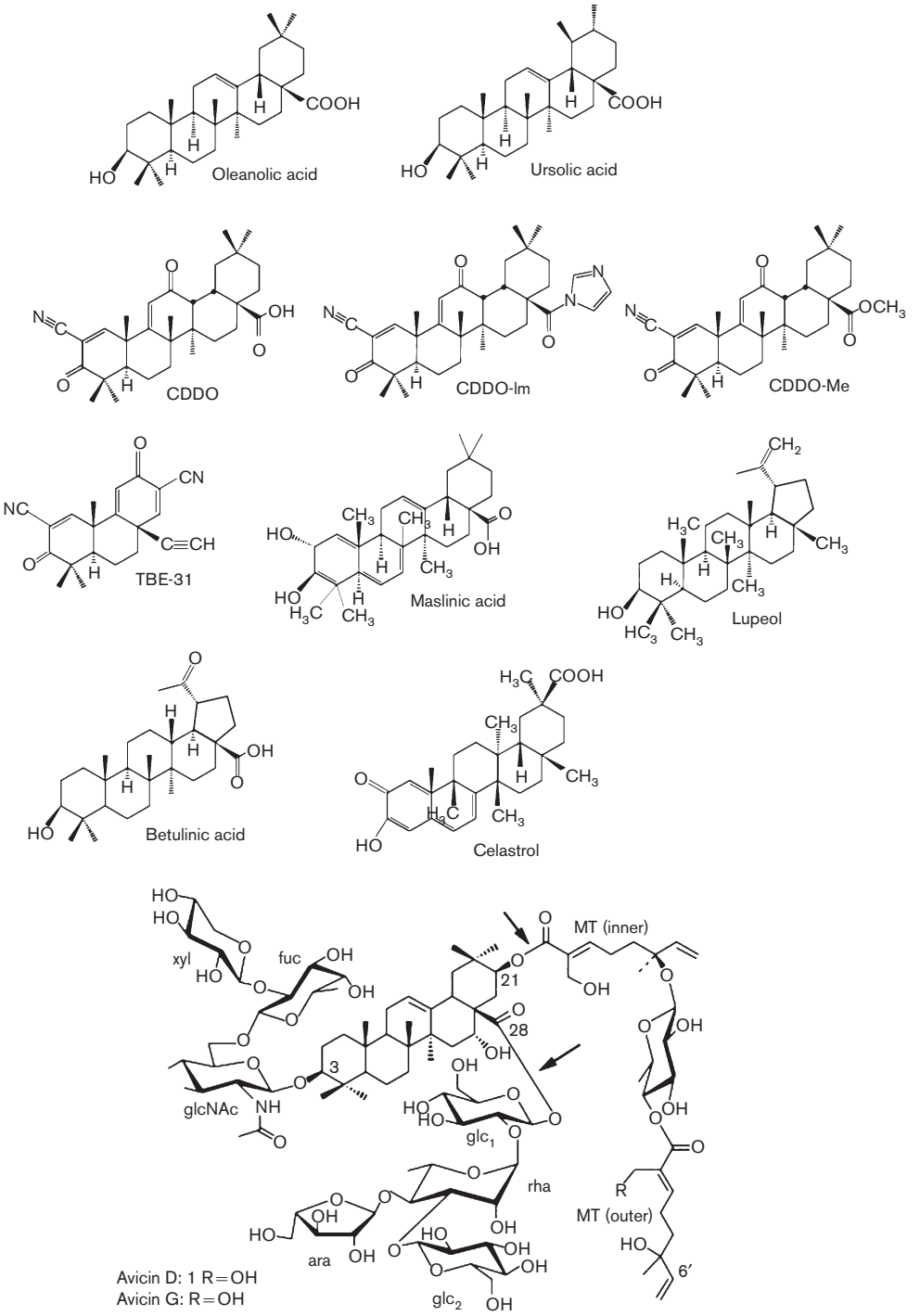
Growth inhibition

CDDO and its derivatives induce both growth inhibition and apoptosis of cancer cells, but the proapoptotic effects seems to be stronger than the effect on cell proliferation.

In many studies, the influence of CDDO and its derivatives on growth inhibition in different solid tumors [27–32] and leukemias was shown [33]. The antiproliferative effect exerted by CDDO-Me in malignant cells seems to be more potent than that induced by either CDDO-Im or CDDO. Moreover, CDDO-Im inhibits the proliferation more strongly than CDDO [34]. The mechanism of this action is independent of the p53 status [35], whereas recruitment of proteins involved in the cell cycle, such as cyclin D1, p21, p27, PCNA, caveolin1, and MYC, seems to be important [4]. However, the role of the nuclear receptor peroxisome proliferator-activated receptor-γ (PPARγ) in CDDO-induced growth arrest is controversial. In fact, CDDO, CDDO-Im, and CDDO-Me bind to and transactivate PPARγ, a transcription factor that controls key differentiation genes, and this activation may inhibit the proliferation of cancer cells by repressing cyclin D1 expression. In fact, in some studies, CDDO induces growth inhibition through PPARγ activation or only partly using this mechanism [27,35,36]. In contrast, other studies showed a PPARγ-independent mechanism [37,38], in fact, utilizing a PPARγ antagonist (GW9662), the receptor activity was inhibited, but not the antiproliferative effect of CDDO, thus showing that the actions of CDDO and CDDO-Im consist in blocking the cell growth independently of PPARγ activation [37].

Lupeol is able to inhibit the growth of tumor cells. In human prostate cancer cells, it was shown that lupeol blocks the cell cycle, inducing G_2/M-phase arrest, and this effect is mediated through the inhibition of the cyclin-regulated signaling pathway. Moreover, lupeol inhibited the expression of cyclin B, cdc25C, and plk1, but induced the expression of 14-3-3sigma genes. However, no changes were observed in the expression of gadd45, p21 (waf1/cip1), and cdc2 genes [39]. A significant arrest of melanoma cells in the G_0–G_1 phase of the cell cycle at 72 h posttreatment with lupeol was observed. The effect of lupeol treatment on the protein expression of the cyclin/cdk complex, which is operative in the G_1 phase of cell cycle and resulted in a dose-dependent decrease in the protein expression of cyclin D1 and cyclin D2 was also

Fig. 1



Chemical structure of the main triterpenoids studied for their antitumor properties.

investigated. The effect of lupeol was also observed on the protein level of cdk2. Treatment with lupeol resulted in a dose-dependent decrease in cdk2 [40]. The inhibition of cell growth was also observed in hepatocellular carcinoma cells [41] and in nude mice implanted with prostate cancer cells [14,42].

Betulinic acid shows an antiproliferative effect in all tested tumor cell cultures, including neuroblastoma, rabdomyosarcoma-medulloblastoma, glioma, thyroid, breast, lung and colon carcinoma, leukemia, and multiple myeloma (MM), as well as in primary cultures isolated from ovarian carcinoma, cervical carcinoma, and glioblastoma

multiforme [43]. Jurkat cells treated with betulinic acid showed an increase in G_0/G_1 phase and a decrease in S phase. The expression of cyclin D3, bcl-X_L mRNA, and protein sharply decreased in Jurkat cells treated with betulinic acid [44].

Moreover, betulinic acid analogs with improved anti-tumor properties were synthesized. Betulinic acid derivatives containing an added cyano-enone functionality (CBA, CBA-Me, and CBA-Im) are potent inhibitors of nitric oxide production in primary mouse macrophages stimulated with IFN-γ (with IC_{50} values of 1 nmol/l). The new betulinic acid derivatives inhibit the growth of Jurkat, U937 leukemia cells, RPMI 8226 myeloma cells, and MCF-7 breast cancer cells, and are markedly more potent than betulinic acid [45].

Apoptosis

CDDO, CDDO-Im, and CDDO-Me have been shown to induce apoptosis of a wide variety of cancer cells in culture [46]. Proapoptotic induction by CDDO was shown in human acute myeloid leukemia (AML), MM, chronic lymphocytic leukemia, lung cancer, breast cancer, prostate cancer, ovarian cancer, and osteosarcoma [46–54].

The extrinsic or death receptor pathway of apoptosis is activated by members of the tumor necrosis factor family of cytokines [e.g. TNF, Fas, and TNF-related apoptosis inducing ligand (TRAIL)]. TRAIL binds to the death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), triggering the recruitment of adaptor proteins that activate either caspase-8 or caspase-10. Active caspase-8 and caspase-10, in turn, cleave and activate downstream effector proteases, including caspase-3 and caspase-7, causing apoptosis [46,55]. Caspase-8 also cleaves Bid and thereby plays a role in the release of cytochrome *c* from mitochondria. The activation of the intrinsic or mitochondria-mediated apoptotic pathway, in response to diverse stimuli, is regulated by the induction of mitochondrial permeability transition (PT) and by the Bcl-2 family of proteins, resulting in cytochrome *c* release, activation of caspase-9 and cleavage of downstream caspases, such as caspase-3. Inhibition of these pathways results from the overexpression of antiapoptotic proteins, altered function of proapoptotic proteins, or defects in signaling pathways, which contribute to the development of the chemoresistant phenotype [55]. It was shown, in several studies, that CDDO, CDDO-Im, and CDDO-Me are able to increase TRAIL-dependent apoptosis through upregulation of the death receptors TRAIL-R1 and TRAIL-R2 [46,47,51–53,56,57]. These synthetic triterpenoids also induce FLICE-like inhibitory protein (FLIP) downregulation. FLIP is an antiapoptotic protein that, similar to procaspases 8 and 10, contains a tandem pair of death effector domains, but lacks a catalytically active protease domain, and thus can operate as a trans-dominant inhibitor of caspases 8 and 10. During death-inducing signaling complex formation, FLIP is preferentially recruited to the death receptor complex, where it binds FADD and thwarts activation of caspases 8 and 10 [52]. Therefore, CDDO and its derivatives, which cause an increase in the expression of DR4 and DR5 and a downregulation of c-FLIP, induce apoptosis through the extrinsic pathway. This proapoptotic mechanism induced by CDDO was shown in leukemia, lung, breast, prostate, and ovarian cancer cells [46,47,51–53,56].

These compounds also operate through the intrinsic pathway and caspase-independent pathways, as shown by experiments based on the use of caspase inhibitors, such as Z-DEVD-fmk and Z-IETD-fmk, inhibitors of caspase-3 and caspase-8, respectively, or general caspase inhibitors, such as BOC-D-fmk or Z-VAD-fmk; in fact, these inhibitors did not or only in part rescue loss of cell viability induced by CDDO-Me and CDDO-Im, suggesting the activation of additional caspase-independent mechanisms. In fact, in prostate cancer cells, caspase-8 and caspase-3 inhibitors prevent DNA fragmentation, but do not provide protection from CDDO-induced apoptosis [58]. Similar results were obtained in ovarian cancer cells, in which it was shown that z-VAD only partly provide protection from the apoptosis induced by CDDO-Im [53]. z-VAD also partly prevents, the decrease in $\Delta\Psi_m$ induced by CDDO-Im, showing that caspase-8 activation is required for CDDO-Im-induced loss of $\Delta\Psi_m$ [49,53]. In fact, caspase-8 leads to mitochondria permeabilization through cleavage of Bid and Bax. Taken together, these data suggest that CDDO and its derivatives induce apoptosis by disrupting mitochondria membrane and releasing citochrome *c*, but require caspase activation for outer membrane permeabilization. Conversely, the disruption of mitochondrial membrane determines, in addition to caspase activation, the release of reactive oxygen species (ROS). In leukemia, however, a caspase-independent induction of loss of mitochondrial membrane potential by CDDO-Me was observed. In fact, z-VAD was unable to prevent the mitochondrial permeabilization, which was Bax-independent [59].

Mitochondria are an important target of these synthetic triterpenoids, and this was hypothesized as a model of permeabilization of mitochondria induced by CDDO and its derivatives, dependent on the thiol status. This hypothesis is supported by the observation that CDDO induces loss of mitochondrial thiol status and the secondary modification of numerous mitochondrial protein thiols. These modifications result in the formation of high-molecular weight protein aggregates that form 'unregulated,' constitutively open, cyclosporin A-insensitive PT pores (PTPs). The formation of such PTPs results in the subsequent generation of mitochondrial superoxide and cell death [60]. This hypothesis is further supported by the observation that GSH directly modulates the opening of the mitochondrial PTP and apoptosis [61].

CDDO decreases GSH levels and, in some cases, increases ROS levels [62]. In line with these findings, many studies have shown that GSH addition completely protects the cells from CDDO-mediated apoptosis. According to these observations, it was postulated that the principal triggering mechanism of CDDO-induced apoptosis is through oxidative stress [47,53,57].

Therefore, CDDO and its derivatives are able to induce apoptosis by both the intrinsic and extrinsic pathways [49,55,59–62], and cooperate for this purpose with TRAIL or proteasome inhibitors (bortezomib and MG132) [47,51,52,56,63].

Hence, like many other chemotherapeutic drugs, CDDO and its derivatives have different targets to induce apoptosis. It is not clear which of these causes the triggering of the apoptotic cascade. NF-κB is a proinflammatory, prosurvival, antiapoptotic transcription factor that is constitutively activated in many tumors. CDDO directly inhibits IKKB and thereby decreases the binding of NF-κB to DNA and subsequent transcriptional activation [34,64,65]. Inhibition of NF-κB induces a decreased expression of the antiapoptotic proteins Bcl-2, Bcl-X_L, and XIAP [34]. It was also reported that the CDDO-mediated activation of JUN NH_2-terminal kinase [62,66] seems to be dependent on the decrease in GSH level and increase in ROS level. It was shown that JNK causes upregulation of the death receptor DR5 [56,65]. Another target involved in CDDO-induced apoptosis is the signal transducer and activator of transcription 3 (STAT3). STAT3-mediated upregulation of c-Myc levels was proposed to be an important mediator of STAT3-induced oncogenesis [67]. The biological activity of CDDO-imidazolide was originally identified by its ability to block the activity of IFN-γ, a known activator of STATs, to stimulate the de-novo production of iNOS. The suppression of STAT3 activity is likely an important mechanism contributing to these activities of CDDO-Me and CDDO-Im [31]. Constitutive activation of the STAT3 pathway was observed in many tumors, including ovarian cancer, and has been shown to confer resistance to chemotherapy-induced apoptosis in epithelial malignancies. Studies carried out in ovarian cancer cell lines showed that CDDO-Im inhibited the constitutive STAT3 activation, and, interestingly, in these cells an inverse correlation between the level of STAT3 constitutive activation and the sensitivity to the proapoptotic effects of CDDO-Im was observed. As STAT3 is an important regulator of several antiapoptotic genes, including c-FLIP, it is tempting to suggest that its inhibition by CDDO-Im may play a potential role in the triggering of apoptosis mediated by this triterpene. Thus, it was proposed that the inhibition of STAT3 activation induced by CDDO-Im determines a decrease in c-FLIP levels, making the tumor cells sensitive to caspase-8-mediated cell death. In line with this interpretation, an earlier study showed that constitutive STAT3 activation protects liver cells from Fas-mediated injury [53].

GSK3 is a multifunctional serine/threonine kinase that was first identified as a critical mediator in glycogen metabolism and insulin signaling. It is now known that GSK3 is an important component of diverse signaling pathways involved in the regulation of cell fate, protein synthesis, glycogen metabolism, cell mobility, proliferation, and survival. There are two GSK3 isoforms: GSK3α and GSK3β. In particular, GSK3β acts as a key regulator, and, when dysregulated, has been implicated in the development of a number of human diseases such as diabetes, cardiovascular disease, some neurodegenerative diseases, and bipolar disorders. The dysregulation of GSK3β has also been implicated in tumorigenesis and cancer progression. However, the mechanisms underlying GSK3β regulation of neoplastic transformation and tumor development are unclear [68]. GSK3β, like GSK3α, is a substrate of AKT and is negatively regulated by phosphorylation at Ser9 by AKT and other kinases, including protein kinase A and protein kinase C. Given that GSK3β has been reported to exert tumor suppressive activity by inhibiting cell proliferation and inducing cell death in a wide variety of stressful conditions, inhibitory phosphorylation by pharmacologic agents, such as lithium chloride, has been shown to determine an apparently tissue-specific cell fate by inducing cell survival or cell death in different tumor cell types. Notably, GSK3 has been linked to prostate cancer progression. CDDO-Me enhanced GSK3β-inactivating phosphorylation, whereas inactivation of GSK3 by pharmacological inhibitors or RNA interference sensitized prostate cancer cells to CDDO-Me cytotoxicity. These data indicate that CDDO-Me interferes with the survival signals propagated downstream of AKT by targeting GSK3 activation [58].

In addition to the apoptosis described here, CDDO and its derivatives are able to induce cell death in different ways, one of which is the induction of autophagic cell death. In chronic myeloid leukemia cells, it was observed that the mitochondrial dysfunction causes either apoptosis, with externalization of phosphatidylserine or a rapid autophagocytosis [33].

Lupeol-mediated apoptosis was studied mainly in prostate cancer cells. Lupeol, like CDDO, induces apoptosis through different pathways. In human pancreatic cancer cells, the predominant effect of lupeol is to activate caspase-8 and to decrease c-FLIP, whereas no significant change was observed in the expression levels of DR4 and DR5. In this study, lupeol was used to overcome resistance to TRAIL-mediated apoptosis in chemoresistant cancer cells [42]. However, another study on human pancreatic adenocarcinoma shows that lupeol reduces the expression of Ras oncoprotein and modulates the protein expression of various signaling molecules involved in PKCα/ODC, PI3K/Akt, and MAPKs pathways, along with

a significant reduction in the activation of NF-κB signaling pathway [69]. However, lupeol was found to induce the cleavage of poly(ADP-ribose) polymerase protein and the degradation of acinus protein, with a significant increase in the expression of FADD protein. Among all death receptor targets examined, lupeol in particular caused a significant increase in the expression of the Fas receptor. The small, interfering, RNA-mediated silencing of the Fas gene and inhibition of caspase-6, caspase-8, and caspase-9 by their specific inhibitors confirmed that lupeol specifically activates the Fas receptor-mediated apoptotic pathway in androgen-sensitive prostate cancer cells [14,70]. These observations indicate that the proapoptotic effects of lupeol are predominantly mediated through activation of the extrinsic apoptotic pathway.

Betulinic acid is able to induce apoptosis in many tumor cell types, such as leukemia, chronic myelogenous leukemia, prostate carcinoma, and neuroblastoma [71–74]. In contrast, in human Burkitt's lymphoma-derived B-cell lines, betulinic acid fails to trigger apoptosis [75]. Apoptosis induced by betulinic acid involves activation of caspases, PARP cleavage, and DNA fragmentation, and was suggested to depend on the mitochondrial pathway. Betulinic acid-induced apoptosis critically depends on the release of cytochrome *c* from the mitochondria and formation of the apoptosome. However, overexpression of Bcl-2 or Bcl-X_L only provides limited protection against betulinic acid-induced apoptosis. More importantly, Bax/Bak-deficient cells are as sensitive to betulinic acid as their wild-type counterparts, suggesting that cytochrome *c* is released in a nonclassical manner. Similarly, pre-incubation with cyclosporin A indicated a crucial role for the mitochondrial PTP in the induction of apoptosis. These observations indicate that betulinic acid affects mitochondria and induces cytochrome *c* release directly through PTP. This event is only transiently prevented by antiapoptotic members of the Bcl-2 family, but is independent of Bax and Bak. These findings help to explain the remarkably broad efficacy of betulinic acid against tumor cells of different origin and its effect in tumor cells that are resistant to other chemotherapeutic agents [76].

In-vitro studies have shown that celastrol was able to induce apoptosis of a variety of tumor cell types. Studies carried out in prostate cancer cells have shown that celastrol induces apoptosis in a dose-dependent manner, resulting in caspase-3 and PARP cleavage [19]. Studies on leukemic cell lines have shown that celastrol induces apoptosis through an inhibition of the expression of the antiapoptotic proteins IAP-1, IAP-2, Bcl-2 Bcl-X_L, c-FLIP, and survivin; these effects were postulated to be mediated through inhibition of NF-κB activity [20]. Interestingly, the proapoptotic effects of celastrol on leukemic cells have also been observed at the level of primary AML cells and are exerted on leukemic stem cells, the cells that initiate and maintain the leukemic process [77].

Avicins activate the intrinsic caspase pathway to induce apoptosis by direct perturbation of mitochondria and by downregulation of Bcl2 family antiapoptotic proteins [78–80]. The cell death induced by avicins is caspase-independent (the pan-caspase inhibitor zVAD-fmk does not provide protection from avicin-induced apoptosis), and is related to induction of autophagy by activation of AMP-activated protein kinase [81]. Recent studies, carried out on cutaneous T-cell lymphoma cells, indicate that avicins, like other triterpenoids, inhibit phosphorylation of STAT3 [82]. These observations have been confirmed in a large number of cancer cell lines, showing also that the inhibitory effects of avicins on STAT3 phosphorylation are dependent on an inhibition of JAK1 and JAK2 phosphorylation and an activation of protein phosphatase-1 [83]. Downregulation of STAT3 activity and expression of STAT3-controlled prosurvival proteins, such as c-myc, cyclin D1, Bcl2, survivin, and vascular endothelial growth factor (VEGF), contribute to the induction of apoptosis in avicin-treated tumor cells [83].

Angiogenesis

Tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste products. The process of angiogenesis plays an important role in many physiological and pathological conditions. Solid tumors depend on angiogenesis for growth and metastasis in a hostile environment [84].

CDDO and its derivatives are effective agents for suppressing angiogenesis, both in cell culture and *in vivo*. The potency of CDDO is particularly striking when tested *in vivo* to inhibit the angiogenic effects of VEGF and TNF-α in Matrigel sponge assays; these compounds show potent antiangiogenic activity at low dosages [84,85]. If the Matrigel sponges are impregnated with CDDO-Me just before implantation in mice, picomolar doses of CDDO-Me will suppress angiogenesis [85]. CDDO-Me also inhibits growth of endothelial cells in monolayer cultures, and suppresses neovascular morphogenesis in three-dimensional cultures, but significantly higher doses are required. The antiangiogenic effects of CDDO-Me on xenografts of Kaposi's sarcoma cells in immunocompromised mice were also shown, using CD31 as a marker [85,86]. In human umbilical vein endothelial cells, these compounds can inhibit the activation of the extracellular signal-regulated kinase ERK1/2 pathway after stimulation with VEGF [86]. Moreover, CDDO is able to prevent NF-κB translocation into the nucleus and thereby the activation of downstream angiogenetic pathways (VEGF, COX-2, and mmp-9) [86,87]. However, the particularly potent antiangiogenic activity observed

in vivo in some experiments suggests that CDDO-Me, as an angioprevention agent, may be interacting with an entire network of molecular and cellular targets, rather than at a single molecular locus or in a single cell type [75]. The suppression of angiogenesis induced by triterpenoids can be enhanced by being combined with other compounds, such as rexinoid. CDDO-Me and rexinoid 268 significantly reduced the formation of ER-negative tumors in MMTV-neu mice, and the combination was synergistic for the prevention of mammary tumorigenesis in this model [88].

It was shown that betulinic acid has antiangiogenic potential [89–91]. A recent study has shown that betulinic acid inhibits in-vitro enzymatic activity of aminopeptidase N (APN), which is known to play an important role in angiogenesis, but betulinic acid did not inhibit in-vivo APN activity in endothelial cells or APN-positive tumor cells. Instead, this compound potently inhibited basic fibroblast growth factor-induced invasion and tube formation of bovine aortic endothelial cells at a concentration that has no effect on the cell viability. In one study, it was shown that antiangiogenic activity of betulinic acid occurs through modulation of mitochondrial function rather than APN activity in endothelial cells [89]. In prostate cancer cells, betulinic acid decreases expression of VEGF and the antiapoptotic protein survivin. The mechanism of betulinic acid-induced antiangiogenic and proapoptotic effects is dependent on the activation of selective proteasome-dependent degradation of the transcription factors specificity protein 1 (Sp1), Sp3, and Sp4 [91].

It is interesting to note that the effect on angiogenesis induced by CDDO-Im and CDDO-Me is 1 log lower than the dosage needed to cause an effect on apoptosis or cell growth (Table 1).

Effect of triterpenoids on cell differentiation

In addition to its effects on cell proliferation and apoptosis, CDDO and its derivatives also have some notable effects on cell differentiation. In this context, the majority of studies were focused on evaluating the effects of CDDO on the differentiation of AML blasts. Experiments carried out on HL60 and U937 cells showed that CDDO, and particularly CDDO-Me, induces granulomonocytic differentiation; similarly, in 2/5 AML cases, pro-monocytic differentiation activity of CDDO and CDDO-Me was reported [92]. Other studies have also explored the effect of the other CDDO derivative, CDDO-Im, on leukemic cell differentiation. In particular, it was shown that CDDO-Im induces monocytic differentiation of HL60 cells, and this effect seems to be mediated through three different effects: activation of the extracellular signal-regulated kinase (ERK) signaling pathway; upregulation of the CCAAT/enhancer-binding protein-β, a transcription factor critical for monocytic differentiation; and activation of the transforming growth factor-β (TGF-β)/SMAD signaling pathway [93]. Another study explored the effect of subapoptotic CDDO doses promoting granulocytic differentiation of HL60 cells, this effect being mediated through increased p42 CEBPA protein synthesis and consequent transcriptional activation of CEBPA-regulated genes [94]. The effect of CDDO on granulocytic differentiation was also confirmed on acute promyelocytic cells; interestingly, CDDO together with ATRA unblocked the differentiation of ATRA-resistant NB4 cells [95]. Other studies have been carried out on osteosarcoma Saos-2 cells, showing that CDDO induces both apoptosis and differentiation (as assessed by alkaline phosphatase activity and osteocalcin production). Interestingly, both the induction of apoptosis and the differentiation of osteosarcoma cells by CDDO required caspase-8 activation [54].

Table 1 CDDO doses required to achieve the main biologic effects

Biological effect	Dosage (μmol/l)
Cell growth	0.1–0.5
Apoptosis	0.1–1
Angiogenesis	0.1
Cell differentiation	0.01–0.1

The dosage is referred to CDDO-Im and CDDO-Me. CDDO requires doses 1 log higher to achieve the same effects as CDDO-Im and CDDO-Me.

Lupane triterpenes were found to promote melanogenesis, a hallmark of B16 2F2 mouse melanoma cell differentiation [96,97]. Studies on the structure–activity relationships showed that the keto function at C-3 of the lupane skeleton played important roles in the melanogenic activities of lupane triterpenes on melanoma cells. The carbonyl group at C-17 of lupane triterpenes was essential for their apoptosis-inducing activity against human cancer cells through the inhibition of topoisomerase I. Melanogenesis, one of the hallmarks of melanoma cell differentiation, has already been shown to be regulated by lupeol through activation of the p38 MAPK signaling pathway [96].

As mentioned above with regard to the angiogenetic effect, the effect on cell differentiation induced by CDDO-Im and CDDO-Me is 1 log lower than the dosage needed to cause an effect on apoptosis or cell growth (Table 1).

NF-E2-related factor 2 activation by triterpenoids

NF-E2-related factor 2 (Nrf2) is a member of a subfamily of the basic leucine zipper family of transcription factors characterized by a conserved cap 'n' collar domain. In response to oxidative stress, Nrf2 controls the fate of cells through the transcriptional upregulation of antioxidant response element (ARE)-bearing genes. Under basal conditions, Nrf2 forms a complex with its inhibitor Keap1, which allows its ubiquitination and proteolytic degradation in the cytoplasm. However, upon exposure of cells to oxidative or chemopreventive compounds, Nrf2

dissociates from its partner Keap1 and translocates to the nucleus, where it interacts with other proteins and activates the transcription of several different types of genes, particularly antioxidative and electrophilic detoxification genes [98]. In an initial study, Liby *et al.* [99] showed that CDDO and CDDO-Im are potent inducers of heme oxygenase-1 and Nrf2/ARE signaling. In particular, it was shown that nanomolar concentration of CDDO-Im marked an increase in Nrf2 levels in monocytic U937 cells.

CDDO-Im greatly increased nuclear accumulation of Nrf2 protein in peripheral blood mononuclear cells and in neutrophils [100]. Subsequent studies have extended these in-vitro observations to in-vivo models showing that CDDO-Im acts as a potent activator of Nrf2 signaling *in vivo* [101,102]. Targeting Nrf2 with CDDO-Im is of potential therapeutic importance in various diseases. Thus, treatment with CDDO-Im provides protection against cigarette smoke-induced emphysema and cardiac dysfunction through activation of Nrf2 signaling [103]. Furthermore, CDDO-Im treatment resulted in effective chemoprevention against aflatoxin-induced hepatic tumorigenesis, with pronounced protection at oral doses as low as 1 μmol/kg body weight and complete inhibition of tumorigenesis at 100 μmol/kg body weight. Protection is achieved through Nrf2-regulated hepatic detoxification and cytoprotective genes, resulting in reduced aflatoxin–DNA adduct formation and inhibition of tumorigenesis [104]. The activation of Nrf2-antioxidant signaling by CDDO attenuates NF-κB inflammatory response and elicits apoptosis [105].

However, some recent data suggest that Nrf2 activation by CDDO could also have a negative impact on the anticancer activity exerted by this triterpenoid. In fact, a series of observations indicate that Nrf2 is abundantly expressed in cancer cells and may be involved in cancer drug chemoresistance [106–108].

Therefore, although the role of Nrf2 activation by CDDO in cancer chemoprevention is clear, the role of Nrf2 activation in anticancer activity of CDDO and its derivatives remains to be shown.

Other triterpenoids were reported to activate Nrf2. Thus, avicinD has been shown to inhibit NF-κB and activate Nrf2in a redox-dependent manner, accounting for its anti-inflammatory and antioxidant properties [79].

Emerging triterpenoid targets in cancer

In addition to the above-mentioned targets of triterpenoids, there is emerging evidence suggesting that these drugs could also affect other cellular pathways or functions. Among them, a possible effect of CDDO on cell adhesion, cell migration, and interaction with other drugs is of particular interest.

The cancer cell environment is a particularly important determinant in the emergence of drug resistance. Soluble factors such as cytokines, hormones, and growth factors, as well as interactions between tumor cells and ECM molecules or adjacent cells, may play a significant role in the pathogenesis and progression of human cancers [109].

Adhesion of MM cells to bone marrow stromal cells (BMSCs) induces IL-6 secretion from BMSCs, which not only regulates the growth of MM cells, but also provides protection against chemotherapy. It was shown that CDDO-Im (0.2 μmol/l) significantly inhibits IL-6 secretion in MM patient BMSCs triggered by MM cell adhesion. Reports that show high serum levels of IL-6 contributing to clinical chemoresistance and treatment failure, coupled with the finding that CDDO-Im decreases the MM adhesion-induced IL-6 secretion from BMSCs, suggest that CDDO-Im may overcome drug resistance in patients with advanced MM [63]. A different group has isolated and characterized the marine, polyether triterpenoid, DT, a natural compound that preferentially induces apoptosis in hormone-independent human breast cancer cell lines. This chemical caused the detachment of cell clusters without losing cell–cell contact, suggesting a potential effect on cell–ECM interactions [110].

CDDO and its derivatives are also able to influence cell migration. TGF family members regulate many cellular functions, including proliferation and differentiation, and TGF-β is a potent apoptotic agent in many cells, including early-stage epithelial tumors. However, in late-stage epithelial tumors, TGF-β becomes a metastatic agent and stimulates epithelial-to-mesenchymal transition and cell migration. CDDO-Im alters TGF-β cell signaling and receptor trafficking, and inhibits cell migration by disrupting cytoskeletal attachments to the polarity complex [111].

The effect of CDDO on the induction of matrix metalloproteinases 1 and 13 (MMP-1, MMP-13) by inflammatory cytokines in human chondrosarcoma cells was reported. CDDO selectively reduced the induction of MMP-1 and MMP-13 both at RNA and protein level. Treatment with CDDO before cytokine stimulation enhanced this inhibition, showing that CDDO functions at the level of transcription. In addition, CDDO reduced IL-1β-mediated invasion of cells through a collagen matrix [112].

In contrast, combining CDDO-Im and bortezomib induces apoptosis even in bortezomib-resistant MM patient cells. Together, these findings provide the framework for clinical evaluation of CDDO-Im, either alone or in combination with bortezomib, to overcome drug resistance and improve patient outcome in MM [63].

Moreover, as mentioned above, CDDO could be used with other drugs for chemoprevention. In particular, CDDO-Me in combination with rexinoid 268 was synergistic for the prevention of mammary tumorigenesis in MMTV-neu mice [88]. CDDO, CDDO-Me, and CDDO-EA are also potent inhibitors of vinyl carbamate-induced mouse lung carcinogenesis in the postcarcinogen phase [113].

As an increasing number of other novel therapeutics is identified and mechanisms of resistance are elucidated, combinations with this drug will be developed to optimize new therapeutic regimens.

Clinical evaluation of CDDO

CDDO is under evaluation in phase I clinical studies. In this context, a first study (NCT00352040) aimed to evaluate the side effects and the best dose of CDDO in treating patients with metastatic or unresectable solid tumors or lymphoma has been carried out. The study was completed at the end of 2008 [114]. In a second study, CDDO-Me was administered to 34 patients at 10 dose levels (5–1300 mg/day). The purpose of the study was to determine the dose limiting toxicity, the maximum tolerated dose, and phase II dose of CDDO-Me in patients with advanced solid tumors or lymphoid malignancies; moreover, the objective was to characterize the pharmacokinetics of CDDO-Me given orally once daily for 21 days every 28 days. The data obtained from this study indicate that: (i) CDDO-Me modulates NF-κB, STAT-3, and Nrf2; and (ii) there are clinical benefits associated with this novel mechanism, including a partial remission and prolonged disease stabilization in several patients. Phase II trials have begun [115]. Another study (NCT00550849) aimed to evaluate the safety and tolerability of CDDO-Me in patients with hepatic dysfunction. The study was terminated at the end of 2007. Finally, a third study (NCT00508807) aimed to evaluate the tolerability, toxicity, and efficacy of CDDO-Me in patients with advanced solid tumors or lymphoid malignancies. The study is estimated to end in April 2009.

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