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Chapter 6

Promising Natural Products As Anti-Cancer Agents against Neuroblastoma

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Abstract

Neuroblastoma is a neuroendocrine tumor arising from neural crest elements of the sympathetic nervous system (SNS). It most frequently originates in one of the adrenal glands, but can also develop in nerve tissues of the neck, chest, abdomen or pelvis, and exhibits extreme heterogeneity, being stratified into three risk categories; low, intermediate, and high.

Low-risk disease is most common in infants and good outcomes are common with observation only or surgery, whereas high-risk disease is difficult to treat successfully, even with the most intensive multi-modal therapies available. Esthesioneuroblastoma, also known as olfactory neuroblastoma, is believed to arise from the olfactory epithelium and its classification remains controversial. However, as it is not a sympathetic nervous system malignancy, esthesioneuroblastoma is a distinct clinical entity and is not to be confused with neuroblastoma. This review describes novel natural products with which neuroblastoma can be treated.

Abbreviations

AIF	Apoptosis-inducing factor
AMPK	Adenosine monophosphate kinase
ATF3	Activating transcription factor 3

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Bcl-2	B-cell lymphoma-2
Bcl-xL	B-cell lymphoma-extra large
$[Ca^{2+}]i$	Intracellular free calcium concentration
CDK	Ccyclin dependent kinase
CDKI	CDK-inhibitor
COX-2	Cyclooxygenase-2
DADS	Diaryl disulfide
DAS	Diaryl sulfide
DNA	Deoxyribonucleic acid
EFSA	European food safety authority
FDA	Food and drug administration
HO-1	Heme oxygenase-1
IL-6	Interleukin-6
MALDI-TOF-MS	Matrix assisted laser desorption/ionization-time of flight-mass
MM	Multiple myeloma
MMP	Matrix metalloproteinase
NCAM	Neural cell adhesion molecule
NF-κB	Nuclear factor-κB
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen
SNS	Sympathetic nervous system
STAT3	Signal transducer and activator of transcription 3
tBid	Truncated Bid
VEGF	Vascular endothelial growth factor
WSWE	*Withania somnifera* water extract

1. Introduction

In the 21st century, the eradication of cancer remains an important long-term goal. Cancer chemotherapy drugs, such as taxol, vincristine, vinblastine, etoposide and irinotecam; have been used extensively for the treatment of certain types of cancer. However, with these treatments, severe gastrointestinal toxicity, including diarrhea and mycosis, and hematological toxicity, including leucopenia and immune suppression, are dose limiting factors. Nonetheless, after removal of malignant tumors by surgery, radiation therapy and/or adjuvant therapy with cancer chemotherapy drugs can be curative.

Neuroblastoma is the most common extracranial solid tumor in children and is derived from cells of the sympathetic nervous system. Despite recent advances in chemotherapy, the prognosis of advanced neuroblastoma remains very poor. Therefore, it is necessary to develop new anticancer agents with antitumor activity but without the serious adverse reactions caused by current cancer chemotherapy drugs. Conventional therapy for this infantile malignancy includes surgery, radiation and chemotherapy; however, treatment in most instances shows low response and poor outcome. Thus, there is an urgent need for the development of new therapeutic strategies for successful treatment of malignant neuroblastoma in young children.

In this review, the anti-cancer effects of natural products isolated from various medicinal plants are discussed.

2. Neuroblastoma

Neuroblastoma, the most common solid extracranial neoplasm in children, originates from embryonic neural crest cells that usually form the sympathetic ganglia and adrenal medulla [1]. Nearly half of neuroblastoma cases occur in children younger than two years. It most frequently originates in one of the adrenal glands, but can also develop in nerve tissues in the neck, chest, abdomen or pelvis. Neuroblastoma is one of the few human malignancies known to demonstrate spontaneous regression from an undifferentiated state to a completely benign cellular appearance [2]. It also accounts for 15% of pediatric tumors and has a poor prognosis in children after 1 year of age [3]. A commonly used staging system for neuroblastoma is described below (Table1).

Stage 1: The cancer is contained within one area of the body (localized) and there is no evidence of it having spread. It can be completely removed by surgery, or there may be very small (microscopic) amounts of tumor left after surgery.

Stage 2A: The cancer is localized and has not begun to spread, but it cannot be completely removed by surgery.

Stage 2B: The cancer is localized and has begun to spread into nearby lymph nodes.

Stage 3: The cancer has spread into surrounding organs and structures, but has not spread to distant areas of the body.

Stage 4: Cancer has spread to distant lymph nodes, bone, bone marrow, liver, skin or other organs.

Stage 4S: (also called special neuroblastoma) This is found in children under one year of age.

Table 1. International neuroblastoma staging system

Stage	Definition
1	Localized tumor confined to area of origin; complete gross excision, with or without microscopic residual
2A	Unilateral tumor with incomplete gross excision; ipsilateral and contralateral lymph nodes microscopically negative
2B	Unilateral tumor with complete or incomplete gross excision, with positive ipsilateral regional lymph nodes; contralateral lymph nodes microscopically negative
3	Tumor infiltrating across midline, with or without regional lymph node involvement; or unilateral tumor with positive contralateral regional lymph nodes; or midline tumor with bilateral regional lymph node involvement
4	Dissemination of tumor to distant lymph nodes, bone, liver or other organs (except as defined in 4S)
4S	Localized primary tumor in infants <18 months of age as defined for stage 1 or 2 with dissemination limited to liver, skin or bone marrow (<10% involvement)

The cancer is localized (as in stage 1, 2A or 2B) but has begun to spread to the liver, skin or bone marrow. In patients over 1 year of age, the tumor is very aggressive and drug-resistant. The 5-year survival rate of patients with such advanced tumors is only 25-30%, despite aggressive chemotherapy. Conversely, in patients under 1 year of age, the tumor usually has a favorable prognosis and frequently regresses or differentiates into ganglioneuroma, requiring little or no therapy. Even after extensive metastasis, some neuroblastomas undergo spontaneous regression (special stage 4S), but the 5-year survival rate of patients with stage IV is as low as 20-25% [4]. Therefore, novel therapeutic strategies are urgently needed to improve the prognosis of these patients.

Although the precise mechanism responsible for this clinical heterogeneity remains unclear, some reports suggest that apoptosis plays a role. In neuroblastomas showing spontaneous regression, extensive apoptosis occurs [5, 6]. On the other hand, disruption of the apoptosis machinery is observed in drug-resistant and/or advanced neuroblastomas, *e.g.*, expression of Bcl-2 [7], p53 mutation or lack of p53 function [8] and loss of caspase-8 expression [9]. Therefore, novel drugs that induce apoptosis in advanced neuroblastoma are required. Apoptosis mainly occurs through two pathways. In the death receptor pathway, receptor ligation leads to activation of caspase-8. In the mitochondrial pathway, on the other hand; various apoptosis-inducing triggers such as DNA damage and oxidative stress cause activation of caspase-9. In these two different pathways, activated initiators, caspase-8 and caspase-9, finally activate caspase-3, the downstream (executor) caspase [10]. The mitochondrial pathway is regulated by Bcl-2 family proteins; the pro-apoptotic Bax and the anti-apoptotic Bcl-2. However, apoptosis is regulated more intricately by a large number of molecules [11]. Therefore, the development of new drugs capable of inducing apoptosis in advanced neuroblastoma, thus achieving a cure without adverse effects, is desirable. In search for novel therapeutic strategies for neuroblastoma, natural products have attracted interest as new approaches with chemotherapy.

3. Secondary Metabolites for Neuroblastoma

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development or reproduction of organisms [12]. However, these classes of compounds also include primary metabolites, so whether a compound is considered to be a primary or secondary metabolite is based not only on its chemical structure but also on its function and distribution within the plant kingdom. Secondary metabolites are often restricted to a narrow set of species within a phylogenetic group. As these compounds are usually restricted to a much more limited group of organisms, they have long been of prime importance in taxonomic research. Secondary metabolites are produced by numerous microbes, plants, fungi and animals, and largely fall into three classes of compound: alkaloids, terpenoids and phenolics. Many thousands of secondary metabolites have been isolated from plants, with many of these having powerful physiological effects in humans, are used as medicines, flavorings and/or recreational drugs.

Alkaloids are a large group of nitrogen-containing compounds, examples of which are known to occur in approximately 20 percent of all flowering plants. Many alkaloids are extremely toxic, particularly to mammals, and act as potent nerve poisons, enzyme inhibitors

or membrane transport inhibitors. Many potentially toxic plant-derived alkaloids have medicinal properties, provided that they are administered in carefully regulated doses. Vincristine and vinblastine from *Vinca rosea* L. (Apocynaceae) are inhibitors of cell division and are used to treat cancers of the blood and lymphatic systems.

Terpenoids are derived from acetyl coenzyme A or from intermediates in glycolysis. They are classified by the number of five-carbon isoprenoid units they contain. Monoterpenes (containing two C_5 units) are exemplified by the aromatic oils (such as menthol) present in the leaves of members of the mint family. In addition to giving these plants their characteristic taste and fragrance, these aromatic oils have insect-repellent qualities. Diterpenes are formed from four C_5-units. Paclitaxel, a diterpene found in the bark of *Taxus brevifolia* Nutt. (Taxaceae), is a potent inhibitor of cell division in animals. At the end of the twentieth century, paclitaxel was developed as a powerful new chemotherapeutic treatment for people with solid tumors, such as ovarian cancer patients. Triterpenoids (formed from six C_5 units) comprise plant steroids, some of which act as plant hormones. While these can also protect plants from insect attacks, their mode of action is different from that of the pyrethroids. For example, the phytoecdysones are a group of plant sterols that resemble insect molting hormones. When ingested in excess, phytoecdysones can disrupt the normal molting cycle with often lethal consequences to the insect. Tetraterpenoids (eight C_5 units) include important pigments such as β-carotene, which is a precursor of vitamin A, and lycopene, which gives tomatoes their red color. Rather than functioning in plant defense, the colored pigments that accumulate in ripening fruits can serve as attractants to animals, which actually aid the plant in seed dispersal.

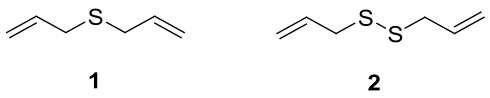
In organic chemistry, phenols, sometimes called phenolics, are a class of chemical compounds consisting of a hydroxyl group (-OH) bonded directly to an aromatic hydrocarbon group. Phenolic compounds are classified as simple phenols or polyphenols based on the number of phenol units in the molecule. Phenolic compounds are also produced by plants and microorganisms, with variation between and within species. Organisms that synthesize phenolic compounds do so in response to ecological pressures such as pathogen and insect attack, UV radiation and wounding. As they are present in the food consumed in human diets and in plants used in traditional medicine of several cultures, their role in human health and disease is a subject of research. For example, some phenols are germicidal and are used in formulating disinfectants.

Phenolic compounds are defined by the presence of one or more aromatic rings bearing a hydroxyl functional group. Salicylic acid concentration increases in the leaves of certain plants in response to fungal attack and enables the plant to mount a complex defense response. Interestingly, aspirin, a derivative of salicylic acid, is routinely used in humans to reduce inflammation, pain and fever. Lignin, a complex phenolic macromolecule, is laid down in plant secondary cell walls and is the main component of wood. It is a very important structural molecule in all woody plants, allowing them to achieve height, girth and longevity. Flavonoids were referred to as Vitamin P (probably because of the effect they had on the permeability of vascular capillaries) from the mid-1930s to the early 1950s, but the term has since fallen out of use. Although there is ongoing research into the potential health benefits of individual flavonoids, neither the Food and Drug Administration (FDA) nor the European Food Safety Authority (EFSA) has approved any health claim for flavonoids or approved any flavonoids as pharmaceutical drugs. Flavonoids have also been proposed to inhibit the pro-inflammatory activity of enzymes involved in free radical production, such as

cyclooxygenase, lipoxygenase or inducible nitric oxide synthase, and to modify the intracellular signaling pathways in immune cells. The widespread distribution of flavonoids, their variety and their relatively low toxicity when compared with other active plant compounds (for instance alkaloids) mean that many animals, including humans, ingest significant quantities in their diet. Foods with a high flavonoid content include parsley, onions, blueberries and other berries, black tea, green tea and oolong tea, bananas, all citrus fruits, Ginkgo biloba, red wine, sea-buckthorns and dark chocolate (with a cocoa content of 70% or greater). Stilbenoids are hydroxylated derivatives of stilbene. They have a C_6-C_2-C_6 structure. An example of a stilbenoid is resveratrol, which is found in grapes and has been suggested to have many health benefits. Food sources of resveratrol include the skin of grapes, blueberries, raspberries and mulberries.

4. Diaryl Sulfide from Garlic (*Allium sativum* L.)

Diallyl disulfide (**2**) is an organosulfur compound derived from garlic and a few other genus *Allium* plants. Along with diallyl trisulfide and diallyl tetrasulfide, it is one of the principal components of the distilled oil of garlic. It is produced during the decomposition of allicin, which is released upon crushing garlic and other plants of the Alliaceae family.



1 **2**

Figure 1. Structures of garlic components.

Karmar et al. reported the use of garlic compounds diallyl sulfide (**1**) and **2** for induction of apoptosis in human malignant neuroblastoma SH-SY5Y cells [13]. Wright staining showed morphological features of apoptosis in SHSY5Y cells treated with 50 and 100 μM **1** or **2** for 24 h. Apoptosis was associated with increases in intracellular free calcium concentration ($[Ca^{2+}]i$), increases in Bax:Bcl-2 ratio, mitochondrial release of cytochrome *c*, increases in cytosolic Smac/Diablo, and downregulation of inhibitor-of-apoptosis proteins and nuclear factor-κB (NF-κB). Garlic compounds **1** and **2** suppressed anti-apoptotic factors and activated calpain and intrinsic caspase cascade for apoptosis, and also downregulated anti-apoptotic proteins, including Bcl-2, BIRC-2, BIRC-3 and NF-κB, to facilitate the apoptotic process in SH-SY5Y cells.

5. Marine Cyanobacterium

Taori et al. reported that marine cyanobacteria are prolific producers of bioactive secondary metabolites, many of which are modified peptides or peptide-polyketide hybrids with promising antitumor activities, such as curacin A (**3**), dolastatin 10 (**4**) and apratoxin A (**5**). *Symploca* species have scarcely been investigated when compared with the more prevalent *Lyngbya* spp., yet a Palauan *Symploca* sp. previously yielded the clinical trial compound **4**. Largazole (**6**) was isolated from *Symploca* sp. The growth of cancer cell lines

derived from colon (HT29) and neuroblastoma (IMR-32) was strongly inhibited by largazole (GI_{50}/LC_{50} 12 nM/22 nM; 16 nM/22 nM) [14].

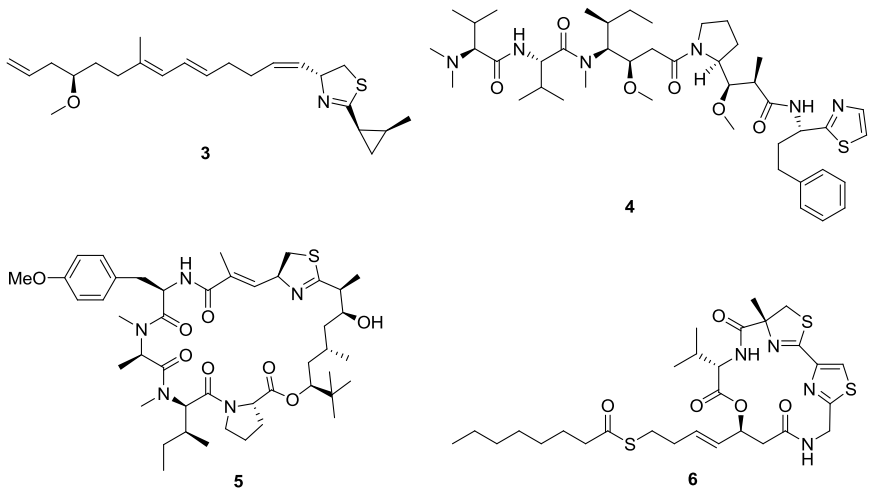


Figure 2. Structures of marine products.

6. Phenolics

6.1. Lignans

Plant lignans are polyphenolic substances derived from phenylalanine via dimerization of substituted cinnamic alcohols. Many natural products, known as phenylpropanoids, are built up of C_6C_3 units derived from cinnamyl units. Some examples of lignans are sesamin (**11**), and podophyllotoxin (**12**). Lignans are one of the major classes of phytoestrogens, which are estrogen-like chemicals and also act as antioxidants. Plant lignans are co-passengers of dietary fiber, and therefore fiber-rich food items are often good sources of lignans. The sources of lignans include cereals (rye, wheat, oat and barley), flax seed and sesame seed, soybeans, cruciferous vegetables such as broccoli and cabbage, and some fruits, particularly apricots and strawberries [15]. Lignans serve an antioxidant role in the plant's defenses against biotic and abiotic factors, and have shown anti-inflammatory and antioxidant activity in basic research models of human diseases [16]. Lignans may also have anticarcinogenic activities, as some epidemiological studies have shown that lignan exposure associates with lower risk of breast cancer [15, 17].

12 is a non-alkaloid, toxic lignan extracted from the roots and rhizomes of *Podophyllum* species [19]. **12** and its synthetic derivatives display a wide selection in medical applications such as purgative, vesicant, antirheumatic, antiviral and antitumor agents. These derivatives include etoposide (**13**), etopophos (**14**) and teniposide (**15**). Their anticancer activity has been under intense study for use in various chemotherapies against lung cancer, lymphomas and genital tumors. For example, **13** is used in chemotherapy for Kaposi's sarcoma, Ewing's sarcoma, lung cancer, testicular cancer, lymphoma, nonlymphocytic leukemia and glioblastoma multiforme [20].

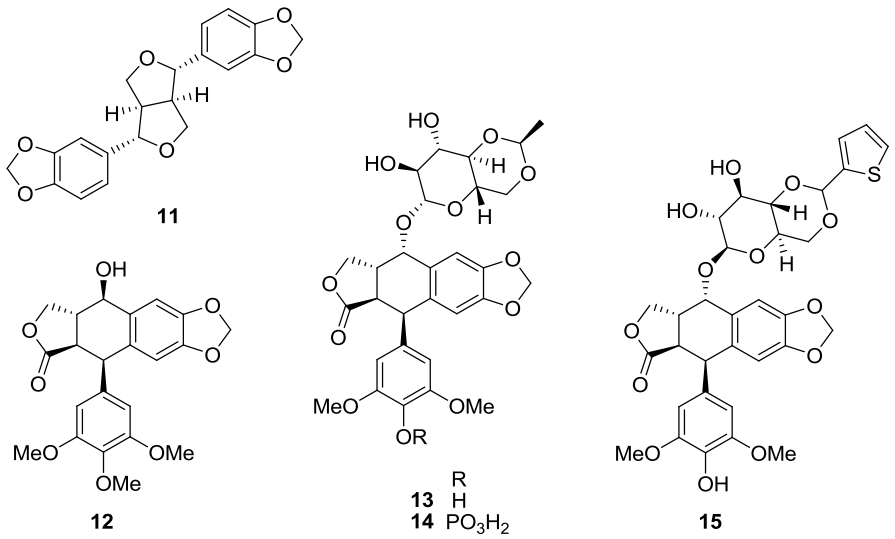


Figure 3. Structures of plant lignans.

Uchiyama et al. reported that the ethanol extract of the *L. puchury-major* seed exhibited cytotoxic activity against human leukemia cells (Jurkat) [21]. Bioassay-guided fractionation of the extract gave ten neolignans (**16**–**20**, and others). Of these, four neolignans (**16**–**18**, **20**) induced apoptotic cell death on flow cytometry. The apoptosis induced by ferrearin C (**16**) is mediated by procaspase-9 processing. Two neolignans, **16** and **18**, showed significant cytotoxicity in neuroblastoma cells [22]. Typical morphologic features of apoptosis were observed for the ferrearin-type neolignans using Hoechst 33342, and apoptotic cytoplasmic membrane inversion was also induced by ferrearin-type neolignans in IMR-32 cells. Furthermore, a Proteome Profiler Array showed that the ferrearin-type neolignans induced the marked expression of heme oxygenase-1(HO-1). The ferrearin-type neolignans strongly trigger apoptotic activity via the induction of HO-1.

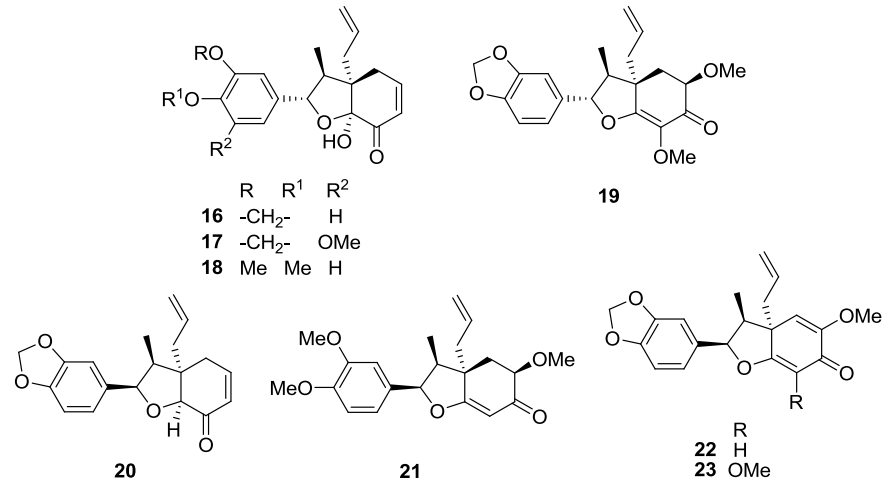


Figure 4. Structures of neolignans from *Licaria puchury-major* (**16**–**20**) and *Aniba gardneri* (**21**–**23**).

6.2. Flavonoids

Das et al. investigated the proteolytic mechanisms for apoptosis in human malignant neuroblastoma SH-SY5Y cells following exposure to flavonoids such as apigenin (**22**), genistein (**23**), (–)-epigallocatechin (**24**), and (–)-epigallocatechin-3-gallate (**25**) [23]. Apoptosis was associated with increases in intracellular free [Ca^{2+}] and Bax:Bcl-2 ratio, mitochondrial release of cytochrome c and activation of caspase-9, calpain and caspase-3. Induction of apoptosis with APG and GST also showed activation of caspase-12. Activation of caspase-8 cleaved Bid to truncated Bid (tBid) in cells treated with **24** and **25**. **24** and **25** induced apoptosis with caspase-8 activation and mitochondria-mediated pathway, whereas **22** and **23** caused apoptosis via an increase in intracellular free [Ca^{2+}] with calpain activation and mitochondria-mediated pathways. Activation of different proteases for cell death was confirmed using caspase-8 inhibitor II, calpeptin (calpain inhibitor), caspase-9 inhibitor I and caspase-3 inhibitor IV. Thus, plant-derived flavonoids cause cell death with activation of proteolytic activities of calpain and caspases in SH-SY5Y cells, and therefore serve as potential therapeutic agents for controlling the growth of neuroblastoma.

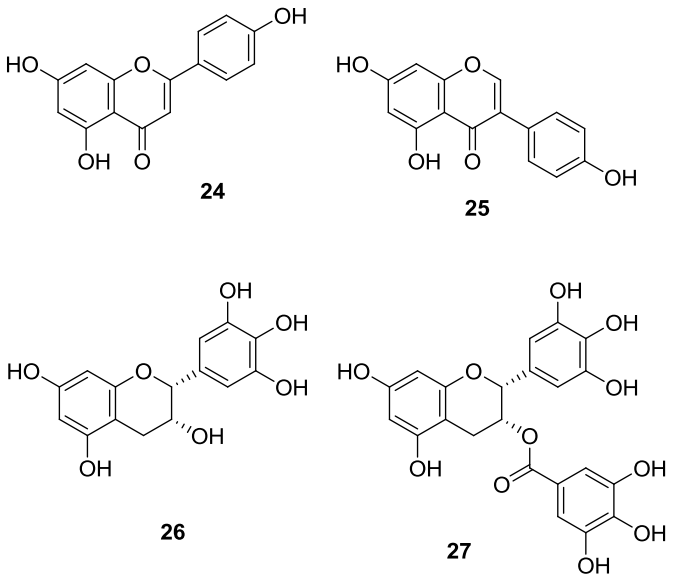


Figure 5. Structures of plant flavonoids.

6.3. Hydroxychalcones

Chalcone is an aromatic ketone and an enone that forms the central core for a variety of important biological compounds, which are known collectively as chalcones or chalconoids.

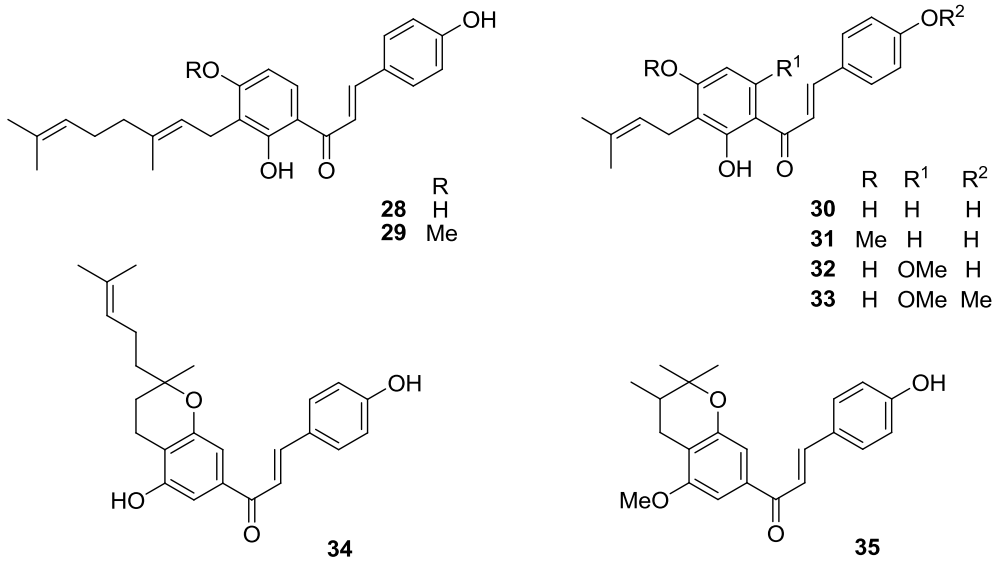


Figure 6. Structures of chalcones from *Angelica keisukei*.

Tabata et al. reported that xanthoangelol (**28**) and isobavachalcone (**30**), major chalcone constituents from stem exudates of *Angelica keiskei*, concentration-dependently reduced the survival rates of neuroblastoma as revealed by trypan blue exclusion test [24]. All chalcones (**28**-**35**) exhibited cytotoxicity against neuroblastoma cells, and two (**30** and xanthoangelol H (**35**)) had no effect on normal cells even at high concentration (10^{-4}M) exposure. Western blot analysis showed that **28** markedly reduced the level of precursor caspase-3 and increased the level of cleaved caspase-3, but Bax and Bcl-2 proteins were not affected. **28** induces apoptotic cell death by activation of caspase-3 in neuroblastoma and leukemia cells through a mechanism that does not involve Bax/Bcl-2 signal transduction. Motani et al. reported that proteomic analysis using 2-dimensional electrophoresis and Matrix Assisted Laser Desorption/Ionization-Time of Flight-Mass (MALDI-TOF-MS) revealed that DJ-1 protein was involved in xanthoangelol-induced apoptosis [25]. DJ-1 was down-regulated by **28**, leading to loss of antioxidant function and acceleration of apoptosis. **28** induces mitochondria-mediated apoptosis via oxidative stress through accumulation of reactive oxygen (ROS) and down-regulation of DJ-1. Through this mechanism, **28** can overcome drug-resistant neuroblastoma. Nishimura et al. reported that Western blot analysis showed that **30** significantly reduced pro-caspase-3 and pro-caspase-9, and subsequently increased the level of cleaved caspase-3 and cleaved caspase-9 in both neuroblastoma cell lines [26]. Moreover, Bax was markedly induced by **30** application. **30** induces apoptotic cell death in neuroblastoma via the mitochondrial pathway and has no cytotoxicity against normal cells.

6.4. Stilbenes

Ito et al. reported that twenty resveratrol (3,5,4'-trihydroxystilbene; **36**) derivatives, which were isolated from stem bark of *Vatica rassak* (Dipterocarpaceae), were evaluated for *in vitro* cytotoxicity against a panel of human tumor cell lines [27]. Among these, seven

compounds displayed marked cytotoxicity. Vaticanol C (**37**) as a major component induced considerable cytotoxicity in all cell lines tested and exhibited growth suppression in colon cancer cell lines at low dose. **37** caused two cell lines (SW480 and HL60) to induce cell death at 4- to 7-fold lower concentrations when compared with **36**. Growth suppression by **37** was found to be due to apoptosis, which was assessed by morphological findings (nuclear condensation and fragmentation) and DNA ladder formation in the colon cancer cell lines. The apoptosis in SW480 colon cancer cells was executed by activation of caspase-3, which was confirmed by Western blot and apoptosis inhibition assay. Furthermore, the mitochondrial membrane potential of apoptotic SW480 cells after 12 h of treatment with **37** was significantly lost, and concurrently, cytochrome c release and activation of caspase-9 were also detected by Western blot analysis. Overexpression of Bcl-2 protein in SW480 cells significantly prevented the cell death induced by **37**. In addition, **37** exhibited cytotoxicity against neuroblastoma SH-SY5Y cells.

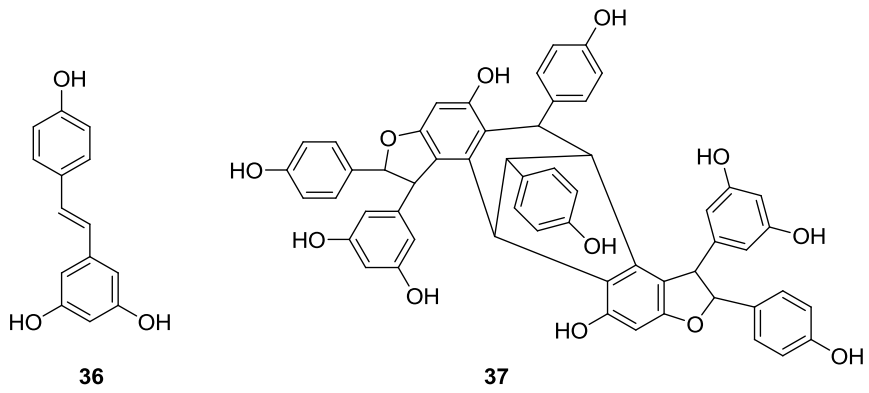


Figure 7. Structures of stilbenes.

6.5. Diarylheptanoids

Diarylheptanoids are a relatively small class of plant secondary metabolites. Diarylheptanoids consist of two aromatic rings (aryl groups) joined by a seven-carbon chain (heptane) and have various substituents. The best known member is curcumin (**64**), which is isolated from turmeric (*Curcuma longa*). They have been reported from plants in a few families, *e.g*., Betulaceae and Zingiberaceae.

Sun et al. reported that bioassay-guided fractionation of the cytotoxic methanol extract from the rhizomes of *Alpinia officinarum* led to the isolation of 17 diarylheptanoids (**38**,**39**,**41**,**43**−**46**,**49**,**51**−**55**,**57**,**58**,**62**,**63**) [28]. The cytotoxic activity of the isolated diarylheptanoids was evaluated against the IMR-32 human neuroblastoma cell line. Among the tested compounds, **41**, **46** and **49** exhibited the most potent activities with IC_{50} values of 0.83, 0.23 and 0.11 μM, respectively. Compounds **41**, **46** and **49** were more potent than the positive control cisplatin and the compound curcumin (IC_{50}: 2.40 μM) which possessed a similar structure and was reported to be cytotoxic to various tumor cell lines.

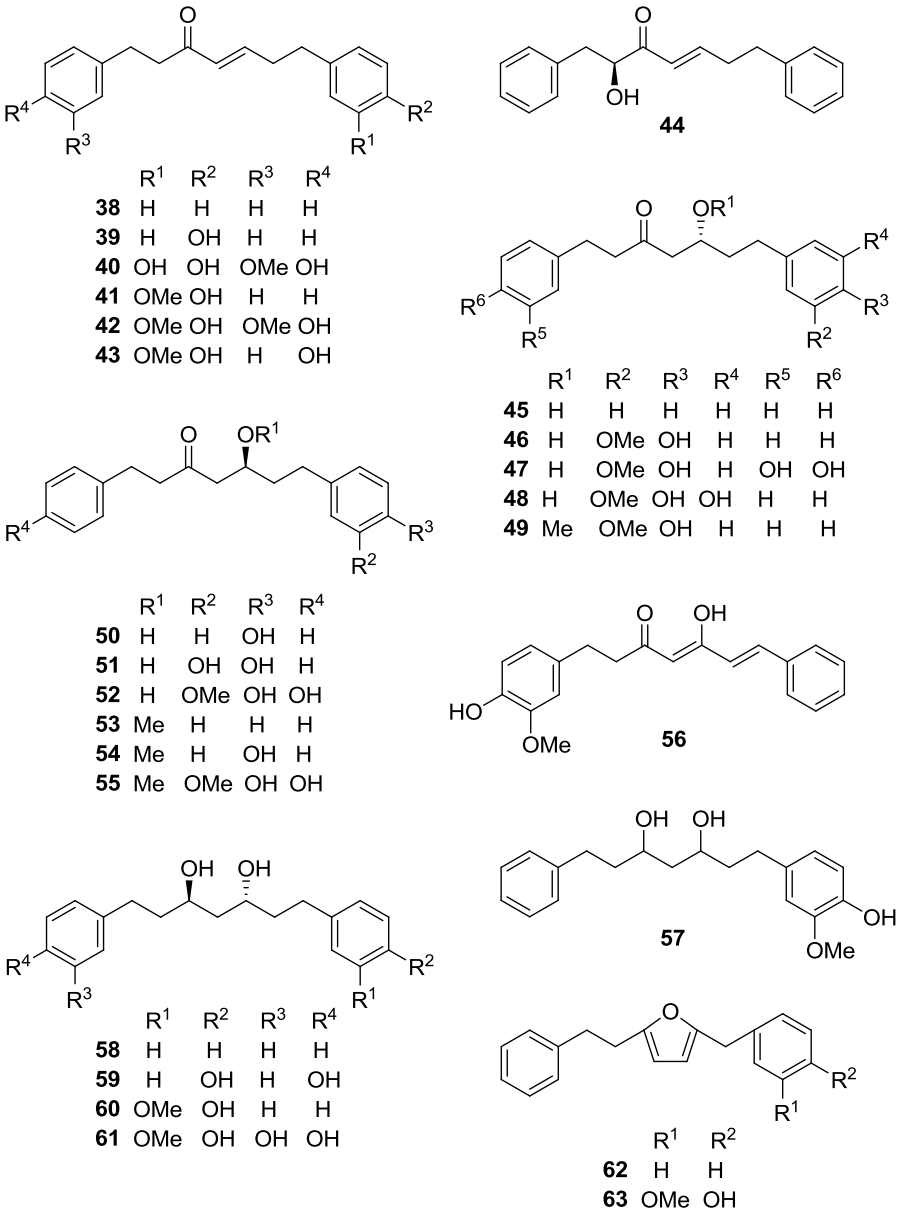


Figure 8. Structures of diarylheptanoids from *Alpinia officinarum*.

Tabata et al. monitored nuclear shrinkage by Hoechst 33342 staining, and activation of caspases-3 and -9 by Western blotting [29]. Two diarylheptanoids (**39** and **49**) showed significant cytotoxicity against neuroblastoma cell lines (IMR-32, SK-N-SH, NB-39). These compounds induced nuclear shrinkage and fragmentation, and activated caspase-3 and caspase-9. Flow cytometry revealed induction of S-phase cell cycle arrest concurrent with an increased sub-G1 cell population. Moreover, a low concentration (10^{-8} M) of **39** induced significant neurite branching in the NB-39 cell line. Both compounds induced mitochondrial apoptosis and S-phase cell cycle arrest, and **39** caused significant neural differentiation when applied at 10^{-8} M.

Tian et al. reported that the most potent diarylheptanoid (**40**) confirmed that cell cycle-related proteins, cyclins, **cyclin** dependent kinase (CDK)s and CDK-inhibitor (CDKI)s, as well as two main apoptosis-related families, caspase and Bcl 2 were involved in S phase arrest and apoptosis in the neuroblastoma cell line SH-SY5Y [30]. Furthermore, following drug treatment, the protein expression of p53, phospho-p53 (Ser20) as well as the p53 transcription-activated genes activating transcription factor 3 (ATF3), puma and Apaf-1 were increased markedly; MDM2 and Aurora A, which are negative p53 regulators were decreased; p53 protein stability was enhanced, while p53 mRNA expression levels decreased slightly and ATF3 mRNA expression apparently increased. Knockdown of the ATF3 gene by siRNA partially suppressed p53, caspase 3, S-phase arrest and apoptosis triggered by compound **40**.

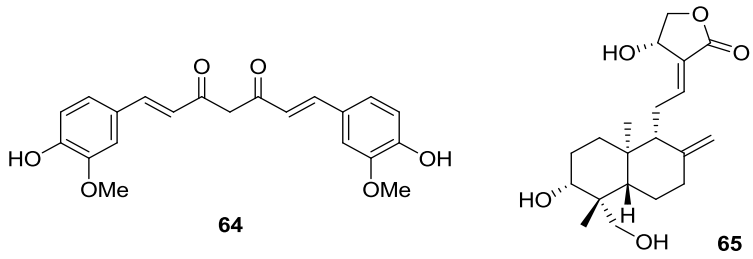


Figure 9. Structures of curcumin and andrographbolid.

Since ancient times, *Andrographis paniculata* (Burn. F.) Wall. ex Ness (Acanthaceae) has been used in the traditional Siddha and Ayurvedic systems of medicine, as well as in tribal medicine in India and other countries for multiple clinical applications. It is widely cultivated in Southern and Southeastern Asia, where it has been traditionally used to treat infections and some diseases. The leaves and roots have primarily been used for medicinal purposes. Andrographolide (**65**) is a labdane-type diterpenoid, which is the main bioactive compound in *A. paniculata* [31]. **65** is an extremely bitter substance isolated from the stem and leaves of *A. paniculata*. **65** is used experimentally in different areas of research including cell signaling, immunomodulation, and stroke [31−33]. Studies have shown that **65** may bind to a spectrum of protein targets by covalent modification in cancer cells, including NF-κB and actin [34].

Sukumari-Ramesh et al. reported a novel cytotoxic role for the dietary compounds curcumin, andrographolide, wedelolactone, dibenzoylmethane and tanshinone IIA in the human S-type neuroblastoma cells SK-N-AS and SK-N-BE(2) [35]. Mechanistically, cell death appeared to be apoptotic on flow cytometry; however, these effects proceeded independently of both caspase-3 and p53 activation, as assessed by both genetic (shRNA) and pharmacological approaches. Notably, cell death induced by both **64** and **65** was associated with decreased NF-κB activity and a reduction in B-cell lymphoma-2 (Bcl-2) and B-cell lymphoma-extra large (Bcl-xL) expression. Finally, **64** and **65** increased cytotoxicity following co-treatment with either cisplatin or doxorubicin, two chemotherapeutic agents widely used in the clinical management of neuroblastoma. Given the proven clinical safety of these compounds, dietary NF-κB inhibitors, such as **64** and **65**, may represent a potent medical adjunct for the treatment of neuroblastoma, warranting further investigation. Coupled

with the documented safety in humans, dietary compounds may represent a potential adjunct therapy for neuroblastoma.

7. Steroids and Terpenoids

Sterols, also known as steroid alcohols, are a subgroup of steroids and comprise an important class of organic molecules. They occur naturally in plants, animals and fungi, with the most familiar type of animal sterol being cholesterol. Cholesterol is vital to animal cell membrane structure and function, and is a precursor to fat-soluble vitamins and steroid hormones. Plant sterols are known as phytosterols and animal sterols are known as zoosterols. Notable phytosterols include campesterol, sitosterol and stigmasterol. More than 200 sterols and related compounds have been identified to date [36].

The terpenoids, sometimes called isoprenoids, are a large and diverse class of naturally occurring organic chemicals similar to terpenes, derived from five-carbon isoprene units assembled and modified in thousands of ways. Most are multicyclic structures that differ from one another in both functional groups and basic carbon skeletons. These lipids can be found in all classes of living things, and are the largest group of natural products. They play a role in traditional herbal remedies and are under investigation for antibacterial, antineoplastic, and other pharmaceutical functions. Well-known terpenoids include citral, menthol, camphor, salvinorin A in the plant Salvia divinorum, the cannabinoids found in cannabis, ginkgolide and bilobalide found in *Ginkgo biloba*, and glycyrrhizin found in licorice. Terpenes are hydrocarbons resulting from the combination of several isoprene units. Terpenoids can be thought of as modified terpenes, wherein methyl groups have been moved or removed, or oxygen atoms added.

Hemiterpenoids, 1 isoprene unit (5 carbons (C))
Monoterpenoids, 2 isoprene units (10 C)
Sesquiterpenoids, 3 isoprene units (15 C)
Diterpenoids, 4 isoprene units (20 C)
Sesterterpenoids, 5 isoprene units (25 C)
Triterpenoids, 6 isoprene units (30 C)
Tetraterpenoids, 8 isoprene units (40 C) (carotenoids)

7.1. Steroids

Withania somnifera (Solanaceae), known commonly as ashwagandha, Indian ginseng, poison gooseberry or winter cherry, is a plant in the Solanaceae or nightshade family [37]. Several other species in the genus *Withania* are morphologically similar. It is used as an herb in Ayurvedic medicine. The plant's long, brown, tuberous roots are used in traditional medicine [38]. In Ayurveda, the berries and leaves are applied externally to tumors, tubercular glands, carbuncles and ulcers. The roots are used to prepare the herbal remedy ashwagandha, which has been traditionally used for various symptoms and conditions [38,

39]. Withaferin A (**66**) is a steroidal lactone that binds to and inhibits vimentin [40], and was isolated from *W. somnifera* [41].

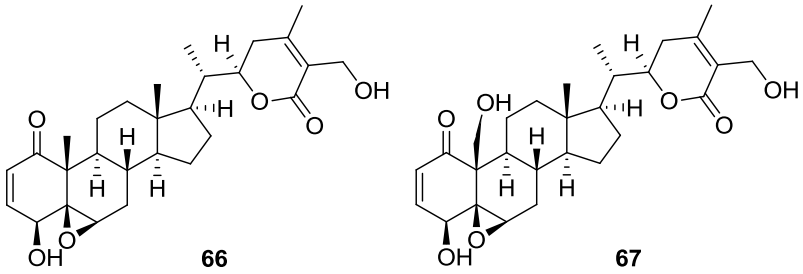


Figure 10. Structures of steroidal lactones from *Withania somnifera*.

Yco et al. found that **66** effectively induces dose-dependent cell death in high-risk and drug-resistant neuroblastoma, as well as multiple myeloma (MM) tumor cells, prevented interleukin-6 (IL-6)-mediated and persistently activated signal transducer and activator of transcription 3 (STAT3) phosphorylation at Y705, and blocked the transcriptional activity of STAT3 [42]. They further provide computational models that show that **66** binds STAT3 near the Y705 phospho-tyrosine residue of the STAT3 Src homology 2 (SH2) domain, suggesting that **66** prevents STAT3 dimer formation similar to BP-1-102, a well-established STAT3 inhibitor. The antitumor activity of **66** is mediated at least in part through inhibition of STAT3 and provides a rationale for further drug development and clinical use in neuroblastoma and MM.

Motiwala et al. reported that semisynthetic acetylated analogues of withalongolide A (**67**) are considerably more cytotoxic than the parent compound [43]. To further explore the structure-activity relationships, 20 new semisynthetic analogues of **67** were synthesized and evaluated for cytotoxic activity against four different cancer cell lines. A number of derivatives were found to be more potent than the parent compound and **66**.

Zhao et al. reported that five new withanolide derivatives were isolated from the roots of *W. somnifera* together with fourteen known compounds [44]. Of the isolated compounds, **66** and 5 compounds showed significant neurite outgrowth activity at a concentration of 1 μM in a human neuroblastoma SH-SY5Y cell line.

Kataria et al., based on the limited toxicity of *W. somnifera*, assessed the efficacy of *W. somnifera* water extract (WSWE) for anti-proliferative potential in neuroblastoma and its underlying signaling mechanisms [45]. WSWE significantly reduced cell proliferation and induced cell differentiation as indicated by morphological changes and NF200 expression in human IMR-32 neuroblastoma cells. The induction of differentiation was accompanied by HSP70 and mortalin induction as well as pancytoplasmic translocation of the mortalin in WSWE-treated cells. Furthermore, WSWE treatment led to induction of neural cell adhesion molecule (NCAM) expression and reductions in its polysialylation, thus confirming its anti-migratory potential, which was also supported by downregulation of matrix metalloproteinase- (MMP-)2 and -9 activity. WSWE treatment led to cell cycle arrest at the G_0/G_1 phase and increases in the early apoptotic population. Modulation of cell cycle marker Cyclin D1, anti-apoptotic marker bcl-xl, and Akt-P, suggest that WSWE may prove to be a promising phytotherapeutic intervention in neuroblastoma-related malignancies.

7.2. Diterpenoids

Tabata et al. reported that the antitumor effects of five phenolic diterpenes (**68**-**71**,**73**) derived from *Hyptis incana* (Lamiaceae), a Brazilian medicinal plant, were examined in neuroblastoma cells [46]. All of the examined compounds (**68**-**71**,**73**) exhibited significant cytotoxicity towards neuroblastoma cells. In particular, 7-ethoxyrosmanol (**70**) had a high degree of efficacy. Nuclear condensation and degradation of procaspase-3 and -9 were observed after treatment of the cells with these compounds. Moreover, phenolic diterpenes induced cell-cycle arrest in the G2/M phase. Rosmanol (**68**) and epirosmanol (**71**) tended to induce differentiation.

On the other hand, Costa-Lotufo et al. reported that two abietane-type diterpenes were isolated from a hexane extract of *Hyptis martiusii* roots and were identified as carnasol (**72**) and 11,14-dihidroxy-8,11,13-abietatrien-7-one (**74**) [47]. Both compounds displayed cytotoxic activity against tumor cell lines, but only **72** was able to inhibit sea urchin egg cleavage.

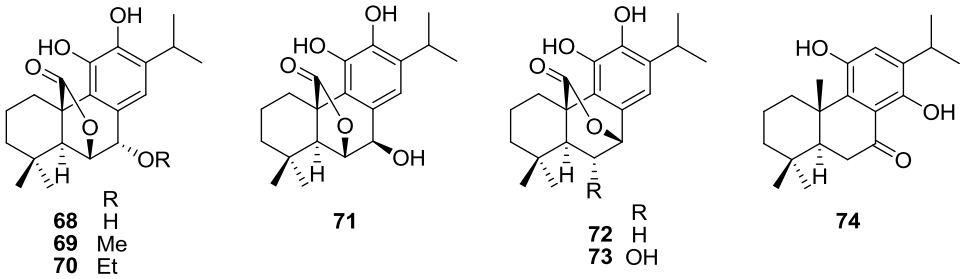


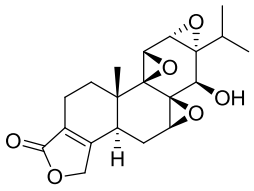
Figure 11. Structures of phenolic diterpenes from *Hyptis incana*.

Tripterygium wilfordii (Celastraceae) is a vine used in traditional Chinese medicine for treatment of fever, chills, edema and carbuncle. *T. wilfordii* has recently been investigated as a treatment for a variety of disorders, Including rheumatoid arthritis, cancer, chronic hepatitis, chronic nephritis, ankylosing spondylitis, polycystic kidney disease and several skin disorders. It is also under investigation for its apparent antifertility effects, which may provide a basis for a male oral contraceptive [48].

Triptolide (75) is a diterpenoid epoxide found from *T. wilfordii*. It has *in vitro* and *in vivo* activities against mouse models of polycystic kidney disease [49] and pancreatic cancer, but its physical properties limit its therapeutic potential [50]. Consequently, a synthetic prodrug, Minnelide, is instead being studied clinically.

Antonoff et al. reported that residual tumors from triptolide-treated mice showed minimal staining with Hsp-70 immunohistochemistry, while control tumors were stained prominently [51, 52]. Tumors from treated mice demonstrated marked staining on transferase-mediated dUTP nick-end labeling (TUNEL) assay, while control tumors showed no evidence of apoptosis. Neuroblastoma is thought to result from failure of embryonal precursors to undergo programmed cell death, and it is believed that altered cellular responses to apoptosis may be involved in the drug resistance seen in more aggressive neuroblastoma tumors. The results demonstrated that exposure of BE(2)-C human neuroblastoma cells to **75** resulted in a reduction in cell growth and proliferation, and the induction of cell death and apoptosis,

together with cell cycle arrest in the S phase. A soft agar assay indicated that **75** inhibited the colony-forming ability of BE(2)-C neuroblastoma cells. Xenograft experiments showed that **75** significantly reduced tumor growth and development *in vivo*.



75

Figure 12. Structure of triptolide from *Triperygium wilfordii*.

Yan et al. demonstrated that **75** not only induced neuroblastoma cell death and apoptosis via caspase-9/caspase-3 pathway activation, but also inhibited cell growth and viability by inducing cell cycle arrest at the S phase [53]. Furthermore, the results showed that **75** inhibited neuroblastoma cell colony-forming capability *in vitro* and tumor progression *in vivo*.

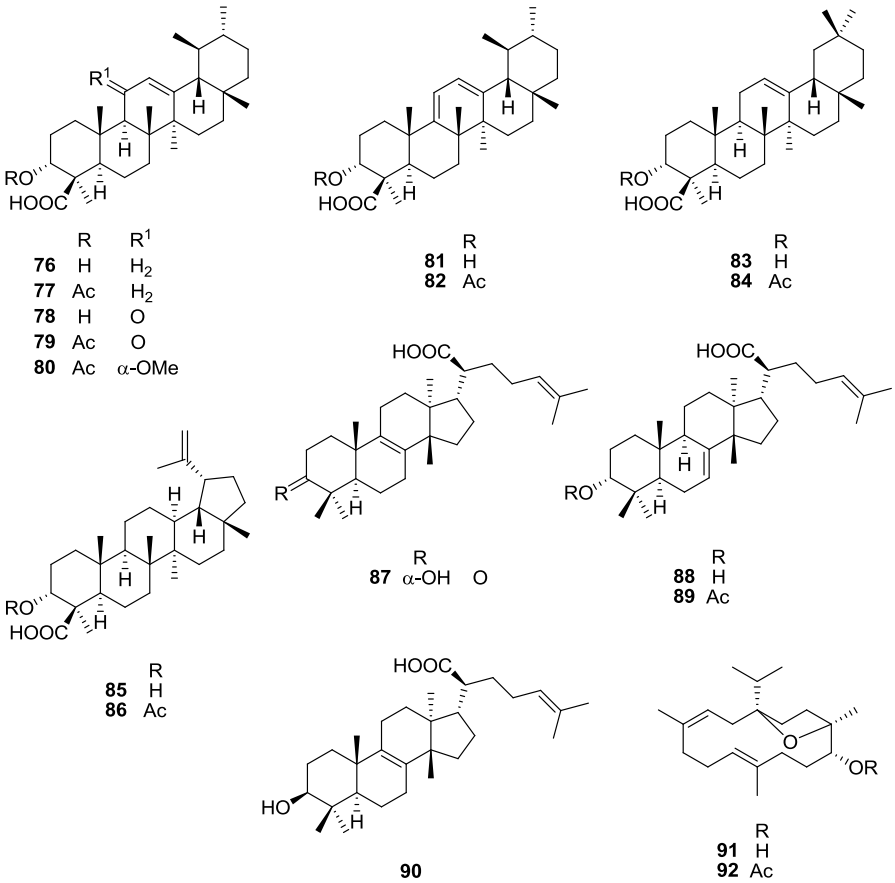


Figure 13. Structures of triterpenoids and diterpenoids from *Boswellia carteri*.

7.3. Triterpenoids

Akihisa et al. reported that fifteen triterpene acids, viz., seven of the β-boswellic acids (ursane-type) (**76**-**82**), two of the α-boswellic acids (oleanane-type) (**83**, **84**), two of the lupeolic acids (lupane-type) (**85**, **86**), and four of the tirucallane-type (**87**-**90**), and two cembrane-type diterpenes (**91**, **92**), isolated from the MeOH extract of the resin of *Boswellia carteri* (Burseraceae), together with a triterpene acid **89** (acetyl derivative of **88**), were examined for their inhibitory effects on cytotoxic activities against three human neuroblastoma cell lines, IMR-32, NB-39 and SK-N-SH *in vitro*[54]. Furthermore, fifteen compounds, **76**-**82**, **84**-**86**, **88**-**90**, **17** and **18**, exhibited potent cytotoxic activities with IC_{50} values of 4.1-82.4 μM against all three human neuroblastoma cells tested.

Tabata et al. reported that kuguaglycoside C (**94**) is a triterpene glycoside isolated from the leaves of *Momordica charantia*. **94** also significantly increased the expression and cleavage of apoptosis-inducing factor (AIF) [55]. Moreover, it was found to induce caspase-independent DNA cleavage in the dual-fluorescence apoptosis detection assay. **94** induces caspase-independent cell death, and is involved, at least in part, in the mechanisms underlying cell necroptosis. In the final phase of the caspase-dependent apoptosis pathway, DFF45 is degraded by caspase-3 and -7 to activate DFF40, a type I DNase, which hydrolyses DNA into oligonucleotides bearing a 3'-OH end.

Pitchakarn et al. focused on the *in vitro* effects of Kuguacin J (**93**), a purified component of bitter melon *M. charantia* leaf extract (BMLE), on the androgen-independent human prostate cancer cell line PC3 and the *in vivo* effects of dietary BMLE on prostate carcinogenesis using a PC3-xenograph model [56]. **93** exerted a strong growth-inhibitory effect on PC3 cells. Growth inhibition was mainly through G1-arrest; but, **93** markedly decreased the levels of cyclins (D1 and E), cyclin-dependent kinases (Cdk2 and Cdk4) and proliferating cell nuclear antigen. Interestingly, **93** also markedly decreased the levels of survivin expressed by PC3 cells. In addition, **93** exerted anti-invasive effects on PC3 cells, significantly inhibiting migration and invasion; **93** also inhibited secretion of the active forms of MMP-2, MMP-9 and uPA by PC3 cells. Furthermore, **93** treatment significantly decreased the expression of membrane type 1-MMP (MT1-MMP) by PC3 cells. Thus, **93** is a promising chemopreventive and chemotherapeutic agent for prostate and breast cancers [14].

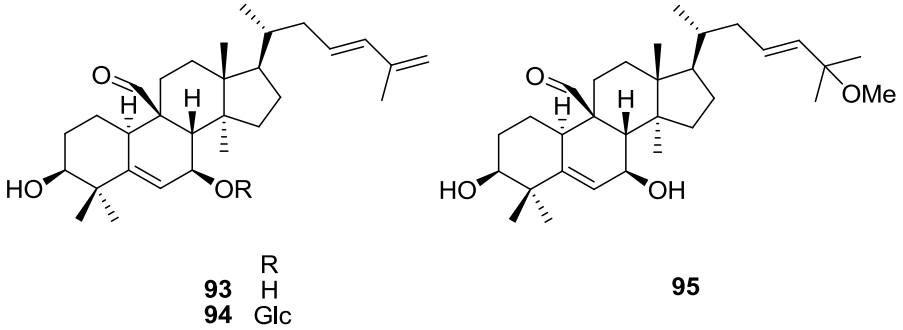


Figure 14. Structures of triterpenoids from *Momordica charantia*.

Weng et al. reported that 3β,7β-dihydroxy-25-methoxycucurbita-5,23-diene-19-al (**95**), a cucurbitane-type triterpene isolated from *M. charantia*, induced apoptotic death in breast

cancer cells through peroxisome proliferator-activated receptor (PPAR)-γ activation. Moreover, **95** inhibited mTOR-p70S6K signaling through Akt downregulation and adenosine monophosphate kinase (AMPK) activation [57].

Acknowledgment

Dr. Keiichi Tabata, co-author and Associate Professor at the School of Pharmacy, Nihon University, passed away on January 13, 2015 (41 years-old). We considered him to be a good researcher and educator. This article is posthumously dedicated to Dr. Tabata with profound gratitude.

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