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Recent Development of Antitumor Agents from Chinese Herbal Medicines; Part I. Low Molecular Compounds

Abstract

A series of low molecular compounds from Chinese herbal medicines which have proved to be, in some cases, highly effective especially in tumor therapy, is listed here (part II will deal with high molecular compounds, to be published in the next issue). In contrast to synthetic agents used in cancer chemotherapy, these natural compounds have relatively low toxicities. Many of the clinical studies referred to in this paper have been carried out on Asians. Because genetic factors influence enzyme levels, sometimes leading to striking differences in metabolism and pharmacokinetics of drugs, results obtained in clinical studies carried out in China are not 100% transferable to the European population. The mechanisms of action of these compounds are manifold, consisting of reactions with DNA bases, intercalation in DNA, inhibition of topoisomerases, inhibition of protein kinases, induction of apoptosis etc. Some of the compounds have interesting structural features, that may be used as lead structures for the development of further antitumor agents.

Key words
Antitumor agents · Chinese herbal medicines · low molecular natural compounds · traditional Chinese medicine

Introduction

As a consequence of the experience gathered over thousands of years, Chinese herbal medicines are considered as a rich source for the discovery of new drugs. In the search of new therapeutic agents, many compounds with new structural features and mechanisms of action have been isolated from Chinese herbal medicines. In recent years, a number of Chinese herbs and their active principles were reported to be antineoplastic and cytotoxic both in experimental and clinical studies. The cytotoxic and antineoplastic mechanisms of these compounds involve DNA intercalation, inhibition of DNA topoisomerases and protein kinases, induction of apoptosis, covalent binding to enzymes of biological importance and some unknown mechanisms. The natural compounds are relatively non-toxic as compared to some synthetic agents. In the present paper, a summary of the most important findings on cytotoxic and antineoplastic compounds of low molecular weight from Chinese herbal medicines is given in order to contribute to the further development of antitumor drugs.

Alkaloids

Phenanthridine Alkaloids

Lycobetaine (ungeremine, AT-1840), a quaternary phenanthridinium alkaloid from some Amaryllidaceous plants, such as *Lycoris radiata* Herb. [1] was reported to be antineoplastic. In reviews and in meeting reports [2], [3], lycobetaine was mentioned to be active in the treatment of cervical, ovarian, gastric and other can-

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Dedication
In memory of Prof. Dr. Dietrich Schmähl, Deutsches Krebsforschungszentrum, Heidelberg

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cers in clinical trials. The overall response in 233 cases of different cancers was reported to be about 35% [3]. No significant myelotoxic, cardiotoxic and hepatotoxic side effects have been observed [2].

In experimental studies, lycobetaine was found to inhibit the growth of S180 [4] and KB [5] tumor cell lines *in vitro* and to be significantly active against Ehrlich ascites carcinoma, ascites hepatoma, leukemias L1210 and P388, Lewis lung carcinoma and Yoshida ascites sarcoma in mice or in rats by *i.p.* injection. In nude mice bearing human gastric cancer xenografts, lycobetaine extended the survival time and decreased the tumor size. Moreover, it was shown to have a direct cytocidal effect on stomach cancer cells *in vitro* even at low concentrations and to arrest carcinoma cells in the G_2/M phase as demonstrated by flow cytometry [6].

A study on the interaction of lycobetaine with calf thymus DNA revealed that lycobetaine intercalates with DNA base pairs, especially the GC-pair [7]. Lycobetaine did not bind covalently to DNA [8]. Studies on structure-activity relationships revealed that the calculated interaction energy of lycobetaine analogues with double-stranded oligonucleotides correlated with their anticancer potency [9], [10]. According to the 3-dimensional structure patterns of the drug-oligonucleotide complex, the quaternary nitrogen atom in lycobetaine plays an important role in the formation of hydrogen bonds between the compound and the oligonucleotide [11]. The betaine and a methylenedioxy group in lycobetaine are supposed to be critical for its antitumor activity [2].

Recently, we have found that lycobetaine is toxic for topoisomerases I and II, and that it strongly inhibits the growth of human tumor xenografts *in vitro* and *in vivo*. The IC_{50} values of lycobetaine in the clonogenic assay against 21 human tumor xenografts of various tumor types range from 0.002 to 27.5 μM with a mean IC_{50} of 0.8 μM. Intraperitoneal administration of lycobetaine at a dose of 30 mg/kg on days 1 – 5 and 8 – 12 to nude mice bearing the large cell lung carcinoma LXFL529 resulted in a significant growth delay of the tumors [12], corresponding to about 40% of the LD_{50} by single *i.p.* injection [13]. Lycobetaine was localized predominately in the nucleus, it competed with ethidium bromide for intercalation into calf thymus DNA and displaced the DNA minor groove binder Hoechst 33258. At growth inhibitory concentrations, lycobetaine inhibited topoisomerases I and II, stabilized the covalent DNA-topoisomerase I intermediate, the so-called cleavable complex, and induced apoptosis. Dose-dependent induction of DNA strand breaks was detected by single cell gel electrophoresis (comet assay) [12].

Lycobetaine is structurally related to the phenanthridine alkaloid lycorine as a major Amaryllidaceous constituent and can be easily obtained in high yield from this constituent by oxidation with SeO_2 [2]. Lycorine was also reported to be cytotoxic [14]. Recently, lycorine has been found to be highly active in inhibiting a number of human tumor cell lines *in vitro*, including LXFL529L, Molt4, HL60, K562, U937 [15], GXF251L, and CXF94L [16]. In contrast to lycobetaine, lycorine did not compete with ethidium bromide for intercalation into calf thymus DNA and did not displace the DNA minor groove binder Hoechst 33258, indicating that its cytotoxic mechanism is different from that of lycobetaine [15], [16]. In addition, lycorine was reported to induce flat morphology in K-*Ras*-transformed fibroblasts *in vitro* [17].

Benzo[*c*]phenanthridine Alkaloids

Among the benzo[*c*]phenanthridine alkaloids, nitidine, fagaronine and ethoxychelerythrine are the major components in Radix Zanthoxyli (Liangmianzhen), the root of *Zanthoxylum nitidum* (Roxb.) DC (Rutaceae) [18] which is listed in the Chinese Pharmacopoeia [19]. Chelerythrine, chelidonine and the related benzo[*c*]phenanthridine alkaloid sanguinarine are known to be the major components in the whole plant of *Chelidonium majus* L. (Papaveraceae), a medicinal herb used in European countries and in China [20].

Nitidine was reported to possess antineoplastic activity against both L1210 and P388 leukemias, Lewis lung carcinoma, and B16 melanoma in mice [21]. It increased the life span of mice inoculated with Ehrlich ascites tumor, caused a decrease in the mitotic index and size of the tumor cells, and inhibited DNA and RNA synthesis in tumors [22]. Ethoxychelerythrine also showed inhibitory activity against Ehrlich ascites carcinoma cells [23]. Nitidine chloride was noted to be effectively used in the clinical treatment of chronic myelocytic leukemia [24].

Nitidine and fagaronine were reported to bind to calf thymus DNA by intercalation [25] and to be toxic to topoisomerases I and II [26]. Nitidine exhibited strong stabilization of the covalent binary complex formed between topoisomerase I and DNA [18]. Yeast cells expressing human DNA topoisomerase I were specifi-

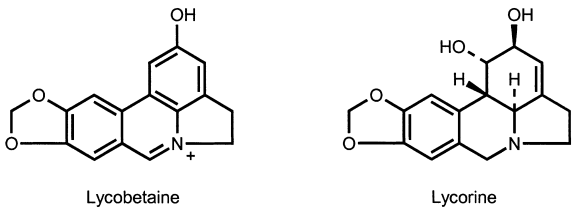


Fig. **1** Lycobetaine and lycorine.

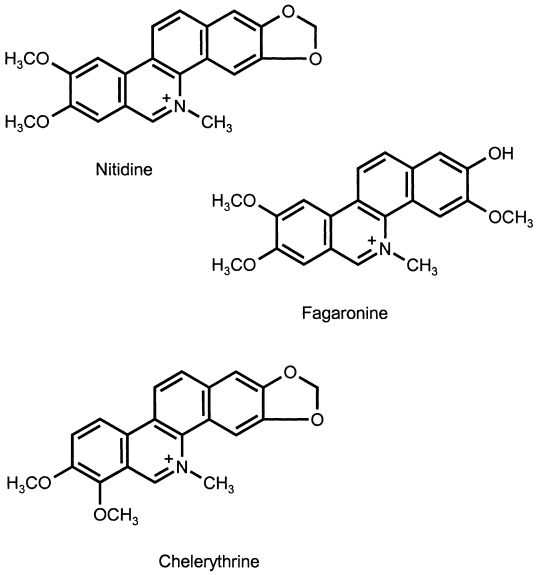


Fig. **2** Nitidine, fagaronine, and chelerythrine.

cally sensitive to nitidine [27]. Unlike camptothecin as reference compound, nitidine and fagaronine bound directly to and mediated the unwinding of B form DNA. Inhibition of topoisomerase II by nitidine was observed at the high concentration of 40 μM in comparison to the inhibition of topoisomerase I at 0.15 – 0.3 μM [28].

The antitumor mechanism of fagaronine is also believed to be due to inhibition of topoisomerases [29], [30]. Fagaronine is a DNA major groove intercalator, does not show any sequence specificity of DNA intercalation, but its highly electronegative oxygen of the hydroxy group is shown to be an acceptor of the hydrogen bond of the amino group of guanine in DNA [31]. In contrast, *N*-demethylfagaronine was neither cytotoxic, nor effectively interacted with DNA, suggesting that the quaternary nitrogen atom is an important prerequisite for its biological efficacy [32].

Chelerythrine was reported to be a specific protein kinase C (PKC) inhibitor [33], [34]. It completely suppressed the growth of GI-101A breast tumor cells stimulated by hydroxychloroquine and prednisone [35], blocked the expression of vascular endothelial growth factor (VEGF) mRNA in GI-101A and HL-60 cells stimulated by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or diethylstilbestrol [36], and inhibited the increased proliferation of MCF-7 cells stimulated by thymeleatoxin [37]. Chelerythrine also inhibited the proliferation of the human prostate cancer cell line, PC3 [38], and gastric cancer cell line, AGS [39]. Incubation of AGS cells with chelerythrine resulted in an arrest of cells in the G_0/G_1 phase, in induction of apoptosis and in an elevation of p53, p21, c-*Myc*, and *Bax* [39]. Chelerythrine was further reported to exhibit cytotoxic activity against a series of radioresistant and chemoresistant human squamous cell carcinoma lines. The carcinoma cells undergo apoptosis rapidly after treatment with chelerythrine *in vitro*. The antitumor effect of chelerythrine was also demonstrated in nude mice against a radioresistant, chemoresistant and p53-deficient human head and neck squamous cell carcinoma line SQ-20B with significant tumor growth delay and minimal toxicity (40). Chelerythrine also inhibited taxol-mediated polymerization of rat brain tubulin and inhibited colchicine and podophyllotoxin binding to tubulin with IC_{50} values of about 60 μM [41].

Sanguinarine was reported to be a potent inhibitor of the catalytic subunit of rat liver protein kinase A (PKA) with an IC_{50} of 6 μM, but a relatively poor inhibitor of PKC with an IC_{50} of greater than 200 μM [42]. Sanguinarine potently inhibited the growth of human keratinocytes with an IC_{50} of 0.2 μM [43]. It decreased concentration-dependently the viability of human epidermoid carcinoma A431 cells at lower concentrations than of normal human epidermal keratinocytes. Sanguinarine treatment of A431 cells resulted in an induction of apoptosis but did not lead to formation of a DNA ladder in normal keratinocytes, even at higher concentrations [44]. Sanguinarine interacted with calf thymus DNA and altered its secondary structure. The maximum of sanguinarine-binding to DNA in buffer of low ionic strength and acidic pH and the number of bound alkaloid molecules per base pair, at saturation, is higher in G-C rich DNA than in A-T rich DNA [44].

Protoberberine Alkaloids

Like benzo[*c*]phenanthridine, protoberberine is also a four-ring system but with one saturated bond. Protoberberine alkaloids occur in numerous plants, including several medicinal plants. Berberine is the most studied protoberberine alkaloid, widely distributed in plants used in traditional Chinese medicine. Plant items listed in the Chinese Pharmacopoeia containing berberine and related protoberberine alkaloids are Rhizoma Coptidis (Huanglian), the rhizome of *Coptis chinensis* Franch., *C. deltoidea* C.Y. Cheng et Hsiao, or *C. teeta* Wall. (Ranunculaceae); Caulis Mahoniae (Ganglaomu), the stem of *Mahonia healei* (Fort.) Carr. or *M. fortunei* (Lindl.) Fedde (Berberidaceae) and Cortex Phellodendri (Huangbo), the stem bark of *Phellodendron chinense* Schneid. or *P. amurense* Rupr. (Rutaceae).

Berberine was reported to possess significant cytotoxicity against some human cancer cell lines, P388 murine leukemia cells [45], and 9L rat glioma cell line [46]. Recently, Iizuka et al. have reported that the extract of Rhizoma Coptidis and berberine significantly inhibited the proliferation of six esophageal cancer cell lines *in vitro* in a concentration-dependent manner [47].

Chi et al. reported on a flow-cytometric study of the effect of berberine on human hepatoma $HepG_2$ cells. Continuous exposure of $HepG_2$ cells to berberine resulted in a concentration-dependent growth inhibition of the cells. Berberine treatment caused a significant reduction of the S phase fraction of $HepG_2$ cells [48] and an arrest of gastric cancer cells in the G_2/M phase [49]. Esophageal cancer cells treated with the extract of Rhizoma Coptidis showed an accumulation in the G_0/G_1 phase and a relative decrease of the S phase [47]. Concentration-dependent effects of berberine were also reported on cell cycle and apoptosis in Balb/c 3T3 cells [50]. Chang et al. reported that berberine downregulated K-*Ras2* gene expression associated with morphologic differentiation in human embryonal carcinoma cells [51]. Furthermore, berberine and some related protoberberine alkaloids induced apoptosis in murine thymocytes [52] and in promyelocytic leukemia HL60 cells [53]. Berberine was also found to be a

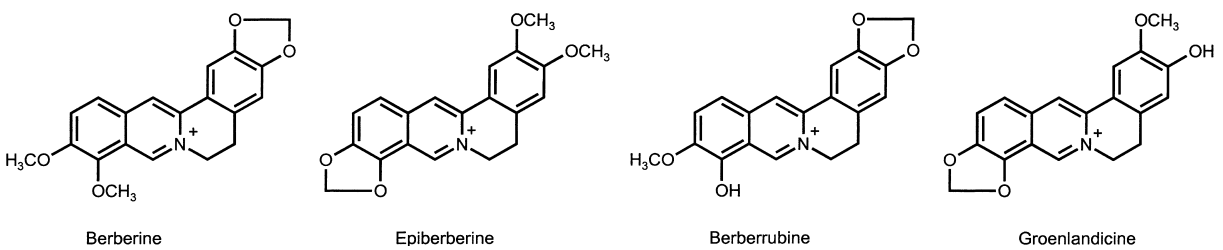


Fig. **3** Berberine, epiberberine, berberrubine, and groenlandicine.

DNA triplex binder [54] and to enhance the cytotoxicity of clinically used antitumor agents, such as nucleotides [55] and nitrosoureas [56] *in vitro*. It was also reported that berberine up-regulated the multidrug-resistant transporter (pgp-170) expression in two oral (KB, OC2), two gastric (SC-M1, NUGC-3) and two colon (COLO 205, CT 26) cancer cell lines [57], [58].

The artifact berberrubine, formed by demethylation of berberine, was found to be highly cytotoxic against P388 leukemia, L1210 leukemia, B16 melanoma and some human cancer cell lines [59]. Makhey et al. reported that berberrubine functions as a potent inhibitor of topoisomerase II *in vitro* [60]. The protoberberine alkaloids epiberberine and groenlandicine were identified as topoisomerase I inhibitors *in vitro* [61]. Topoisomerase II-mediated DNA cleavage assays showed that berberrubine poisons the enzyme by stabilizing the enzyme-DNA complexes. Berberrubine induced DNA cleavage in a site-specific and concentration-dependent manner. Comparison of the cleavage pattern of berberrubine with that of etoposide revealed that berberrubine represents a new class of antitumor agent with topoisomerase II poisoning activity as well as catalytic inhibition. The step at which berberrubine induces a cleavable complex is possibly different from that of etoposide [62].

1,4-Benzoquinone Derivatives

From the seed of *Iris pallasii* var. *chinensis* (Iridaceae), a 1,4-benzoquinone derivative with an unsaturated aliphatic side chain (irisquinone A), has been isolated [63]. The seed of *I. pallasii* var. *chinensis* is used in Chinese folk medicine as a fertility regulating agent and for the treatment of malignant diseases.

Irisquinone A was found to be cytotoxic and antitumorigenic. In mice tumor xenografts, it showed growth inhibitory activity against cervical cancer U_{14} and Ehrlich carcinoma by *i.p.* application and against lymphosarcoma by *i.p.* and oral administration [64]. Oral treatment of mice bearing U_{14} tumor with irisquinone A at a dosage of 100 mg/kg or *i.v.* treatment at a dosage of 5 mg/kg once every other day for 5 cycles, starting 24 h after implantation of the tumor, caused a tumor inhibition rate of 35 – 55 % [65]. The LD_{50} of irisquinone A in mice was about 28 mg/kg after *i.p.* and about 2.8 g/kg after oral administration. The chemotherapeutic indices (LD_{50}/ED_{50}) of irisquinone A were estimated to be 5 by *i.p.* administration and 14 by oral administration.

Radiosensitizing effects of irisquinone A were found *in vitro* against the tumor cell lines U_{14} [64], S-180V [65], HeLa [66], against Ma 7373 breast cancer cells in mice, and against human intestinal mucoadenocarcinoma in nude mice [66]. The mechanism of this effect was considered to be an inhibition of oxygen consumption and depletion of glutathione in tumor cells [66]. In a clinical trial, irisquinone A, given orally to 558 patients with cancer of the lung and esophagus, or with superficial metastatic cancer during radiotherapy, significantly contributed to the reduction of tumor size and to the prolongation of survival time of the patients [64].

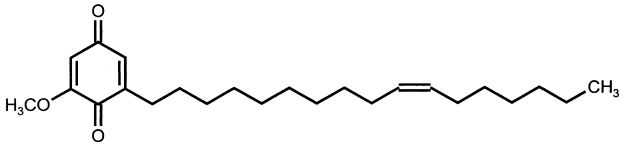
Related 1,4-benzoquinone derivatives were isolated from the Chinese medicinal plant *Ardisia japonica* (Thunb.) Bl. (Myrsinaceae). One of them is embelin, a 2,5-dihydroxy-1,4-benzoquinone with a saturated side chain. Embelin was found to exhibit significant antitumor activity in rats against autochthoneous fibrosarcomas induced by methylcholanthrene and it prolonged the survival time of the animals [67]. An *in vitro* study with embelin using a fibrosarcoma cell line showed a concentration-dependent decrease in thymidine uptake and glutathione levels of the tumor cells [68].

Diterpenes

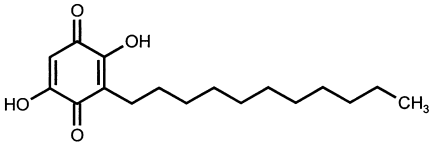
Rabdosia diterpenes

Several *Rabdosia* species (Lamiaceae) native to China are used in folk medicine as antitumor or anti-inflammatory agents. Studies on the chemical constituents of the leaves and stems and on their biological activities have been carried out with various *Rabdosia* species, especially *Rabdosia rubescens* (Hemsl.) Hara. Diterpene compounds, such as oridonin and ponicidin, are derived from kaurane. The diterpenes were found to be the cytotoxic principles in *Rabdosia* species [69].

115 patients suffering from inoperable esophageal carcinoma were treated with chemotherapy alone (group A) or with chemotherapy plus *R. rubescens* (group B); in group A, 10 out of 31 patients (32.3 %) treated with chemotherapy alone, responded to the treatment, including 2 partial responses (greater than 50 % tumor regression) and 8 minimal responses. In group B, 59 out of 84 patients (70.2 %) responded to the treatment, including 10 complete responses (100 % tumor regression), 16 partial and 33 minimal responses. The one-year survival rates of group A and group B were 13.6 % and 41.3 %, respectively. No significant differences were observed between the two groups regarding the side effects alopecia, anorexia, nausea and hyperpyrexia which occurred in more than 30 % of patients [70]. Between August 1974 and January 1987, 650 patients with moderate and advanced esophageal carcinoma were treated with a combination of chemotherapy and *R. rubescens* or *R. rubescens* plus different traditional Chinese patent medicines. Forty patients survived for over 5 years (5-year survival rate 6.15 %); 32 for over 6 years; 23



Irisquinone A



Embelin

Fig. **4** Irisquinone A and embelin.

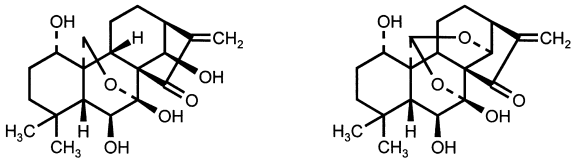


Fig. **5** Oridonin and ponicidin.

for more than 10 years, and 5 for more than 15 years [71]. PC-SPES, a combination of eight herbs including *R. rubescens* has been tested for the treatment of prostate cancer showing significantly decreased serum prostate specific antigen (PSA) of the patients [72], [73]. In a review, it was mentioned that oridonin and ponicidin were tested in clinical trials for the treatment of esophageal cancer [74].

Oridonin was reported to be cytotoxic against Ehrlich ascites carcinoma [75] and L1210 leukemia [76] in mice after *i.p.* injection. The cell killing rates of oridonin (15 mg/kg *i.p.*) on day 5 and day 8 in L1210 cells were 73 % and 39 %. The G_2 and S phases of L1210 cells were prolonged, while the G_1 phase was unchanged [76]. In *in vitro* investigations, oridonin exhibited inhibitory effects on the proliferation of the human gastric adenocarcinoma cell line (MGc80 – 3) and esophageal cancer cell line (CaEs-17) at concentrations below 15 μg/ml [77]. An extract of *Rabdosia rubescens* exerted a cytotoxic effect on cisplatin-sensitive human ovarian cancer A2780 cells with an IC_{50} of about 0.6 mg/mL [78]. Oridonin was found to inhibit DNA, RNA, and protein syntheses in L1210 cells *in vitro* in a concentration-dependent manner. Inhibition of DNA and RNA synthesis was fast but reversible, whereas the inhibition of protein synthesis was strong and long lasting [79]. Oridonin inhibited DNA synthesis also in a cell-free system [80].

Oridonin (7.5 mg/kg for 7 days) and cisplatin (0.4 mg/kg for 4 days) showed synergistic antineoplastic effects in mice bearing Ehrlich ascites carcinoma, S180 or P388 leukemia and there was a distinct growth inhibition of Ehrlich ascites carcinoma cells *in vitro*. In the S180 cell culture, the IC_{50} values of cisplatin were reduced to 1/3.4 and 1/6.7 by cotreatment with oridonin at concentrations of 0.5 and 1 μg/mL. A greater amount of DNA cross-links and DNA-protein cross-links in S180 cells was detected when the cells were treated with cisplatin plus oridonin instead of cisplatin alone [81]. Synergistic antitumor effects of oridonin with bleomycin A_5 were also reported [82]. Several diterpenes which are structurally related to oridonin, were also reported to exert cytotoxic and antitumor activities [83], [84].

The mechanism of oridonin activity was postulated to be due to covalent binding of oridonin to a specific site of enzymes in tumor cells [85]. Oridonin and related diterpenes bearing an α-methylenecyclopentanone structure are electrophilic and can be considered as weak alkylating agents [86]. The reactivity of the α-methylene group was examined using the reaction of oridonin with adenosine and cytidine as nucleic acid model compounds and with thiols, L-cysteine, L-lysine and L-serine as model compounds representing relevant constituents of active centers of enzymes. The reaction with thiols proceeded easily under mild conditions to give the corresponding thioether adducts, the adduct with l-cysteine being formed nearly quantitatively. However, oridonin did not react with adenosine and cytidine under the same conditions [87].

Quassinoids

Fructus Bruceae (Yadanzi), the mature fruit of *Brucea javanica* (L.) Merr (Simaroubaceae), is listed in the Chinese Phamacopoeia and is used as an antimalarial and antidysenteric agent and for the treatment of warts and corns by external application. A number of diterpene quassinoids characterized by an epoxy binding between positions 13 and 20 and a hydroxy function at position 5, were isolated from the fruits of *B. javanica*. Bruceantin and brusatol are two important representatives of these constituents possessing antileukemic activity. Structurally, bruceantin and brusatol differ from each other only in the acid moiety [88].

Kupchan et al. have discovered the antileukemic activity of bruceantin [89]. Bruceantin exerted growth inhibitory effects *in vitro* and *in vivo* against a series of tumor cell lines but did not show significant effects in clinical studies against solid tumors [90], [91], [92].

In contrast, an oil emulsion of the fruits of *B. javanica* was recently reported to show clinical efficacy. From 35 patients with recurrent metastatic gastrointestinal cancers treated with the oil emulsion, 24 patients (46 %) showed a reduction of tumor size and improvement of clinical symptoms [93]. A clinical trial on 68 patients with brain metastasis in lung cancer treated with 10 % oil emulsion combined with radiotherapy resulted in an improvement of living quality and in prolongation of median survival time (15 months) compared to radiotherapy alone (10 months) [94]. Clinical *i.v.* application of the oil emulsion was reported to improve the intracranial hypertension caused by brain metastasis from lung cancer [95].

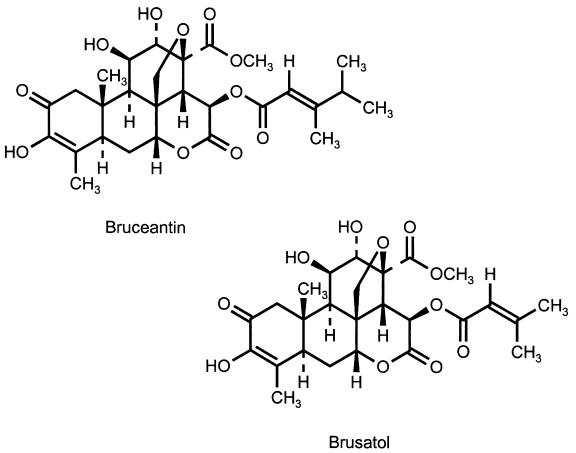


Fig. **6** Bruceantin and brusatol.

Flavones

***Scutellaria* Flavones**

Radix Scutellariae (Huangqin), the root of *Scutellaria baicalensis* Georgi (Lamiaceae), is known to contain a number of flavones,

namely baicalein, wogonin and the corresponding 7-*O*-β-D-glucopyranosiduronides baicalin and wogonosides [96], [97]. It is listed in the Chinese Pharmacopeia and is a well known traditional Chinese medicine.

Baicalin and baicalein were reported to exhibit growth inhibitory activity towards the human hepatoma cell lines PLC/PRF/5 and Hep-G2 as well as the human pancreatic cancer cell line BxPC-3 with an IC_{50} of 20 μg/mL for baicalin and 50 μg/mL for baicalein [98]. Cytotoxic effects of baicalein, baicalin, and wogonin were also observed on the human bladder cancer cell lines KU-1 and EJ-1, and on a murine bladder cancer cell line (MBT-2). All three substances inhibited cell proliferation *in vitro* in a dose-dependent manner with baicalin as the most active compound. An *in vivo* study showed that the root extract of *S. baicalensis* at an oral daily dose of 10 mg per mouse for 10 days significantly inhibited the growth of MBT-2 cells implanted to C3H/HeN mice [99]. We have also found that baicalein, baicalin, wogonin, wogonoside and skullcapflavone II (neobaicalein) inhibited the growth of the human tumor cell lines LXFL 529L (large cell lung carcinoma) and HL60 at a micromolar range [100].

Baicalein strongly inhibited DNA topoisomerase II, probably by stabilizing the covalent enzyme-DNA intermediate in a ternary complex. It also inhibited the proliferation of three human hepatocellular carcinoma cell lines [101]. A structure-activity analysis revealed that flavones with hydroxy groups at the 5, 7, 3′ and 4′ positions favored topoisomerase II-mediated DNA cleavage. Recently, we have found that baicalein also inhibits topoisomerase I by stabilizing the otherwise cleavable complex [100]. Formation of the cleavable complex might contribute to the growth inhibitory activity of flavones on human tumor cell lines [102]. Baicalin was also found to inhibit cell proliferation and to induce apoptosis in several human prostate cancer cell lines [103].

Baicalin, wogonin, skullcapflavone II and wogonoside exhibited only weak inhibitory properties on tyrosine kinase activity of the epidermal growth factor receptor (EGFR) with IC_{50} values exceeding 60 μM. In contrast, we found that baicalein is a potent inhibitor of EGFR with an IC_{50} of 1.1 μM. In A431 cells overexpressing EGFR, baicalein was significantly more active than in other cell lines [100]. Baicalein strongly inhibited human T-lymphoid leukemia cell proliferation with an IC_{50} of about 5 μM. Protein tyrosine kinase activity in human T-lymphoid leukemia cells was significantly reduced by baicalein [104].

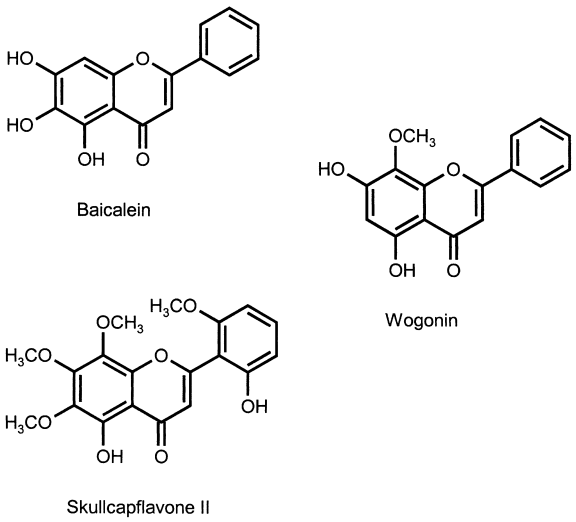


Fig. **7** Baicalein, wogonin, and skullcapflavone II.

Moreover, baicalein has been reported to inhibit the activity of cAMP phosphodiesterases (PDE). We confirmed the PDE inhibitory activity of baicalein and further showed that baicalein is able to inhibit the cAMP-specific isoenzyme family PDE4 with an IC_{50} of 10 μM. The respective glucuronide baicalin is only a weak PDE4 inhibitor. However, when LXFL 529L whole cells were incubated with baicalein, no inhibition of the intracellular PDE activity was observed and the intracellular cAMP level remained unchanged [100].

Baicalein potently inhibited the growth of a human breast carcinoma cell line, MDA-MB-435 with an IC_{50} of about 6 μg/mL and was more active than flavones isolated from citrus fruits including hesperetin and naringenin [105]. Baicalein and related flavones also inhibited the proliferation of estrogen receptor-positive MCF-7 human breast cancer cells; the inhibition was not reversible by an addition of estrogen [106]. Baicalein and wogonin strongly inhibited xanthine oxidase, indicating that they might be useful for the remission of brain tumors, since xanthine oxidase serum levels are increased in tissues of brain tumors [107]. Baicalein as an α-glucosidase inhibitor suppressed *in vitro* invasion and *in vivo* metastasis of mouse melanoma cells [108].

Polymethoxylated Flavones

Fructus Aurantii (Zhiqiao) and Fructus Aurantii immaturus (Zhishi), the immature fruits of *Citrus aurantium* L. (Rutaceae) and its cultivated variants are listed in the Chinese Pharmacopoeia. They contain polymethoxylated flavones such as tangeretin, nobiletin, sinensetin and auranetin.

The polymethoxyflavones from *C. aurantium* were found to possess a wide spectrum of biological activities. Tangeretin, nobiletin and related polymethoxyflavones were reported to exert antiproliferative activities against a number of tumor cell lines [109] and to induce differentiation of HL60 cells *in vitro* in a concentration-dependent manner [110]. HL60 cells treated with these flavones differentiated into mature monocyte/macrophage. A structure-activity study revealed that the *ortho*-catechol moiety in the 2-phenyl nucleus of flavones is a prerequisite for their antiproliferative activity and for the induction of differentiation in HL60 cells. A hydroxy group at C3 and a methoxyl group at C8 enhanced the activity [111]. Tangeretin and nobiletin also inhibited the invasion of mouse MO4 cells into embryonic chick heart fragments *in vitro* [112].

An ethyl acetate extract of orange juice did not affect the initial uptake rate of [^3H]vinblastine by Caco-2 cells but significantly increased the steady-state uptake of cyclosporin A as a P-glycoprotein inhibitor. 3,3′,4′,5,6,7,8-Heptamethoxyflavone, tangeretin and nobiletin were found to be active compounds. They all increased the steady-state uptake of [^3H]vinblastine by Caco-2 cells in a concentration-dependent manner. The ethyl acetate extract and the polymethoxyflavones also increased steady-state [^3H]vinblastine uptake by LLC-GA5-COL300 cells, a cell line

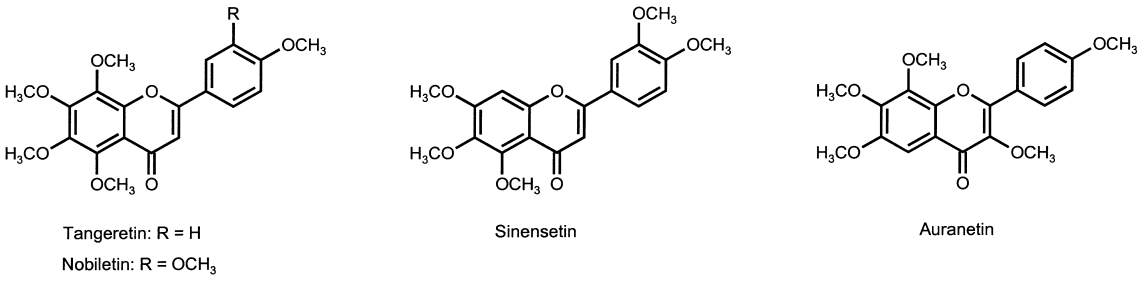


Fig. **8** Tangeretin, nobiletin, sinensetin, and auranetin.

transfected with human MDR1 cDNA [113]. The ethyl acetate extract of grapefruit juice, orange juice and the polymethoxyflavones was also reported to increase the uptake of [^3H]vincristine into adriamycin-resistant human myelogenous leukemia K562/ADM cells [114].

Indigoid Bisindoles

In the 1960's, Chinese scientists began to study the active principle in the Chinese patent medicine "Danggui Longwei Wan" which had been effectively used as an antileukemic agent [115]. This official patent medicine contains 11 ingredients, including *Indigo naturalis*. The antileukemic activity was found to be associated with indirubin, a minor component in *Indigo naturalis* [116]. Indirubin is a bisindole derivative with a 3,2′-linkage, while indigo and isoindigo bisindoles possess 2,2′- and 3,3′-linkages, respectively. In experimental studies, indirubin inhibited Lewis lung carcinoma in mice and W256 sarcoma in rats [117].

Treatment of 314 patients suffering from chronic myelocytic leukemia (CML) with indirubin at daily oral doses of 300 – 450 mg per patient resulted in 82 complete remissions (26%), 38 partial remissions (12%) and 87 beneficial effects (28%). The overall response was 87% [118]. In a further study, treatment of CML patients with indirubin for 1.5 – 6 months markedly increased 5′-nucleotidase activity of leukocytes in some cases with a palliative effect [119]. In another clinical trial including 57 cases of CML, treatment with indirubin resulted in a median survival of 31.5 months without obvious side effects [120].

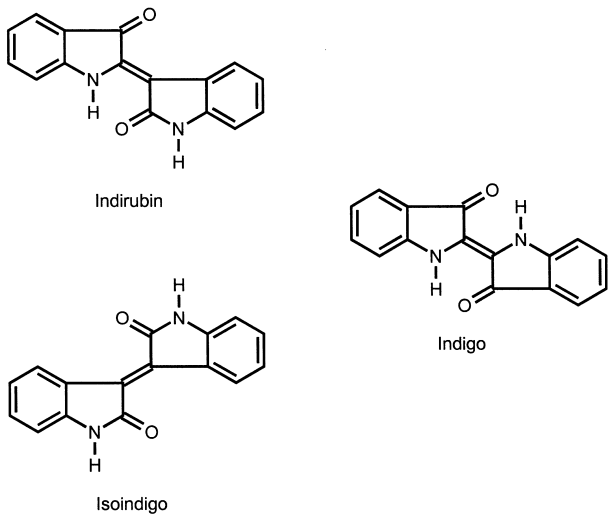


Fig. **9** Indirubin, indigo, and isoindigo.

Because of the manifold findings on antileukemic effects of indirubin, different studies were carried out on its mechanisms of action. Indirubin turned out to inhibit cell-free DNA synthesis catalyzed by partially purified DNA-dependent DNA polymerase from Ehrlich ascites tumor cells in a concentration-dependent manner. This inhibition could not be reversed by increasing the DNA concentration, but could be abolished by increasing the enzyme concentration. The strongest inhibition resulted after preincubation with the enzyme and DNA, suggesting that indirubin, DNA and the enzyme formed a tertiary complex [121]. The DNA polymerase 1 activities of chronic myelocytic leukemia cells from the peripheral blood of patients decreased significantly after treatment with indirubin. Similar results have been observed with *Escherichia coli* DNA polymerase 1 *in vitro* [122].

Intraperitoneal or *s.c.* administrations of indirubin at a daily dose of 200 mg/kg partially inhibited the incorporation of [^3H]thymidine into the DNA of W256 sarcoma cells but not the incorporation of [^3H]uridine into RNA and of [^{14}C]phenylalanine into protein [123]. [^3H]Thymidine incorporation into DNA of liver and spleen of healthy control mice was not affected by *i.p.* injection of indirubin, whereas hepatic and splenic [^3H]thymidine incorporation was inhibited in mice bearing L7212 leukemia [124]. After incubation of indirubin with calf thymus DNA, the λ_{max} 207 nm of indirubin shifted towards a longer wavelength with decreasing absorbance. The binding of indirubin to DNA was rather weak, since indirubin molecules were easily released during precipitation with alcohol or gel filtration. The amount of bound [^3H]indirubin was concentration dependent. Calf thymus DNA bound 46 indirubin molecules per 1000 nucleotides [125].

Electron microscopic examination of peripheral blood samples and leukocytes of bone marrow from patients with chronic granulocytic leukemia treated with indirubin showed a swelling of the cell nucleus membrane, swelling and degeneration of the rough endoplasmic reticulum, and condensation of chromosomes [126]. Measuring the labelling index of bone marrow cells from patients with chronic myelocytic leukemia *in vitro* using [^3H]thymidine before and after indirubin treatment revealed a decrease of labelling index, the decrease being most significant in myelocytes and polychromatophilic erythroblasts [127].

Electron spin resonance spectrometry demonstrated that the C:C double bond obviously plays an important role in the anticancer activity of indirubin and indigo [128]. Recently, we have found that indirubin and its analogues are potent inhibitors of cyclin-dependent kinases (CDKs). The crystal structure of CDK_2 in a complex with indirubin derivatives showed that indirubin interacts with the kinase's ATP-binding site through van der Waals interactions and three hydrogen bonds [129], [130], [131]. In fact, a number of structures closely related to indirubin were reported to be cyclin-dependent kinase inhibitors [132]. Cell cycle molecular targets are being used for the development of new anticancer drugs [133].

Subacute toxicity testing of indirubin administered orally at 100 – 400 mg/kg per day to rats for 30 days showed no effects on leukocytes or on liver or renal function. Oral application of 80 mg/kg of indirubin per day for 1 – 3 months to rats caused anorexia and diarrhea, but no effects on bone marrow. Long-term administration of high doses of indirubin did not affect the hematopoietic stem cell production or DNA formation [134]. A subacute toxicity study of indirubin in dogs receiving three different doses showed no adverse effects at a low daily dosage (20 mg/kg). At a middle daily dosage of 100 mg/kg, mild diarrhea occurred within 10 – 30 days. Serum glutamic-pyruvic transaminase (GPT) levels were slightly elevated after 5 months in one of three dogs treated. At a high daily dosage (200 mg/kg), serious diarrhea and hematofecia occurred during 40 – 60 days and serum GPT levels were elevated after 3 months of treatment. Pathological changes were also observed in all tissue sections obtained after 6 months [135]. Bone marrow and blood indices, renal function, and electrocardiogram were not affected by any of the three dosages [136]. Therefore, indirubin was believed to be safe at clinical daily doses of 150 – 200 mg. The slight liver toxicity observed in dogs receiving 100 mg/kg corresponds to a daily dose of 2240 mg in humans [135]. In human peripheral lymphocytes [137] and in bone marrow cells of patients with chronic myelocytic leukemia [138], [139], indirubin did not increase sister chromatid exchange (SCE).

Several *N*-substituted indirubin derivatives [140], [141], indirubine acyl derivatives, indirubin oximes [142], and halogenated indirubins [143] were synthesized and tested as potentially antineoplastic agents. Some halogen-substituted indirubine derivatives were reported to exhibit higher antitumor activity against L7212 in mice and W256 sarcoma in rats than native indirubin. The most active compound was found to be 5-iodoindirubine which was active against W256 sarcoma, L7212, L7712, L759 leukemias, and Lewis lung carcinoma. It showed increases in lifespan of mice bearing L7212 leukemia by 41 – 73%, and of rats bearing W256 sarcoma by 48 – 83%. The antitumor activity of 5-iodoindirubin against W256 sarcoma and L7212 leukemia was significantly higher than that of indirubin at the same dosage. *In vitro*, the IC_{50} value of 5-iodoindirubin on [^3H]thymidine incorporation into DNA of P388 cells was 6.4 μg/mL compared to 17 μg/mL of indirubin [143], [144].

Bisindole derivatives with 2,2′-, 3,3′- or 3,2′-linkages were compared for their effects on nucleic acid and protein synthesis. They all formed complexes with calf thymus DNA as measured by UV and visible spectrometry and inhibited nucleic acid and protein synthesis on cultured W256 sarcoma cells and *in vivo*. Isoindigo derivatives showed the highest potency in inhibiting DNA synthesis in cancer cells and in cell-free systems, whereas indigo derivatives had the lowest activity [145], [146]. *N*-Methylisoindigo (meisoindigo) was more potent than indirubin against W256 sarcoma in rats [143]. It was suggested that the improved absorption of meisoindigo is one of the major reasons for the enhanced antitumor activity as compared to indirubin. Studies on the mechanism of action of *N*-methylisoindigo indicated that it strongly inhibited DNA biosynthesis in L1210 cells and caused an arrest of S phase cells [147]. At a non-toxic concentration of 0.7 μg/mL *N*-methylisoindigo induced differentiation of ML-1 human myeloblastic leukemic cells as the most pronounced effect accompanied by the down-regulation of c-*Myb* gene expression, suggesting that the antitumor mechanisms of *N*-methylisoindigo involve these effects. *N*-Methylisoindigo was reported to be a second generation derivative, and was chosen for clinical treatment of chronic myeloid leukemia [148].

Conclusion

A series of low molecular compounds from Chinese herbal medicines, which proved to be in some cases highly effective especially in tumor therapy, has been listed here (part II will deal with high molecular compounds, to be published the next issue). These medicinal plants have been used since hundreds of years in China. In contrast to synthetic agents used in cancer chemotherapy, natural compounds have relatively low toxicities. Obviously, because of genetic factors that influence enzyme levels accounting for sometimes striking differences in metabolism and pharmacokinetics of drugs, results obtained in clinical studies carried out in China are not transferable to 100% to the European population. Since many of the clinical studies referred to in this paper have been carried out on Asians; the outcome of such studies in Caucasians or Africans might not be the same.

The mechanisms of action of these compounds are manifold, consisting in reactions with DNA bases, intercalation in DNA, inhibition of topoisomerases, inhibition of protein kinases, induction of apoptosis, etc. Some of the compounds presented here have interesting structural features, which have been or can be used as lead structures for the development of further antitumor agents.

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