12 Advances in Cancer Chemotherapeutic Drug Research in China

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12.1 Introduction of Background of Anticancer Drug Research in China

Anticancer agents have a long history, in particular some herbs used for cancer patients can be found in the old literature. But cancer chemotherapy as a science began in the 1940s; nitrogen mustard was discovered at that period of time to treat lymphoma effectively.[1] It attracted much attention in medical circles and promoted further development of many anticancer drugs including synthetic compounds, medicinal plants, and antibiotics. At present, cancer chemotherapy has formed one of the very important disciplines for cancer treatment.

In China, before 1949 cancer chemotherapy was not established. After the foundation of People's Republic of China in 1950s, the government paid attention to the cancer control problem. At the end of 1955, under the auspices of Chinese Academy of Sciences, the international conference on antibiotics was held in Beijing[2] and the task of investigating new anticancer antibiotics was proposed by some scientists. In 1956, a 12-year plan (1956−1967) for the advancement of science and technology in China was initiated and anticancer drug research was also included. Many units affiliated with research institutions, medical schools, pharmaceutical companies, as well as hospitals, began to join in this research program. At the end of 1958, the movement of the Great Leap Forward encouraged people to search methods and drugs for combating cancer, which was considered a very dangerous disease and a very difficult research problem. Since then, the massive screening of Chinese medicinal herbs and folk remedies for cancer treatment was

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Table 12.1 National Conferences of Tumor Pharmacology and Chemotherapy[3]

Date of Opening	Place	Participants
1983, 9	Dalian	148
1987, 11	Hangzhou	234
1989, 11	Nanning	240
1992, 10	Zhengzhou	404
1995, 10	Tianjin	370
1998, 9	Taian	300
2001, 10	Fuzhou	246

spread. After cooperative work, some effective agents for certain kinds of cancer patients were proved. Meanwhile in Beijing, the National Bureau for cancer prevention and treatment, affiliated with the Ministry of Health of the People's Republic of China was organized. It consisted of more than 10 branch organizations, one of which was a cooperative group of anticancer drug research, and Dr. B. Xu was appointed as a head of this group. In order to organize collaborative tasks and exchange information of varying antitumor agents, periodic meetings and conferences were held in different cities in China. For instance, the National Conference of Tumor Pharmacology and Chemotherapy was begun at that time and continued up to recent years. Some records of these conferences may be seen in Table 12.1.

Several promising antineoplastic agents found to be effective and six cooperative research programs were organized, including (1) *Camptotheca acuminata*, (2) *Cephalotaxus fortunei*, (3) *Cantharidines*, (4) *Colchicum autumnale*, (5) *Crotalaria sessiliflora* L., (6) *Curcuma zedoaria* (Berg) Rose, and so on. After joint efforts of different units, a number of therapeutic drugs were studied in detail both experimentally and clinically. Some of them were recommended for clinical use and production. New anticancer drugs commonly used in other countries were also investigated in China using native material and methods such as alkaloids from *Vinca rosea*, nitrogen mustard derivatives, antimetabolites, and others (at that time China was isolated from abroad). In this period, which was thought to be the early stage of anticancer drug research in China, many specialists in this area gained much knowledge and experience and also learned a great deal from foreign literature. Meanwhile, the fundamental basis of research, education, and production of anticancer drugs was established.

Later, in the 1970s to the 1990s, under the influence of the national policy of reform and opening up, China's door opened more and more, and some specialists and scientists had the opportunity to go abroad and learn new achievements in the world enriching their knowledge of cancer chemotherapy. New disciplines such as cell kinetics, molecular tumor pharmacology, standard evaluation procedures of new anticancer agents both in the laboratory and clinic, efficient screening methods and qualified clinical trials, combination chemotherapy for different cancers, and so forth were introduced to China. Some leading institutions in this field such as the Shanghai Institute of Materia Medica (SIMM), the Chinese Academy of Sciences (CAS), the Institute of Materia Medica at Beijing (IMM), the Chinese

Table 12.2 Basic Antitumor Drugs Amounted to 47 Approved by Chinese Ministry of Health in 1993[4]

1. Alkylating agents: chlormethine, cyclophosphamide, ifosfamide, formylmerphalan,* glyciphosphoramide,* melphalan, carmustine, lomustine, semustine, nitrocaphane,* altretamine, chlorambucil, thiotepa, busulfan
2. Antimetabolites: methotrexate, mercaptopurine, fluorouracil, tegafur, cytarabine, hydroxycarbamide
3. Anticancer antibiotics: actinomycin D (Gengshengmeisu*), mitomycin C, pingyangmycin,* daunorubicin, doxorubicin, aclacinomycin A
4. Plant antitumor drugs: vinblastine, vincristine, etoposide, homoharringtonine,* hydroxycamptothecin,* teniposide
5. Endocrine antitumor agents: tamoxifen, aminoglutethimide, medroxyprogesterone acetate
6. Other antitumor agents: procarbazine, dacarbazine, cisplatin, carboplatin, L-asparaginase, interferon, calcium folinate, mitoxantrone, ondansetron, mesna, cyclosporin A, lentinan
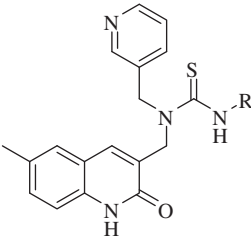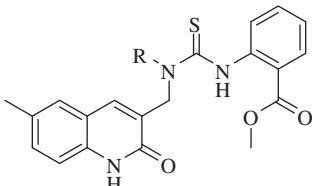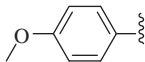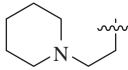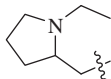
*Invented in China.

Academy of Medical Sciences (CAMS), Institute of Pharmacology and Toxicology of Chinese Academy of Military Medical Sciences, Shanghai Institutes of Pharmaceutical Industry, Institute of Antibiotics in Beijing, Tumor Hospitals located in Beijing, Shanghai, and Guangzhou all played an important role in raising the level of cancer chemotherapy in Chinese practice and actively making exchange of knowledge for searching and producing new antitumor drugs. Within a short period of time, the majority of well-known anticancer preparations in the world could be already produced in China and satisfied clinical needs. Some drugs, including a number of China's own developed agents, were exported to other countries such as vinca alkaloids, camptothecins, and so on. Table 12.2 shows the basic antitumor drugs used clinically at that time. It also meant that the most popular antitumor agents could be manufactured and supplied by Chinese pharmaceutical companies.

Afterward, the basic antitumor drugs changed periodically. Some old drugs were replaced by new agents or more effective compounds. Recently, a number of targeted drugs and bio-therapeutic agents have also been included.

In recent years, the knowledge concerning the nature of cancer has increased greatly, and molecular oncology, molecular pharmacology, genetic engineering, and other disciplines make the scope of anticancer drug research much broader. In particular, many new targets interfering with tumor growth and metastasis have been disclosed. Based on new findings and by means of new methods and techniques, many new drugs including cytostatic agents, tyrosine-kinase inhibitors, angiogenesis inhibitors, anti-signal transduction drugs, antineoplastic antibodies, differentiation-inducing agents, new generations of antimetabolites, and cytotoxic agents have been developed. Some Chinese research laboratories and pharmaceutical plants actively take part in collaborative work with foreign pharmaceutical companies and other organizations; many new findings and drug preparations already have been discovered. At present, nearly all new anticancer drugs can be found in Chinese pharmaceutical markets and clinical hospitals. Thousands of scientific papers related to investigation and production of different antitumor agents are published

Table 12.3 Chemical Structures of Selected Compounds and Their Activity on Enzyme and Cell Levels

Number	R	Biological Assays	
		Enzyme Assay[a] Inhibition (%)	Tumor Cell Inhibition Rate or IC_{50} (μmol/l)[b]
1		0	60.5
3		0	45.5
4a		8.6	6.1
4b		3.7	7.3

[a]Inhibition percentage of kinase activity was generated by measuring the inhibition of phosphorylation of a peptide substrate added to the enzyme reaction in the presence of 10 μmol/l inhibitor.
[b]Human lung adenocarcinoma cell line SPAC1 with overexpressed EGFR. Dose−response curves were determined at five concentrations. IC_{50} values are the concentrations in μmol/l needed to inhibit cell growth by 50% as determined from these curves.

in journals, books, or online in network annually by Chinese specialists. Many important contributions in this field can be found through different channels. Here, we will review some achievements obtained in China which are more closely related to our work .

12.2 Natural-Derived Anticancer Agents Developed in China

12.2.1 *Gengshengmeisu (Actinomycin K, D)*

In the 1950s, actinomycins (actinomycin C, J, and so on) were reported to have antineoplastic action.[5] In SIMM, some active substances isolated from the culture

Table 12.4 Chemical Structures of Selected Compounds and Their Enzyme Inhibitory Activities

5a–5b, 5e–5g   5c–5d, 5h

Number	R$_1$	R$_2$	Enzyme Assays Inhibition at 10 μmol/l (%)[a]			
			EGFR	**FGFR**	**VEGFR**	**PDGFR**
5a	CH$_2$CO$_2$Et	H	20.2	1.7	38.6	11.5
5b	CH$_2$CO$_2$Et	Br	29.5	NA	32.4	16.1
5c	CH$_2$CO$_2$Me	H	35.5	10.4	35.3	16.8
5d	CH$_2$CO$_2$Me	Br	10.1	5.8	36.7	8.0
5e	CH$_3$	H	18.4	19.4	38.6	25.4
5f	CH$_3$	Br	12.1	1.1	38.6	17.4
5g	CH$_3$	NO$_2$	44.2	10.5	27.6	9.4
5h	CH$_3$	H	36.7	18.12	4.6	2.0

[a]The percent inhibition of the kinase activity was generated by measuring the inhibition of phosphorylation of a peptide substrate added to enzyme reaction in the presence of 10 μmol/l inhibitor.

media of *Streptomyces melanochromogenes* (including No. 1779) which were obtained from the soil sample collected in Dalian region were investigated in detail.[6] After systematic study, one of the substances called antibiotic No. 1779, or actinomycin K, was proved to have anticancer property.[7] Pharmacological experiments showed that it could inhibit sarcoma 180, Ehrlich ascites carcinoma, spindle cell sarcoma, and sarcoma AK in mice. Its antitumor effect was stronger than actinomycin C. After toxicological and mechanistic studies, actinomycin K was recommended for clinical trials. Clinical data showed that this antibiotic exerted remarkable therapeutic effect for choriocarcinoma and malignant lymphoma. Afterwards, actinomycin K was approved for manufacture named Gengshengmeisu. Later work showed that actinomycin K consisted of two components, K1 and K2, K1 being a new substance (in very little amount), while K2 was identified with actinomycin D, which was confirmed as an antitumor agent in the US at the same time. After several years, the purified actinomycin K produced in Chinese pharmaceutical factories was equal to actimomycin D and provided for clinical use.

It was found in our laboratory that actinomycin K could inhibit RNA synthesis inducing nucleolus segregation. By means of quantitative electron microscope method it was proved that actinomycin K inhibited transcription process from rDNA to rRNA in tumor cells providing new data for elucidation of its mechanism of action.[8]

In SIMM, the chemists synthesized dozens of new derivatives of actinomycin K and tested their antitumor activity but none was found better than actinomycin K.[9] The data of relationship between chemical structure and activity of those compounds were meaningful for further studies.

12.2.2 Hydroxycamptothecin

In 1966, the plant alkaloid camptothecin[10] isolated from a famous Chinese tree *C. acuminata* was reported to have a strong antitumor activity against murine leukemia cells L1210. At that time, the researchers in SIMM paid much attention to this tree isolating different active principles from its bark, trunk, leaves, and fruits, and testing their antitumor activity *in vitro* and *in vivo*. From 1970 to 1974 several investigators in the US reported that camptothecin could cause severe bladder toxicity and weak therapeutic effect,[11] thus the drug was dropped from clinical trials. In our laboratory, we persisted in this study, finding some antidotes for the toxicity. Meanwhile, we found that 10-hydroxycamptothecin (HCPT)[12] isolated from the fruits of this tree possessed higher antitumor activity and lower toxicity than camptothecin. After a series of studies, HCPT was put into production for clinical use. When we reported such results at international conferences and published a series of papers related to hydroxycamptothecin, new attention was paid to the analogs of camptothecin, promoting novel investigations of its derivatives. In the last 20 years, irinotecan (CPT-11) and topotecan, the analogs of hydroxycamptothecin have become more popular anticancer drugs widely used in tumor hospitals in different countries. It is already known[13] that the active metabolite of CPT-11 is 7-ethyl-10-hydroxycamptothecin or SN38 that possesses much higher potency (more than 100 times) than CPT-11. It suggests that a great potential of exploring better drugs than HCPT exists and more work should be pursued. In China, HCPT as a basic common anticancer drug produced by several pharmaceutical companies has been used clinically for different cancer patients mainly with gastrointestinal carcinoma, tumors of the head and neck with marked therapeutic effect.[14] But some investigators pointed out that the lactone-ring opened form of HCPT, i.e., its sodium preparation, should be inactive. According to our experimental data,[15] both lactone-ring closed and opened forms of HCPT were effective against tumor growth, the latter being less potent, but it could be converted into closed form under certain conditions.

At present, some new findings concerning the derivatives of camptothecins achieved in our institute will be described later.

12.2.3 Homoharringtonine

Cephalotaxus is a famous tree having 10 species in Asia; 8 of them are distributed in China. In 1972, some principles isolated from the tree including harringtonine and its analogs were reported to have antitumor action in murine leukemia model.[16] Afterward, no clinical result of cancer treatment was reported. In China, at that time several research groups of different institutions systematically investigated *Cephalotaxus*-derived alkaloids. Harringtonine (HRT) and homoharringtonine (HHRT) were independently isolated in SIMM and IMM at Beijing and subjected to

preclinical and clinical studies. As a result of collaborative work with hospitals and pharmaceutical plants, both HRT and HHRT got approval for clinical use and production.[17−19] Although HHRT being included in the list of basic cancer therapeutic drugs in China for many years and clinical trials in the US confirmed the definite therapeutic action of this drug for leukemia patients, it is not yet recommended for production in other countries.

12.2.4   Polysaccharide Preparations

It can be seen from Table 12.2 that lentinan is a popular anticancer adjuvant agent frequently used in cancer chemotherapy. This drug was prepared from *Lentinus edodes* (berg.) Sing and contains polysaccharides. A number of Chinese traditional medicines such as *Polyporus versicolor*, *Grifolia umbellata*, *Pachyman*, *Ginseng*, and *Ganoderma lucidum* consist of different kinds of polysaccharides that can enhance body immunity acting like biological-response modifiers. Polysaccharide-peptide (PSP)[20] isolated from *Coriolus versicolor* cov-1 showed certain antitumor activity similar to PSK without noticeable side effects.[21,22] Experimental study[23] of the mixture of Lucid Ganoderma and Lucid Ganoderma spore (MLGLGS) showed that at high dosages it could produce inhibitory action on the growth of human lung tumor xenograft LAX-83 in nude mice. Lin and coworkers[24] reported that the mechanism of antitumor action of *G. lucidum* was mainly related to the inmuno-enhancing activity modulating many components of immune system such as the antigen-presenting cells, NK cells, T, and B lymphocytes. Clinically, the preparations of *G. lucidum* are always used in combined therapy playing a supportive role for body function and decreasing toxicities caused by cytotoxic drugs.

12.2.5   Some Meaningful Anticancer Substances from Traditional Chinese Medicine (TCM)

As mentioned in Section 12.1, after massive anticancer screening work was completed in the 1950s and 1960s, certain effective agents were found. In the literature,[25] according to the six therapeutic principles in TCM for treatment of cancer patients, more than 70 remedies (most of them are medicinal herbs) have been found to possess antitumor activity. In our laboratory, we screened some of them finding about 10 active substances.[26] In other units some useful agents were also obtained. Few active substances are briefly described.

Lycobetaine (Ungeremine) is a semisynthetic derivative of lycorine isolated from *Lycoris radiata*.[27] In the 1960s, we began to study the family Amarylidaceae plant from which several alkaloids were proved to have antineoplastic activity such as hemanthamine, pretazettine, narciclasine, and so forth. Only lycobetaine (LBT) was found to be the strongest antitumor substance. Clinical trials also indicated that LBT could exert therapeutic effect on ovarian carcinoma and gastric carcinoma without marked toxicity. It preferentially inhibited chromatin activity and did not induce single- or double-strand breakage or cross-links. LBT could intercalate into DNA and its DNA-binding property might be classified to a new group of DNA

intercalators of plant origin. Unfortunately, when it was injected into cancer patients intraperitoneally or intravenously, side effects of local irritation occurred frequently. Thus, this drug ceased manufacture. Further study to improve its preparation or taking it as a leading compound is still to be considered.

In TCM, cantharidin prepared from a kind of beetle (*Mylabris phalerata* Pall) was employed for treatment of neoplastic disease. Pharmacological study[28] showed that it had a definite anticancer action on animal tumors. In liver carcinoma patients, it exhibited certain beneficial effect. At present, its derivatives such as disodium cantharidate,[29] norcantharidin, hydroxycantharidimide, methylcantharidimide, and its compound preparations are used clinically. Several active principles[30,31] isolated from traditional Chinese herbal medicines such as oridonine from *Rabodosia rubesens* for esophageal carcinoma in Henan province; indirubin from *Indigo naturalis* for chronic myelocytic leukemia in Beijing; β-elemine from *Curcuma aromatica* for cervical carcinoma, irisquinone from the seeds of *Iris palasii* for radiosensitizing effect in Tianjin city are also used for treatment of different kinds of cancer patients. Detailed investigations are still warranted.

In TCM, some minerals such as arsenic compounds were also employed for cancer treatment. In the 1990s,[32] intravenous injection of arsenic trioxide (As_2O_3) could exert therapeutic effect on acute promyelocytic leukemia with remission rate of 73.3%. When it was used in combination with all-trans retinoic acid (ATRA), the efficacy was increased more markedly with complete remission rate more than 90%.[33,34] Such remarkable effectiveness has been confirmed in other countries and gained international recognition. In recent years, oral administration of arsenic sulfide preparations (As_2S_3, As_4S_4) derived from old Chinese remedies were also demonstrated to have antileukemia effect.[35]

For treatment of different kinds of cancer patients some medicinal formulae or compound prescriptions are frequently used in traditional Chinese medical practice; it is rather difficult to determine which the most important element for efficacy is and/or what the mode of action is. Recently, *Realgar-Indigo naturalis* formula (RIF)[36] was employed for treating promyelocytic leukemia and investigated in detail indicating that tetraarsenic tetrasulfide is the principal component, indirubin and tanshinone IIA (included in the formula) are adjuvant ingredients. When the above-mentioned elements mixed in one formula could produce synergistic effect that was better than a single agent separately. Such work as a model for analyzing mechanisms of TCM formulae or compound recipes is very helpful and encouraging to conduct more detailed studies related to traditional Chinese formulae or recipes.

12.3 Synthetic Anticancer Drugs

12.3.1 Alkylating Agents

It is well known that nitrogen mustard (mechlorethamine, HN2), the first synthetic anticancer drug, possesses definite antineoplastic action with a narrow antitumor spectrum and high toxicity. In the 1950s to the 1970s, hundreds of thousands of its

derivatives were synthesized and tested for antitumor activity. Only a small number of effective compounds have been proved to be safe and useful for the treatment of cancer patients such as cyclophosphamide, melphalan, lomustine, thiotepa, chlorambucil, lomustine, busulfan, and so on. These drugs[37] mainly act on DNA and proteins of cancer cells, causing cross-linking of DNA strands and interfering with the replication of DNA and transcription of RNA which are classified as alkylating agents. At that period of time synthetic chemists and pharmacologists in SIMM actively joined to search for better synthetic anticancer drugs. We tested more than 2000 synthetic compounds and other agents with different chemical structures and varying mechanisms of actions and discovered about 20 effective agents. After systematic preclinical work, more than 10 compounds were recommended for clinical trials such as 3P, Ho-14, AT-16, AT-222, AT-290, AT-346, AT-581, AT-1258, Sb-57, Sb-71, and so forth. In other Chinese institutions, a number of effective compounds with anticancer activity have also been successfully developed. In this section, some effective drugs are presented briefly.

12.3.1.1 Mecaphane (Methoxysarcolysin, 3P)[38,39]

Methoxysarcolysin (designated as 3P) was synthesized in our institute by Pan and others. It is a derivative of sarcolysin with chemical structure of *p*-bis-(chloroethyl)-amino-*o*-methoxy-phenylalanine. Pharmacological studies showed that 3P exhibited marked inhibition on sarcoma 180, Ehrlich ascites carcinoma, spindle cell sarcoma in mice, and Yashida ascites carcinoma, Guerin's carcinoma, Walker carcino-sarcoma in rats. 3P was used orally or intraperitoneally and easily absorbed; the highest blood concentration was attained 30 min after oral administration. The concentration dropped steadily and the higher concentrations were found in bone marrow, kidney, and liver. This drug was distributed throughout many internal organs, about 40% of which was excreted in the urine during the 24 h and smaller amounts were detected in feces. It inhibited mitosis and nucleic acid metabolism in cancer cells, and the mechanism of action was similar to that of other alkylating agents. Clinical data showed that it was effective against chronic myelocytic leukemia, Hodgkin disease, seminoma, and other tumors. Of 40 cases with chronic myelocytic leukemia, 37 were evaluated as effectively treated, among them 10 cases achieved complete remission, in some cases no recurrence was found during a 10-year follow-up period. This drug was included in 1977 and 1985 editions of Chinese Pharmacopeia.

12.3.1.2 N-*formyl Sarcolysin*

N-formyl sarcolysin (NF)[40] DL-*p*-bis(2-chloroethyl)amino-*N*-formyl-phenylalanine was synthesized in the IMM−CAMS. Han and coworkers reported that NF possessed strong antitumor action on Yashida sarcoma, Walker tumor, reticulum-cell sarcoma, Krebs-2 ascites carcinoma, and others. NF inhibited protein synthesis and the incorporation of 3H-thymidine into nucleic acid. It caused abnormalities in the chromosomes of tumor cells. By means of electron microscopy, progressive

degeneration in the mitochondria and an increase in the number of lysosomes were noticed. NF was administered orally, but the absorption in the gastrointestinal tract was incomplete. Clinical studies demonstrated that this drug could exert a marked therapeutic effect on seminoma patients. The 5-year survival rate reached 71% when NF was administered alone. It was also employed in combined therapy with radiation or surgery.

12.3.1.3   Nitrocaphane (AT-1258)

In SIMM, we systematically studied the antitumor activity of para-, ortho-, and meta-isomers of phenylalanine derivative of HN2 by substituting one hydrogen atom of the methyl group of HN2 with phenylalanine.[41] Experimental results showed that the ortho-isomer (AT-581 or ocaphane) possessed a strong antitumor effect on a number of animal tumors including rabbit tumor model, that is, Brown−Pearce carcinoma.[42] Clinically, it was found effective for treatment of patients with malignant pleural effusion and cancer of head and neck, but it was fairly toxic to hemopoietic organs. It is already known that the cytotoxic action of HN2 bears some relation to the chemical reactivity of the chlorine atoms of the mustard grouping. We postulated that the high chemical reactivity of ocaphane might be related to the chlorine atom; an introduction of electronegative group such as nitro group into the benzene ring could possibly deactivate the mustard grouping. Thus, AT-1258 (nitrocaphane), 2-bis-(2-chloroethyl)-aminomethyl-5-nitro-phenylalanine was synthesized and compared with AT-581 in respect to their antitumor activity and toxicity. The experimental data revealed that AT-1258 had remarkable therapeutic effect on different animal tumors better than AT-581.[43−45] The intraperitoneal injection of 4 mg/kg of AT-1258 produced 82−99% inhibition of Ehrlich solid carcinoma and solid hepatoma in mice. The therapeutic effect was nearly the same when it was given orally at 8−10 mg/kg. In rats bearing 5- to 6-day-old Jensen sarcoma with $2-5 \text{ cm}^2$ of tumor mass, AT-1258 produced tumor regression in a majority of the rats 12−15 days after seven injections of the drug, the cure rate being 62−86%. When nitrocaphane and ocaphane were used at the same tolerated dose, ocaphane (2 mg/kg) caused 67% and nitrocaphane (4 mg/kg) 96% ($p < 0.01$) of tumor inhibition in mice bearing Ehrlich carcinoma. Nitrocaphane at 1.5 mg/kg inhibited Brown−Pearce carcinoma in 66−78% of rabbits inoculated intraocularly, but the inhibition caused by ocaphane was only 49−65%. Both drugs prolonged the survival of rabbits (from 110% to 120%) when the tumor was inoculated intravenously. The experiments with ^{14}C-nitrocaphane showed that the radioactivity was distributed throughout many internal organs after oral administration. The highest content of ^{14}C was noted in the kidney, intestine, liver, tumor, and lung. In normal rats, the biological half-life of the drug in plasma was about 13 min. It was excreted mainly in the urine and feces, 65% of the total being excreted within 24 h.

Clinical data[44] demonstrated that this drug is more effective against nasopharyngeal carcinoma, malignant lymphoma, and lung cancer. The therapeutic efficacy frequently is observed in carcinomas of the squamous cell and undifferentiated cell

types. It can be used intravenously, orally, and locally. The side effects including anorexia, vomiting, and bone marrow depression were not severe and could be ameliorated or abolished by symptomatic treatment. Since the 1970s, this drug has been manufactured and used in many hospitals for a variety of cancer patients as one of the powerful alkylating agents. In China, nitrocaphane is included in the list of basic antitumor drugs for cancer patients and is easy to use.

12.3.1.4 Glyciphosphoramide (M25)

Han reported that in his institute a series of cyclophosphamide derivatives were synthesized and screened for anticancer activity. Among them M25, *N,N*-di (2-chlorethyl)-*N,N*-diethoxy carbonyl phosphoramide was proved to be the strongest compound exhibiting significant inhibitory action on Yoshida sarcoma and Walker carcino-sarcoma 256, and some rats bearing these tumors were cured completely.[19] Clinical studies indicated that this drug was effective in treating lung cancer (anaplastic form), breast cancer, in particular the ulcerating form, and Hodgkin disease. M25 could inhibit the mitosis of HeLa cells that was different from cyclophosphamide. In China, this drug is used locally for treatment of cancerous ulcers of advanced patients with remarkable effect.

12.3.1.5 Bimolane, Probimane, and Sobuzoxane (MST-16)

In the 1970s to the 1990s, ICRF-154 (1,2-bis-(3,5-dioxypiperazinyl) ethane) and ICRF-159 (propylenediamine tetra-acetylimide or razoxane) were frequently used in cancer chemotherapy for anti-metastasis activity. In order to search for better drugs, Ren[46] at SIMM synthesized biomolane (1,2-bis (4-morpholinomethyl-3, 5-dioxypiperazin-l-yl) ethane), a new compound, and found that it possessed a definite therapeutic effect against lymphoma, breast carcinoma, and other tumors. It was also used for treatment of patients with psoriasis or uveitis.[47,48] Probimane (1,2-bis (*N*4-morpholinomethyl-3,5-dioxopiperazin-l-yl) propane; designated as AT-2153 or MST-02), an analog of bimolane, was discovered to have better antitumor action than bimolane.[49,50] It produced marked antitumor action on Lewis lung carcinoma, B16 melanoma, colon adenocarcinoma 38, and hepatoma in mice, and was also active against spontaneous lymphoma and human bronchial adenocarcinoma heterotransplanted into nude mice. Moreover, it exhibited a potentiating effect to irradiation against sarcoma 37 and S-180 in mice. It did not show mutagenic activity in Ames test. ^{14}C-labeled probimane studies showed that it was easily absorbed in oral administration and widely distributed in the majority of tissues. Probimane was excreted mainly in urine and partly in feces. When ^{14}C was labeled at central dioxopiperazine or methyl morpholine group of probimane and injected intravenously in mice bearing Lewis lung carcinoma by whole body autoradiography, the drug was broken into at least two parts: a central part and a methyl morpholine group. The central part of the compound hardly penetrated through the blood−brain barrier but accumulated in the urinary bladder. The methyl morpholine group revealed a high affinity to tumor tissue and accumulated in spleen, bone,

and liver.[51] In mice bearing tumors, probimane could decrease the serum sialic acid level that was thought to be related to its anti-metastatic effect.[52] After long-term administration of bimolane, the incidence of acute leukemia in several cases was reported, thus biomolane was stopped. Ren and Cai designed and prepared many analogs of probimane, among them MST-16 (4,4′-(1,2-ethanediyl)-bis (1-isobutoxycarbonyloxymethyl-2-6-piperazinedione) or sobuzoxane) was found to be the best one and was studied in detail.[50−55] In cooperation with Japanese colleagues, sobuzoxane was subjected to systematic preclinical and clinical studies. It was proved that this drug was effective for treatment of leukemia and lymphoma patients.[56−58] At present, sobuzoxane has obtained approval in Japan for clinical use and production. It can be administered orally and can ameliorate the toxicity induced by doxorubicin[59] that is useful for combination therapy.

12.3.2 Metal Anticancer Agents, Antimony-71 (Sb-71), Sb-57, and so forth

In cancer chemotherapy, antimony compounds were little studied in comparison with other metal anticancer drugs. In 1960s, at SIMM a systematic investigation was conducted on the antitumor action of antimony and other metal chelating compounds. Chou et al.[60] designed and synthesized several classes of these compounds including (1) various salts of Sb-EDTA chelating agents; (2) N-substituted analogs of antimonial chelate of ethylene diaminetriacetic acid (EDTA); (3) metal chelates of EDTA; (4) analogs of antimonial chelate of propylene diamine tetraacetic acid (PDTA); (5) metal chelates of ammonia triacetic acid (ATA); (6) chelating agents to ATA; (7) antimonial chelates related to ATA; (8) antimonial chelates of ATA analogs; (9) nickel and copper complexes of methionine; (10) chelating agents (e.g., EDTA, PDTA, ATA). After screening and pharmacological studies, [61] we found that (1) the chelates of 10 metals including Hg, Bi, Pb Zn, Mn, Cu, Co, Ni, Sn, Ba, and EDTA, PDTA, and ATA had no antitumor activity on Ehrlich carcinoma; (2) complexones of several metals did not inhibit the growth of sarcoma 180 in mice; (3) several aminocarboxylic complexones of antimony could produce marked inhibition on animal tumors. Among tested compounds, Sb-71 (antimony ammonia triacetic acid) and Sb-57 (antimony sodium PDTA) were found to have better therapeutic action on a number of experimental tumors.[62] Both compounds significantly prolonged the survival time of mice bearing Ehrlich ascites carcinoma or spindle cell sarcoma and obviously retarded the growth of Guerin carcinoma in rats with 58−70% inhibition. Sb-71 also exerted therapeutic effect against sarcoma 180. Experimental results showed that Sb-71 inhibited all four stages of mitosis of cancer cells, and the interphase cells showed also definite changes, such as the conglutination of the structure of nuclear chromatin. It was proved that its therapeutic effect was closely related to the inhibitory action on mitotic process.[63] The toxicological studies in mice, rabbits, and monkeys showed that they had no severe side effects, only flattening of T-wave of EKG, nausea, vomiting, and decrease in body weight were observed in the group receiving high dosages. Subcutaneous injection

of sodium dimercaptosuccinate, BAL-glucoside, BAL, and cysteine could produce a significant protective action upon mice intoxicated by Sb-71, decreasing the mortality rate of mice. These sulfhydryl drugs could also antagonize the tumor-inhibitory action of Sb-71, but the antitumor effect could recur when the dose level was increased.[64] Later, Sb-71 and Sb-57 were recommended for clinical trials and exhibited therapeutic effect on some patients with gastrointestinal carcinoma, fibrosarcoma, breast carcinoma, and other tumors. In 1971, Sb-71 as a new anticancer drug was approved in China for production and used in many hospitals. The mechanistic study revealed that Sb-71 inhibited significantly the incorporation of ^{65}Zn into tumor cells[65] and in mice bearing Ehrlich ascites carcinoma, zinc chloride could exert antagonistic action against the therapeutic effect of the drug.[66] The experiments of distribution and excretion of Sb-71 indicated that Sb could combine with cancer cells with high affinity and sulfhydryl compounds (sodium dimercaptosuccinate or cysteine) decreased obviously the Sb content in tumor cells.[67] Such findings provided us new approaches for searching for novel anticancer agents. Unfortunately, at that period of time due to political movements such work was interrupted, the working team was disorganized as well and no more concern was considered about the clinical use and production of Sb-71. The study of the second generation of this drug was discontinued. In the 1970s to the 1980s, the metal anticancer drugs cisplatin and carboplatin appeared and their remarkable therapeutic efficacy for cancer patients caused great interest for chemical and medical researchers. Cisplatin was recognized as one of the most successful drugs in cancer chemotherapy.[68,69] In the late 1980s to 1990s, Hu et al. at Xiamen University systematically investigated antimony and other metal compounds synthesizing many derivatives and elucidating the structure—activity relationships (SAR).[70−72] He also conducted collaborative work with Russian scientists[73] to determine the crystal structures by chemical analysis, X-ray diffraction, infrared (IR) spectroscopy, and other methods. The absolute configuration of antimony chelates including Sb-71 and Sb-57 were also studied. Hu pointed out that the work with antimony chelating compounds published in 1960s is farsighted compared with other metal chelating agents in its design, synthesis, and activity assay. It is speculated that antimony preparations should have less toxicities than other metal drugs possessing alkylating activity, because Sb-71 has a different mode of action that could interact with trace metals affecting the tumor growth and metastasis. Tiekink in his review article[74] indicated that the exploration of anticancer potential of antimony and bismuth compounds is not as well developed as for other metal-containing species, but they deserve more research effort. Based on the findings about the toxicity, therapeutic effect, and action mechanism, it seems to us that Sb-71 and analogs should have a bright future and further investigations are necessary.

12.3.3   Other Effective Compounds and Preparations

In SIMM, some compounds were found to have antitumor activity such as oxalysine,[75] anordrine,[76] cheliensisin A[77] and studied in detail regarding their SAR and

action mechanisms, but these agents lacked good clinical results and served only as leading compounds for further studies.

Taxol is a very important anticancer agent first isolated from the bark of the Pacific yew *Taxus brevifolia* by Wani et al. in 1963,[78] and in the 1970s to the 1980s, its chemical structure, unique disruptive action on microtubules, and suitable clinical formulation were discovered.[79−81] Then the drug was used widely for treatment of different cancers. It was reported[82] that in IMM−CAMS a systematic study of taxol isolated from *Taxus chinensis* was conducted and the national preparation of this drug with similar efficacy as imported taxol was approved in 1990s for clinical use and production. In recent years, some new preparations such as taxol-liposome was prepared by a number of laboratories[83] and clinical data showed that it has good therapeutic action with fewer side effects.[84] 5-Fluorouracil (5-FU) implants, a new sustained-release preparation of 5-FU, was invented in the Hefei Technology University, An-Hui Province.[85] Clinical study indicated that such sustained-release preparation can be located directly into tumor mass increasing and prolonging the local drug concentration. This drug preparation can enhance therapeutic action and alleviate the side effects remarkably.[86] 5-FU implant has obtained SFDA approval for production as a new preparation of 5-FU, which is frequently employed in many hospitals, in particular in surgical oncology departments in China.[87] As mentioned earlier, ATRA can exert prominent therapeutic action on acute promyelocytic leukemia, and its new analogs, that is, R1 = 4-(ethoxycarbophenyl)-retinamide and R2 = *N*-4-(carboxylphenyl) retinamide could be used as cancer chemopreventive agents for the treatment of precancerous lesions such as leucoplakia of the mouth and the vulva with a cure rate of 69.2%; some patients with squamous skin cancer were also cured.[19]

Other new drugs as well as preparations discovered in a number of Chinese institutions, clinical hospitals, medical schools, pharmaceutical companies, and TCM-related organizations might be found elsewhere.

12.4   New Inhibitors of Topoisomerases and Molecular-Targeted Anticancer Agents

12.4.1   New Inhibitors of Topoisomerases

12.4.1.1   Introduction to Topoisomerases and Their Inhibitors

DNA replication, transcription, and chromosome segregation are critical cellular events for cells to properly proliferate, grow, and execute different functions. In these processes, the double-strand DNA becomes entangled and this topological problem needs solving in a timely and efficient manner. DNA topoisomerases are such cellular enzymes that are responsible for the problem. Topoisomerases function via introducing transient breaks in DNA by cleaving single or double strand(s) and rejoining the broken ends following rotation or passage of the strand(s).[88−91]

Topoisomerases are classified into two types: type I and II.[88,90] Type I topoisomerases include nuclear topoisomerase I (Top1), mitochondrial topoisomerase I, topoisomerase 3α, and topoisomerase 3β, all of which introduce single-strand breaks in DNA without the requirement of ATP;[90] type II topoisomerases, generating double-strand breaks (DSB) by hydrolyzing ATP, contain topoisomerase II (Top2 that is subtyped into Top2α and Top2β) and SPO11. Top1 is essential in mammals while Top2 is absolutely required for DNA replication. In addition, both Top1 and Top2 are frequently hyperactivated in tumor cells. Therefore, targeting Top1 and Top2 becomes an important successful strategy for cancer therapy.[89,90]

Camptothecins are the only Top1 inhibitors used for cancer therapy at present. In 1966, Wall and Wani[10] isolated a pentacyclic alkaloid camptothecin from *C. acuminata*, which then became the prototype for further modification and optimization. In the 1970s, 10-hydroxycamptothecin came into clinical use in China as the first camptothecin derivative; in the 1990s, another two well-known camptothecin derivatives topotecan and irrinotecan were approved for clinical anticancer uses in Japan, the US, and then worldwide. Camptothecins became one of the most important classes of anticancer drugs in clinical use, which are extensively used in the treatments of various solid tumors, including lung and colorectal cancers (CRCs). However, the current camptothecins in clinical use show several serious drawbacks including unstable lactone, reversible drug−target interaction, severe toxicity, and drug resistance.[90,92,93] Therefore, considerable efforts have been applied to the discovery of novel camptothecin derivatives.

In contrast to Top1 inhibitors, a lot of Top2 inhibitors have been used in clinical cancer treatments.[89] Top2 inhibitors are divided into Top2 poisons and Top2 catalytic inhibitors. Top2 catalytic inhibitors inhibit Top2 activity but do not cleave DNA strands, few of which have entered clinical anticancer uses. Most of the clinically used Top2 inhibitors including etoposide, doxorubicin, and mitoxantrone are Top2 poisons, characteristic of their cleaving DNA double strands and increasing Top2−DNA covalent complexes. Top2 inhibitors (primarily the Top2 poisons) have long been the first-line drugs in the treatments of various solid tumors and are important components in many therapeutic regimens containing molecular-targeting drugs. However, these drugs bear intolerable toxicities, especially bone marrow suppression. Other defects also include the generation of drug resistance and limited efficacy against metastatic tumors.[89] Consequently, there is an increasing interest in searching and developing anticancer agents targeting human Top2, especially those with new chemical scaffold and new mode of action.

Investigators in SIMM have put intensive efforts into the discovery of new inhibitors of topoisomerases, primarily focused on natural products. In this section, we will describe the anticancer activities and possible molecular mechanisms of several promising topoisomerase inhibitors with special emphasis on their unique modes of action. These inhibitors include Top2 inhibitors salvicine, gambogic acid (GA), MFTZ-1, and Echinoside A and Top1 inhibitor chimmitecan derived from camptothecin.

12.4.1.2   The Top2 Inhibitor Salvicine

Salvicine is a novel diterpenoid quinone compound (Figure 12.1) that was structurally modified from a natural product lead isolated from the Chinese medicinal plant *Salvia prionitis* Hance (Labiatae). This plant has been used as a folk medicine for its antibacterial, antitubercular, and antiphlogistic actions.[94] Salvicine has been
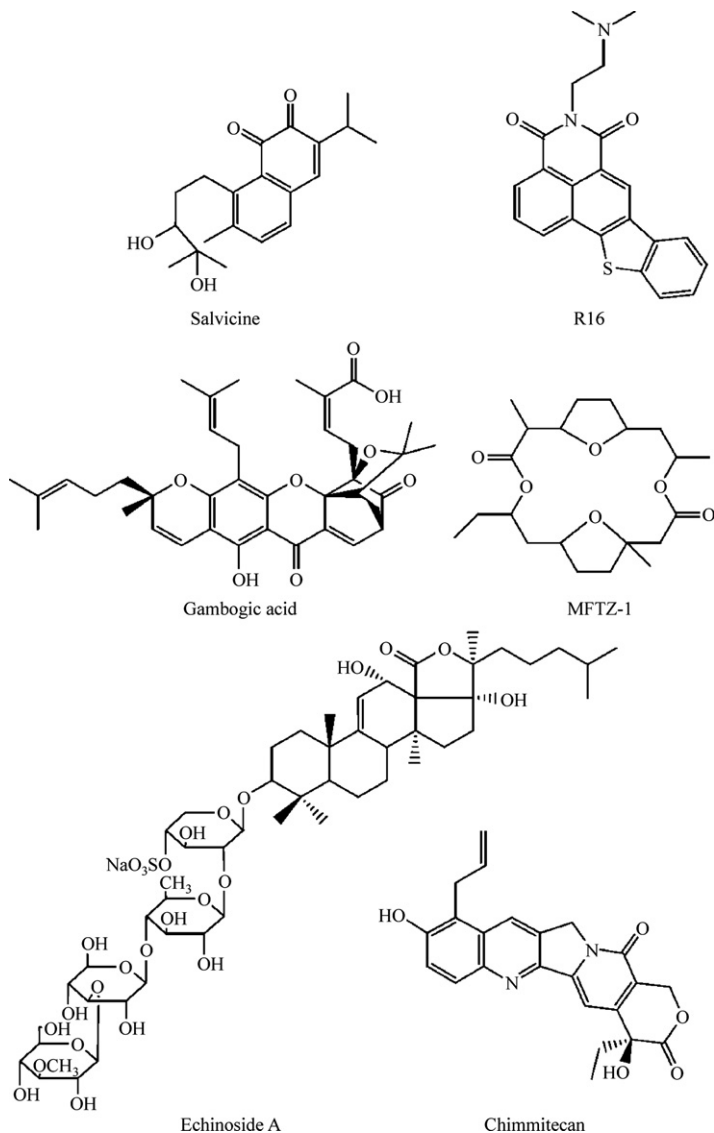
Figure 12.1 Chemical structures of the new inhibitors of topoisomerases discussed in this section.

demonstrated to be a multiple-targeting anticancer drug candidate with Top2 as its primary cellular target.[95] Salvicine is distinguished with its novel chemical structure, distinct profile of anticancer activity, low toxicity, new mode of action, and promising pharmaceutical perspective. At present, salvicine is undergoing phase II clinical trials in China with a promising perspective of translating into lifesaving therapeutic options.

Anticancer Activity

Anticancer Activity *in vitro* and *in vivo* Salvicine displays potent growth inhibitory activity against a panel of human tumor cell lines *in vitro* and in mice bearing human tumor xenografts.[94,96] Salvicine is as cytotoxic as the classical anticancer drug etoposide and weaker than vincristine in three leukemia cell lines, but 4.2- and 5.4-fold more potent than etoposide and vincristine against 12 solid tumor cell lines.[96] Noticeably, salvicine shows relative selectivity in its anticancer activity, particularly against gastric and lung carcinoma cells. The anticancer effect of salvicine has been found to be associated with its ability to induce apoptosis, as indicated in K562 and SGC-7901 cells.[97] Salvicine possesses a significant antineoplastic activity against murine S-180 sarcoma and Lewis lung cancer, and human lung adenocarcinoma xenografts A-549 and LAX-83.[94] Consistent with the result from the *in vitro* study, salvicine elicits significant inhibition on lung and gastric adenocarcinoma including A-549, SPC-A4, SGC-7901, MKN-28, and MKN-45 xenografts, while salvicine has no growth inhibitory effects on IBC, BEL-7402, HO8910, and HCT-116 xenografts in nude mice.

Distinguished Activity Against Multidrug-Resistant Tumor Cells Tumor multidrug-resistance (MDR) is considered to be one of the most important impediments to the effective chemotherapy of cancer. MDR is primarily involved in the anticancer drugs of natural origins.[98,99] It has always been the focused area to develop new strategy to circumvent MDR. Therefore, it is especially noteworthy that salvicine is able to circumvent MDR caused by P-glycoprotein (P-gp) overexpression. Salvicine effectively kills tumor cells overexpressing P-gp with a mean resistance factor of 1.42, which is much lower than that of vincristine, doxorubicin, and etoposide. Salvicine induces similar levels of apoptosis in MDR K562/A02 and parental K562 cells, promising its activity against MDR. Unlike other MDR modulators that inhibit the drug efflux by P-gp, salvicine downregulates mdr-1 and P-gp expression in K562/A02 MDR cells.[95,100−103]

Significant Anti-Metastatic Activity Tumor metastasis is the main threat to the lives of patients with cancer under most circumstances. Metastasis refers to the dissemination of cancer cells from initial tumor to distant sites and involves a series of processes, including loss of adhesion, acquisition of cell motility, extracellular proteolysis, and angiogenesis.[104] Salvicine significantly reduces the lung metastatic foci of MDA-MB-435 orthotopic xenografts, without obviously affecting primary tumor growth[105] indicating that salvicine possesses prominent inhibition on tumor metastasis.

Mechanism of Action

Salvicine Inhibits Top2 with a Distinct Mechanism Salvicine is a non-intercalative Top2 inhibitor that is different from other classical Top2 inhibitors in its mode of action.[95] Specifically, salvicine promotes Top2−DNA non-covalent binding; it has been shown to inhibit Top2-mediated DNA religation though not affecting the Top2-mediated DNA cleavage.[106,107] Moreover, salvicine has been shown to bind to the ATPase domain of human Top2 with high affinity and to inhibit the activity of Top2 by competing with ATP.[108] In addition, it is noteworthy that the Top2 inhibition of salvicine can be abrogated by glutathione (GSH), which is a reactive oxygen species (ROS) scavenger, suggesting that its inhibitory effect on Top2 might be due to ROS generation.[109] Together, salvicine emerges as a novel Top2 inhibitor with a distinct mode of action with ROS generation, competitively binding to the ATP pocket, promoting Top2−DNA binding, and inhibiting Top2-mediated DNA religation.[95]

Salvicine Induces Gene-Specific DNA Damage Non-selective DNA damage induced by DNA-damaging agents is the main cause for their severe toxicity and side effects. Most DNA-damaging agents used in clinical use attack DNA without selectivity. In this aspect, salvicine is potentially advantageous because it induces gene-specific DNA damage in tumor cells, with preferential damage occurring in the P2 promoter region of the oncogene *c-myc*. No obvious DNA damage was found in the 3' region of the same gene.[110] It appears possible that DNA damage within such genomic regions is an early event, which could lead to growth inhibition mediated by alterations of the expression of selected proliferation regulatory genes, such as *c-myc*.

Activation of Transcription Factor c-Jun, Downregulation of mdr-1 Expression and Inhibition of DNA Repair The transcription factor c-Jun is involved in extensive pathophysiological processes. Salvicine was found to stimulate c-jun gene expression and to inhibit mdr-1 gene expression in MDR K562/A02 cells. Salvicine enhanced levels of the active forms of JNK and c-Jun and raised the DNA-binding activity of AP1. Inhibition of c-jun expression disrupted enhancement of c-Jun and p-c-Jun by salvicine and simultaneously prevented the reduction of mdr-1 mRNA and P-gp protein levels, confirming that c-Jun activation is a prerequisite for reduction of mdr-1 mRNA and P-gp protein levels by salvicine. Most importantly, downregulation of c-jun expression inhibited apoptosis and cytotoxicity induced by salvicine in both MDR and parental K562 cells.[101] Another study reveals that activation of JNK, an enzyme responsible for the phosphorylation of c-Jun, directly contributes to suppression of mdr-1 gene expression.[111] So a clear molecular pathway through which salvicine reduces mdr-1/P-gp expression can be drawn: salvicine stimulates JNK phosphorylation and activated JNK phosphorylates serines 63 and 73 of c-Jun resulting in increased transcription activity. Phosphorylated c-Jun promotes expression of c-jun itself, thus increasing c-Jun levels. Furthermore, the transcription-factor complex containing c-Jun binds to the consensus

AP1 target element in the mdr-1 gene promoter and represses transcription leading to reduction of mdr-1 mRNA and P-gp expression and killing MDR tumor cells.

On the other hand, salvicine inhibits DNA-PK-dependent DNA repair. DNA-PK-dependent DNA repair abrogates DNA damage (DNA DSB) that triggers apoptosis and cell killing.[112] Repairing broken DNA generally reduces anticancer activities of DNA-damaging agents. Enhancement in DNA repair is one of the key elements resulting in drug resistance. Thus, interference with DNA repair emerges as a new approach to circumvent tumor drug resistance.[112] Salvicine significantly inhibits DNA-PK-dependent DNA repair by reducing the kinase activity and the protein level of the catalytic subunit of DNA-PK, a critical component of non-homologous end joining.[113] Thus, salvicine bears an interesting characteristic featured by its triggering DNA DSB and inhibiting DNA repair simultaneously, which possibly favors its direct cell killing against MDR cells.

Another characteristic of salvicine is that salvicine itself does not induce MDR. By persistent induction in combination with one-step selection, a salvicine-resistant A-549/SAL subline was established. Compared with parental cells, A-549/SAL cells display 8.91-fold resistance to salvicine and an average of 6.70-fold resistance to the antimetabolites. The cells, however, are not resistant to alkylating agents, platinum compounds, and other naturally derived antineoplastics indicating that persistent exposure to salvicine itself does not induce a typical tumor multidrug-resistant (MDR) phenotype.[114] Taking the facts that salvicine overcomes MDR and itself does not induce MDR together, it appears possible that salvicine would be effective against MDR tumors in clinic.

Salvicine Inhibits Tumor Metastasis by New Mechanisms Tumor metastasis is one of the most common causes leading to death. Salvicine possesses significant activity against tumor metastasis. A comparison of gene expression profiles of primary tumors and lung metastasis of salvicine-treated and untreated groups revealed that genes involved in tumor metastasis, particularly those closely related to cell adhesion and motility, were obviously downregulated, including fibronectin, integrin alpha3, integrin beta3, integrin beta5, FAK, paxillin, and RhoC.[105] Salvicine was further shown to downregulate RhoC at both mRNA and protein levels, to inhibit stress fiber formation and invasiveness of MDA-MB-435 cells, and to block translocation of both RhoA and RhoC from cytosol to membrane. In addition, salvicine specifically inhibits the adhesion of human breast cancer MDA-MB-435 cells to fibronectin and collagen. The fibronectin-dependent formation of focal adhesions and actin stress fibers is also inhibited by salvicine. Salvicine downregulates β1 integrin ligand affinity, clustering and signaling via dephosphorylation of focal adhesion kinase and paxillin. Moreover, salvicine induces extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) activation. Salvicine also promotes the production of ROS that contributes to the salvicine-induced activation of ERK and p38 MAPK. Salvicine and its generated ROS inactivate β1 integrin function and results in cell adhesion inhibition. Collectively, the anti-metastatic activity of salvicine is closely related to the Rho-dependent signaling pathway.[105,115]

Inhibitory Effect of Salvicine on the Telomere−Telomerase System The length of telomeres, which are shortened with cell divisions, limits cells to a fixed number of divisions. Telomerase is able to maintain the length of telomeres and is very important for cells to divide unlimitedly. Telomerase is hyperactivated in most tumor cells, which is critical for the immortalization of tumor cells. Thus, telomere and telomerase have been recognized as potential anticancer targets.[116]

Salvicine-induced downregulation of telomerase activity in HL-60 cells, which preceded a decrease in expression of the telomerase catalytic subunit (hTERT) and telomerase-associated protein (TP1) at the mRNA level. The protein phosphatase inhibitor okadaic acid prevented the downregulation of telomerase activity by salvicine. The significant increase in protein phosphatase 2A (PP2A) activity induced by salvicine treatment was blocked completely by okadaic acid. The specific caspase-3 inhibitor Z-DEVD-FMK did not reverse the decrease in telomerase activity or the increase in PP2A activity in HL-60 cells exposed to salvicine. Thus, the salvicine-induced decline in telomerase activity may be primarily due to the dephosphorylation of telomerase components mediated by PP2A activation.[117] The result was also confirmed in the solid tumor A-549 cell line.[118]

Salvicine has further been demonstrated to induce cell cycle-independent telomere erosion independent of its inhibition on telomerase activity but dependent on its disruption of the telomere repeat binding factor 2 (TRF2). Salvicine induces telomeric DNA damage and telomere erosion in lung carcinoma A-549 cells, which is independent of cell cycle. Salvicine not only downregulates TRF2 protein level but also impedes TRF2-telomere binding. By overexpressing the full-length trf2 gene and transfecting TRF2 small-interfering RNAs, TRF2 protein is confirmed to protect both telomeric and genomic DNA from the salvicine-elicited events. In addition, it is noteworthy that although both the ataxia-telangiectasia-mutated (ATM) and the ATM- and Rad3-related (ATR) kinases respond to the salvicine-induced DNA damages, only ATR is essential for the telomere erosion. Thus, the activated ATR augments the salvicine-triggered TRF2 disruption, whereas the TRF2 reduction in turn enhances ATR function.[119]

Low Toxicity and Side Effects

Preclinical and phase I clinical studies revealed that salvicine did not cause any dose-limiting toxicity and serious bone marrow suppression, which differentiates it from the first-line clinically used Top2 inhibitors. The major toxicities of salvicine belong to I∼αgrades, including leukopenia, neutropenia, elevation of transaminases, nausea, vomiting, mucositis, sweating, coughing, and so forth. There was no irreversible toxicity observed and no drug-related death was found.

Future Prospects

Salvicine reveals several unique features, including its novel chemical structure, distinct profile of anticancer activity, new mode of action, and low toxicity. These features make it distinguished as a multiple-targeting anticancer drug candidate with Top2 as its primary cellular target (Figure 12.2).[95] There are some important questions on the mechanisms of action of salvicine remaining to be clarified, for
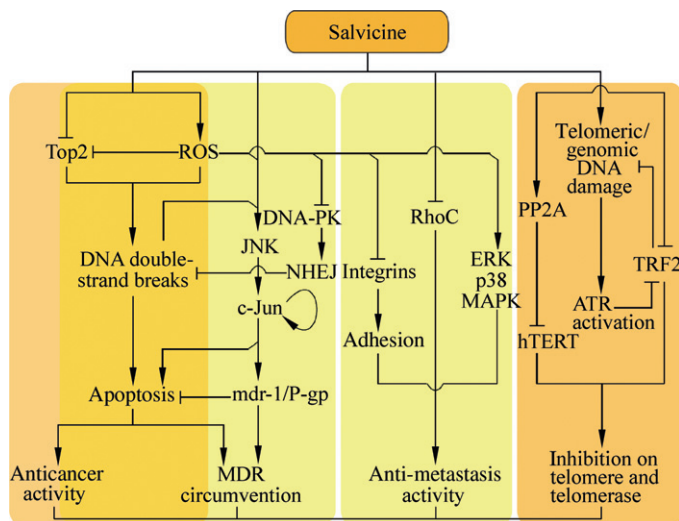
Figure 12.2 Salvicine is a multiple-targeting anticancer drug candidate with Top2 as its primary anticancer target. Symbols: →, promotion; ⊥, inhibition. Abbreviations: Top2, topoisomerase II; ROS, reactive oxygen species; DNA-PK, DNA-dependent protein kinase; NHEJ, non-homologous end joining; P-gp, P-glycoprotein; MDR, multidrug resistance; ERK, extracellular signal-regulated kinase; p38 MAPK, mitogen-activated protein kinase; PP2A, protein phosphatase 2A; hTERT, human telomerase catalytic subunit; ATR, ATM- and Rad3-related; TRF2, telomere repeat binding factor 2.

example, how does salvicine activate c-Jun and inhibit DNA-PK, TRF2, and RhoC? What intrinsic relationship(s) or link(s) are there between its activities of anticancer, anti-metastasis, and MDR circumvention? Are these activities of salvicine independent of each other or inseparable? The answers to those questions could greatly help accelerate its undergoing phase II clinical trials and guide its potential clinical uses in the future.

12.4.1.3 Other New Top2 Inhibitors

The Naphthalimide Analog R16

Amonafide, a naphthalimide derivative, although selected for exploratory clinical trials for its potent anticancer activity, has long been challenged by its unpredictable side effects.[120−122] Clinical studies found that amonafide was extensively metabolized to *N*-acetyl-amonafide via N-acetylation by *N*-acetyltransferase 2. This metabolite caused a high-variable, unpredictable toxicity because of the interindividual differences in N-acetylation and greatly obstructed its clinical development. Scientists in the East China University of Science and Technology (Shanghai, People's Republic of China) synthesized 2-(2-dimethylamino)-6-thia-2-aza-benzo[def]chrysene-1,3- diones (R16) by substituting 5′-NH$_2$ of the naphthyl

with a heterocyclic group to amonafide, with additional introduction of a thiol group (Figure 12.1). This substitution eliminates the potential toxicity threat of amonafide because R16 has no NH_2 group at 5′ position.

R16 is more cytotoxic than its parent compound amonafide in human tumor cell lines. It is also effective against MDR cells. Importantly, R16 inhibits tumor growth in mice implanted with S-180 sarcoma and H22 hepatoma. Mechanistic study shows that R16 functions as a Top2 poison via binding to the ATPase domain of human Top2α. Using a Top2 catalytic inhibitor aclarubicin, ATM/ATR kinase inhibitor caffeine and Top2-deficient HL-60/MX2 cells, it was shown that R16-triggered DNA DSB, tumor cell cycle arrest, and apoptosis were in a Top2-dependent manner.[123]

R16 induces G2 arrest via an ATM-activated Chk2-executed pathway. R16 triggers phosphorylation of the DNA-damage sensor ATM responding to γ-H2AX-indicated DNA DSB. Inhibition of ATM using both the pharmacological inhibitor caffeine and the specific small interference RNA (siRNA) rescues G2 arrest elicited by R16 indicating an ATM-dependent manner of the naphthalimide-driven G2 arrest. Furthermore, depletion of Chk2 but not Chk1 with their corresponding siRNAs reverses the R16- and amonafide-triggered G2 arrest. Moreover, both analogs phosphorylate Chk2 more persistently than Chk1 in an ATM-dependent manner. Therefore, R16 as well as amonafide could preferentially employ Chk2, which could be accounted for further by differential phosphorylation of Chk1 and Chk2 by ATM.

At the same time, R16 was demonstrated to trigger time and concentration-dependent Chk1 reduction which was unrelated to the mRNA level and HSP90-involved degradation. Proteasome inhibitors MG-132 or lactacystin can prevent Chk1 decline induced by R16 accompanied by significant accumulation of ubiquitinated Chk1 protein indicating the involvement of ubiquitin−proteasome pathway. R16 also results in loss of Chk1 function. By site specifically mutating the phosphorylation sites of Chk1 protein at Ser317 or at Ser345, R16-triggered Chk1 reduction was demonstrated to be associated with its apoptotic induction and cell killing. Thus, the novel Top2 inhibitor R16 induces degradation of Chk1 via the ubiquitin−proteasome pathway, impairing the function of Chk1 and thus contributing to the anticancer activity of R16.[124]

Gambogic Acid

GA (Figure 12.1), a natural product isolated from the amboges resin of *Garcinia hurburyi* tree, was approved for testing in clinical trial as a wide spectrum antitumor drug in 2004 and now is undergoing its phase II clinical trial in China.[125] GA has shown to exert its antitumor effects via the induction of apoptosis,[126] which is dependent on caspases[127] but independent of the cell cycle in breast cancer.[128] Specifically, caspase-8 acts as a key executor in the GA-induced apoptosis.[127] GA targets transferrin receptor and triggers rapid apoptosis in tumors.[129] GA was also revealed to downregulate telomerase and to directly interact with c-MYC in human hepatoma[130,131] and lung cancer cells,[132] to reduce CDK7 kinase activity,[126] to

inhibit activation of NF-kB, and target the microtubulin-associated protein stathmin 1 (STMN1).[125]

However, the above targeting activities of GA seem to be hard to account for its non-selective anticancer activities. Recently, Top2 has been identified as the primary cellular target of GA.[123] GA significantly inhibits the catalytic activity of Top2. Although not trapping and stabilizing covalent Top2−DNA cleavage complexes, GA inhibits DNA cleavage and ATP hydrolysis. Downregulation of Top2α prevents GA-induced apoptosis and restores cell proliferation. Moreover, GA directly binds to the ATPase domain of Top2α−DNA and may share common binding sites with ATP. GA also inhibits Top2α-mediated DNA cleavage and modulates the activity of Top2α poisons.[123] The finding of Top2 targeting by GA gives deep insight into its molecular anticancer mechanism and could guide its undergoing clinical evaluation.

MFTZ-1

14-Ethyl-2,5,11-trimethyl-4,13,19,20-tetraoxa-tricyclo[14.2.1.17,10]eicosane-3,12-dione (MFTZ-1) is a macrolide compound isolated from *Streptomyces* by researchers in the Kunming Institute of Botany of the Chinese Academy of Sciences (Kunming, People's Republic of China) (Figure 12.1). MFTZ-1 displays *in vitro* and *in vivo* anticancer activities. It is also effective against MDR tumor cells showing that it is a poor substrate of drug transporter(s). MFTZ-1 functions as a non-intercalative Top2 poison via binding to ATPase domain of Top2, characterized by its strong inhibition on the decatenation and relaxation of Top2. The capacity of MFTZ-1 to stabilize Top2−DNA covalent complexes is comparable with that of the classic Top2 poison, etoposide. MFTZ-1 triggers DNA DSB and apoptosis in a Top2-dependent manner.[133]

MFTZ-1 reduces HIF-1α accumulation driven by hypoxia or growth factors in human cancer cells. However, MFTZ-1 does not affect the degradation of HIF-1α protein or the level of HIF-1α mRNA. By contrast, MFTZ-1 apparently inhibits constitutive and inducible activation of both phosphatidylinositol-3-kinase (PI3K)-Akt and p42/p44 MAPK pathways. Moreover, MFTZ-1 abrogates the HIF-1α-driven increase in VEGF mRNA and VEGF protein secretion. MFTZ-1 also lowers the basal level of VEGF secretion. Therefore, an important feature emerges that MFTZ-1 can reduce constitutive, HIF-1α-independent VEGF secretion and concurrently antagonize inducible, HIF-1α-dependent VEGF secretion. Consequently, MFTZ-1 disrupts tube formation of human umbilical vein endothelial cells (HUVECs) stimulated by hypoxia with low-concentration serum or by serum under normoxia and inhibits HUVECs migration under normoxia. MFTZ-1 also prevents microvessel outgrowth from rat aortic ring. Thereby, MFTZ-1 can elicit potent antiangiogenesis under different conditions. By using specific small-interfering RNA targeting Top2α or Top2-defective HL-60/MX2 cells, MFTZ-1 was revealed to affect HIF-1α accumulation and HUVECs tube formation irrelevant to its Top2 inhibition. Significantly, MFTZ-1 at sub-cytotoxic concentrations ($<1\,\mu M$) reduces constitutive and inducible HIF-1α accumulation and VEGF secretion via
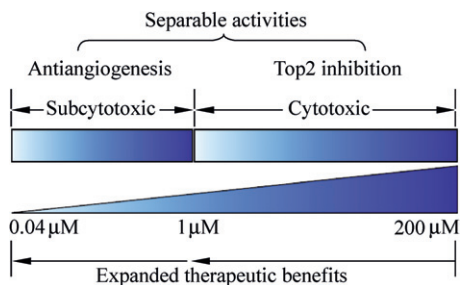
Figure 12.3 Separable activities of MFTZ-1.

PI3K-Akt and MAPK pathways, eliciting antiangiogenesis independently of its Top2 inhibition at cytotoxic concentrations (>1 μM).[134]

Therefore, MFTZ-1 has dual targets of antiangiogenesis and Top2 inhibition in a separable manner. This unique feature might offer more therapeutic benefits in its potential clinical settings (Figure 12.3).

Echinoside A

The potential novelty and diversity of chemical structures of marine-derived natural products have persistently been driving the search for new types of such anticancer agents, which has resulted in the discovery of several drug candidates with unique structures and mechanisms of action, including ecteinascidin 743, squalamine, and psammaplin A. Some of them in clinical trials display excellent therapeutic effectiveness in treating recurrent or refractory cancers.[135−137] Echinoside A (Figure 12.1) is an antifungal marine-derived saponin isolated from sea cucumber.[138] Echinoside A shows potent antitumor activities by inhibiting the growth of S-180 sarcoma and H22 hepatoma in standard mouse models and human prostate carcinoma PC-3 xenografts in nude mouse models. Echinoside A is a DNA non-intercalative Top2α inhibitor revealing the unique characteristics of inhibiting the non-covalent binding of Top2α to DNA by competing with DNA for the DNA-binding domain of the enzyme and for interfering predominantly with the Top2α-mediated pre-strand-passage cleavage/religation equilibrium over with the post-strand-passage one. These features distinguish Echinoside A from other known Top2α inhibitors such as etoposide and doxorubicin. Moreover, Echinoside A induces DNA DSB in a Top 2-dependent manner. Together with its new chemical entity and potent *in vitro* and *in vivo* antitumor activities, the unique action mode of Echinoside A on Top2 makes it a new prototype for further modification and optimization in developing new anticancer drugs.

12.4.1.4 The Top1 Inhibitor Chimmitecan, a Novel Camptothecin Derivative

Chimmitecan, 9-allyl-10-hydroxycamptothecin, is a novel small alkyl 9-position substitution for camptothecin[139] (Figure 12.1) with potent Top1 inhibition, outstanding anticancer activities *in vitro* and *in vivo*, a salient anti-MDR activity, good stability in human serum albumin, improved solubility, and availability. Those features favorably promise its therapeutic potential in clinical settings.

The Anticancer Activity of Chimmitecan and Its Molecular Mechanism

The Anticancer Activities Chimmitecan exerts potent *in vitro* antitumor activity over a wide variety of human tumor cell lines originated from different tissues, including leukemia, lung, gastric, hepatocellular, colon, breast, ovarian, and cervical cancers. Comparative studies reveal the enhanced anticancer potency of chimmitecan with an averaged IC_{50} against 20 human tumor cell lines of 83 nM, significantly lower than that of topotecan (281 nM) or SN38 (191 nM). Chimmitecan displays similar selectivity in its anticancer effect to topotecan and SN38. Notably, chimmitecan is more effective in three pairs of MDR tumor cell lines than topotecan and SN38 indicating its potential anti-MDR activity. In addition, the cytotoxicity of chimmitecan is unaffected by human serum albumin.[140]

Chimmitecan was tested for its *in vivo* anticancer activities in nude mice by using human tumor cell lines of different tissue origins, including lung, gastric, hepatocellular, colon, and pancreatic cancers. The results showed that human cancers from lung, colon, and pancreas were highly sensitive to chimmitecan, as evidenced by almost total disappearance of tumor xenografts in nude mice treated with chimmitecan, while gastric and hepatocellular cancers displayed medium sensitivity.

The Molecular Mechanism As with other camptothecins, chimmitecan also produces anticancer effects by inhibiting Top1 catalytic activity and trapping and stabilizing covalent Top1−DNA complexes. Nanomolar levels of chimmitecan cause impressive DNA damage, G2-M-phase arrest, and apoptosis in human leukemia HL-60 and colon cancer HCT-116 cells.[140,141] Chimmitecan as well as camptothecin was found to induce the repairable DSB in human colon cancer HCT-116 cells. The cellular disposal of DSB was reflected as the progressive dispersal of γ-H2AX foci, reduction of comet tails, dynamic activation of RAD51-mediated homologous recombination (HR) repair, and reversible G2-M arrest. In this process, the differential kinetics of Chk1 and Chk2 activation was characterized by the progressively increased phosphorylation of Chk2 until 72 h, the degradation of Chk1, and the disappearance of phosphorylated Chk1 48 h after drug removal. Using RNA interference, we further showed that Chk2 was essential to G2-M arrest, whereas Chk1 was mainly required for HR repair in CPT-treated HCT-116 cells. Moreover, Chk2, rather than Chk1, predominated over the control of cell survival in this model. The differential roles of Chk1 and Chk2 in regulating HR repair and G2-M-phase arrest were also confirmed in HT-29 colon cancer cells. The data provide critical evidence to further explore checkpoint modulation, especially Chk2 inhibition as a therapeutic strategy in combination with chimmitecan and other camptothecins.[141]

12.4.2   Molecular-Targeted Anticancer Agents

12.4.2.1   Introduction to Molecular-Targeted Anticancer Agents

The clinical success of the protein tyrosine-kinase (PTK) inhibitor Gleevec established a milestone for anticancer therapy. Since then, a lot of molecular-targeted

anticancer agents specifically against PTKs including epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), platelet-derived growth factor receptor (PDGFR), and vascular endothelial growth factor receptor (VEGFR) have become the components in standard regimens of anticancer combination therapy.[142,143] Among them, inhibitors of tumor angiogenesis are their important representatives.

Angiogenesis plays a critical role in tumor progression, particularly in the growth and metastasis of solid tumors.[144−147] An avascular tumor can rarely exceed $1-2$ mm^3. Once vascularized, a tumor grows rapidly and nearly exponentially. Moreover, the vascular density of a tumor is closely associated with its metastatic potential, and thus with its malignancy. Inhibition of tumor angiogenesis has been one of the most important strategies for cancer therapy. Several angiogenesis inhibitors including bevacizumab (Avastin), sorafenib (Nexavar), and sunitinib (Sutent) have been applied successfully in clinical therapy of solid tumors in combination with chemotherapy.[147] However, the current antiangiogenic agents are facing the challenge of tumor drug resistance, including evasive and intrinsic resistance.[148] Therefore, new inhibitors of angiogenesis, particularly with new chemical skeletons or new mechanisms of action, are necessary to be developed.

Compounds derived from natural products are generally characteristic of their diverse chemical structure and possible new modes of action.[135−137] With this in mind, Chinese investigators have taken advantage of their rich natural resources in order to discover new types of antiangiogenic agents. A lot of natural compounds have been distinguished for their apparent antiangiogenic activities and new mechanisms of action. In this section, we will describe several representative compounds including pseudolaric acid B (PAB),[149−151] oligomannurarate sulfate (JG3),[152] philinopside E (PE),[153,154] and *Grateloupia longifolia* polysaccharide (GLP).[155]

Current cellular signaling kinase-targeted anticancer agents, specifically, those with single targets are generally of relatively low therapeutic effectiveness and predispose tumors to drug resistance. Therefore, there has been increasing interest in those agents targeting specific signaling cascades or multiple molecular targets. In addition, efforts have been put in the discovery of agents that circumvent tumor resistance to current molecular-targeted anticancer therapeutics.[156] In the following section, we will briefly introduce three representative agents including the PI3K-Akt-mTOR cascade inhibitor S9,[157] the multi-targeted PTK inhibitor marine-derived oligosaccharide sulfate (MDOS),[158] and the new EGFR inhibitor BB.[227] In addition, another two new cellular signaling kinase-targeted anticancer compounds, Y31 and AL3810, also display prominent preclinical anticancer activities. Y31, a derivative of rapamycin, is a novel, specific inhibitor of mTOR with improved water solubility and *in vivo* anticancer activity. On the other hand, AL3810 is a synthetic multi-targeted PTK inhibitor targeting KDR, Flt1, PDGFR, FGFR1, and c-Kit with IC$_{50}$s in the nanomolar range, with more potent *in vivo* anticancer activity than other commercially available multi-targeted PTK inhibitors Sorafenib and Sutent.

12.4.2.2 Angiogensis Inhibitors

Pseudolaric Acid B

PAB (Figure 12.1) was first isolated by the scientists in SIMM from the root bark of *Pseudolarix kaempferi* Gorden and used as an antifungal agent and as an agent that causes the early termination of pregnancy in folk medicine in China.[151,159] Recently, PAB was further proved to possess potent anticancer activity by targeting microtubulin and neoangiogenesis.[149,151,160] Detailed structure−activity studies show that a hydrophobic group (-CO2Me or -Me) at C-7, a Δ^7 double bond, an acyloxy (OAc) at C-4,3 and the side chain with a conjugated double bond and a hydrophilic terminal group are essential for its anticancer activity.[161,162]

PAB Inhibits Neoangiogenesis PAB reveals prominent antiangiogenesis activity as evidenced by its reducing VEGF-stimulated HUVEC migration, tube formation of HUVECs, neovascularization of chicken chorioallantoic membranes.[149] The mechanism is involved in its inhibiting the angiogenesis potential of human endothelial cells and downregulates the levels of HIF-1 protein by promoting proteasome-mediated degradation in human tumor cells.

Hypoxia-inducible factor 1 (HIF-1) is an initiation factor of neoangiogenesis that has become an attractive anticancer target. PAB displays potent *in vitro* antiangiogenic activity shown by inhibiting VEGF-stimulated proliferation and migration and fetal bovine serum-stimulated tube formation of human umbilical vascular endothelial cells in a concentration-dependent manner. Moreover, PAB (10 nmol per egg) significantly suppressed *in vivo* angiogenesis in the CAM assay. On the other hand, PAB abrogated hypoxia-induced VEGF secretion from MDA-MB-468 cells via reducing HIF-1α protein. The selective proteasome inhibitor MG-132 completely reversed the reduction of HIF-1α protein in the PAB-treated MDA-MB-468 cells. In conclusion, PAB displays the dual antiangiogenic activities of directly inhibiting endothelial cells and abrogating paracrine stimulation of VEGF from tumor cells due to reducing HIF-1α protein by promoting its proteasome-mediated degradation in MDA-MB-468 cells, which has potential clinical relevance.[149] On the other hand, the antiangiogenic activity of PAB may also be associated with its ability to inhibit MAPK- and AKT-driven antiapototic{aq} signaling and thus to antagonize the antiapoptotic effect of VEGF.[150]

PAB Suppresses the Polymerization of Microtubulin via Binding to Its Colchicine Site One prominent characteristic of PAB is its resulting in dramatical G2/M-phase arrest followed by apoptosis, either in HMEC or in tumor cells.[151,160] This effect of PAB is directly subsequent to the disruption of cellular tubulin and thus to the interference with mitotic spindle assembly. These effects of PAB have been shown further to suppress the polymerization of microtubulin by direct interaction with the colchicine-binding site on tubulin.[160] The findings provide cancer therapy with a novel chemical class targeting the colchicine-binding site on tubulin.

JG3

Heparanase, a mammalian endo-h-D-glucuronidase, capable of partially depolymerizing heparan sulfate chains at a limited number of sites, is thought to help promote cancer invasion and metastasis. Heparanase is also tightly involved in angiogenesis, where it acts to release heparan sulfate−sequestered heparin-binding angiogenic factors, such as basic fibroblast growth factor (bFGF) and possibly other endothelial cell growth factors, from the basement membrane and extracellular matrix. Thus, it is perhaps unsurprising that heparanase expression levels are closely correlated with the metastatic and angiogenic potentials of tumor cells. Because of its involvement in the development and metastasis of malignant tumors, heparanase has recently become an attractive target for the treatment of highly malignant tumors. Oligomannurarate sulfate (JG3) (Figure 12.7), a newly semisynthesized, structurally novel sulfated oligosaccharide derived from marine oligomannurarate blocks, has been found to be a new inhibitor of heparanase.[152]

JG3 significantly inhibits tumor growth, angiogenesis, and metastasis. It can abolish heparanase-driven invasion and suppress the release of heparan sulfate−sequestered basic fibroblast growth factor (bFGF) from the extracellular matrix and subsequent angiogenesis. Moreover, JG3 inactivates the bFGF-induced bFGF receptor and the phosphorylation of ERK1/2 and blocks bFGF-triggered angiogenic events by directly binding to bFGF. On the other hand, JG3 combats heparanase activity via binding to the KKDC and QPLK domains of the heparanase molecule. The JG3−heparanase interaction is competitively inhibited by low-molecular-weight heparin but not by other glycosaminoglycans. Thus, JG3 seems to inhibit heparanase activities by acting as a competitive inhibitor of heparan sulfate. In conclusion, JG3 inhibits both major heparanase activities by simultaneously acting as a substrate mimetic and as a competitive inhibitor of heparan sulfate, collectively contributing to its anticancer, anti-metastasis, and antiangiogenesis activities.[152]

Philinopside E

Marine-derived cancer therapeutics have been intensively investigated for their novel chemical skeletons and unique anticancer potentials. Trabectedin (Yondelis, ET-743) from a tropical sea-squirt is the first marine drug approved for cancer therapy (soft-tissue sarcoma in the European Union, 2007) and several marine natural products have been taken into clinical trials.[163] PE (Figure 12.1), isolated from the sea cucumber *Pentacta quadrangularis*, elicits potent antiangiogenesis and antitumor activity by a unique molecular mechanism. PE specifically interacts with the extracellular domain of KDR, which is distinct from conventional small-molecule inhibitors targeting its cytoplasmic kinase domain to block its interaction with VEGF and the downstream signaling molecules. PE also markedly suppresses alpha (v) beta (3) integrin-driven downstream signaling as a result of disturbance of the physical interaction between KDR and alpha (v) beta (3) integrin in HMECs, followed by disruption of the actin cytoskeleton organization and decreased cell adhesion to vitronectin. All of these findings substantiate PE to be an unrecognized therapeutic class in tumor angiogenesis and, more importantly, reveal

the therapeutic potential in angiogenesis and cancer development via targeting integrin−KDR interaction.[153,154,164]

Grateloupia longifolia Polysaccharide

GLP is another marine natural product, a new type of polysaccharide isolated from the alga *G. longifolia*. GLP inhibits cell proliferation, migration, and tube formation of HMEC-1 cells, and reduces CAM neovascularization, which result in obvious *in vitro* and *in vivo* antiangiogenesis effect.[155] However, the antiangiogenesis effect of GLP is not associated with the classical VEGF−VEGFRs signaling. In contrast, GLP significantly decreases tissue factor at both mRNA and protein levels suggesting that GLP inhibits angiogenesis by downregulating the expression of tissue factor.[155]

12.4.2.3   Cellular Signaling Kinase-Targeted Anticancer Agents

The PI3K-Akt-mTOR Cascade Inhibitor S9

S9, a hybrid of alpha-methylene-gamma-lactone and 2-phenyl indole compound (Figure 12.4), is a novel dual inhibitor of the PI3K-Akt-mTOR axis and tubulin polymerization,[157,165] possesses potent activity against this pathway. On the one hand, S9 abrogates the EGF-activated PI3K-Akt-mTOR signaling cascade and Akt translocation to cellular membrane in human tumor cells. S9 inhibits both PI3K and mTOR but minimally affects the 30 other tested kinases. Notably, S9 completely impedes hyper-phosphorylation of Akt as a feedback of inhibition of mTOR by rapamycin. On the other hand, S9 arrests cells in M phase other than G1 phase, distinct from compounds targeting the PI3K-Akt-mTOR pathway. This unexpected phenomenon results from the fact that S9 inhibits tubulin polymerization via binding to the colchicine-binding site of tubulin. Molecular modeling further supports the conclusion that S9 concurrently targets the PI3K-Akt-mTOR pathway and tubulin by revealing that S9 potentially binds to the kinase domains of PI3K p110alpha subunit and mTOR, and shares similar hydrophobic interactions with colchicine in the complex with tubulin. S9 induces rapid apoptosis in tumor cells, further reflecting the synergism between its blocking the PI3-Akt-mTOR signaling and inhibiting tubulin cytoskeleton. Finally, S9 exerts potent antiproliferative activity in a panel of tumor cells originated from different tissue types including drug-resistant cells and in nude mice bearing human tumor xenografts. Collectively, S9 targets both the PI3K-Akt-mTOR signaling and the microtubule cytoskeleton, which combinatorially contributes to its antitumor activity and provides new clues for anticancer drug design and development.

The EGFR Inhibitor BB

The new synthetic quinonazoline derivative BB (*N*-(3-bromophenyl)-7-methoxy-6-(3-(3-methoxypyrrolidin-1-yl)propoxy)-quinazolin-4-amine; Figure 12.4) is a selective EGFR inhibitor.[227] BB selectively inhibits EGFR with an IC_{50} value of 50 ± 37 nM, at least 32-fold more potent than it suppresses all other 10 tested receptor tyrosine kinases (RTK). Consequently, BB effectively inhibits autophosphorylation of the EGF-stimulated EGFR and phosphorylation of its key
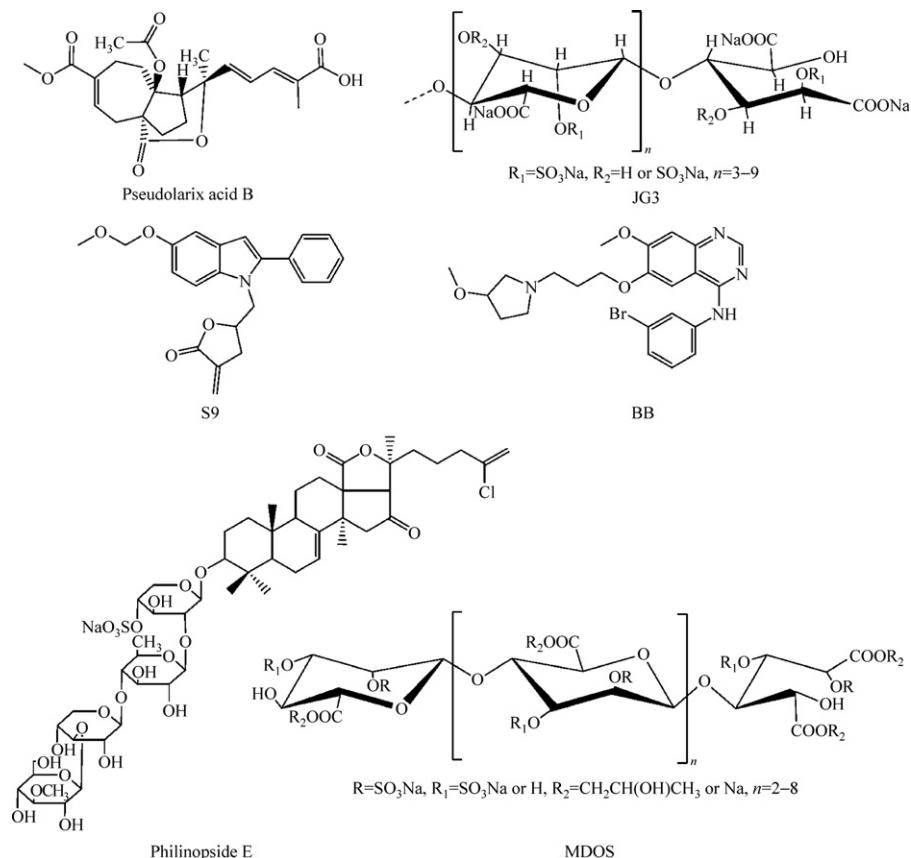
Figure 12.4 Chemical structures of the new molecular-targeted anticancer agents discussed in this section.

downstream signaling molecules ERK and AKT in A-549 cells. BB not only suppresses the EGF-stimulated proliferation of A-549 cells but also inhibits the EGF-independent proliferation of various tumor cells. BB also exhibits antiangiogenesis activity, as evidenced by antagonizing EGF-induced HMEC-1 migration *in vitro*, blocking HMEC-1 tube formation, and inhibiting microvessel sprouting from rat aortic rings. Most importantly, BB prominently inhibits *in vivo* tumorigenesis of NIH3T3 cells specifically driven by the activation-mutated EGFR genes (A750P or L858R) and reduces the number of microvessels in the xenografts. Therefore, BB could be a promising EGFR-targeted anticancer candidate.

The Multi-Targeted Protein-Tyrosine Kinases Inhibitor Marine-Derived Oligosaccharide Sulfate

The novel MDOS (Figure 12.4) is a multi-targeted PTK inhibitor that inhibits various RTK and non-receptor tyrosine kinases (NRTK).[158] At enzymatic levels,

MdOS suppresses RTKs including HER2, EGFR, VEGFR, PDGFR, c-Kit, and FGFR1, and the NRTK c-Src, with minimal impact on FGFR2. Consistently, MdOS inhibits phosphorylation of PTKs, exemplified by HER2, EGFR, and VEGFR2, and downstream molecules of Erk1/2 and AKT at cellular levels. MdOS suppresses PTKs in a unique ATP-competitive mode of action via directly binding to the residues of entrance rather than those of the ATP-binding pocket. Consequently, MdOS exerts excellent antiangiogenic activity revealed by inhibiting proliferation and tube formation of HMECs, arresting microvessel outgrowth of rat aortic rings, and hindering the neovascularization of chick allantoic membrane. Collectively, as a new multi-targeted PTK inhibitor with a novel unique scaffold, MdOS could be a promising agent for further evaluation in PTK-associated cancer therapy.

12.5 Recent Work on Design, Synthesis, and Antitumor Evaluation of Several Series of Derivatives

12.5.1 N-Substituted-Thiourea Derivatives

12.5.1.1 Design and Synthesis

RTKs play crucial roles in signal transduction pathways that regulate cell differentiation and proliferation.[166] Overexpression of certain growth factor, receptor kinases is strongly associated with carcinogenesis.[167] The epidermal growth factor receptor (EGFR/Her-1/ErbB-1), which belongs to the ErbB receptor family, is a 170 kDa glycoprotein that contains an extracellular ligand-binding domain, a transmembrane region, and an intracellular domain with kinase activity.[168] A strong correlation has been found between solid tumors with high levels of EGFR and poor prognosis.[169] Thus, EGFR is an attractive target for the design and development of compounds that can specifically bind the receptor and inhibit its tyrosine-kinase (TK) activity and its signal transduction pathway in cancer cells. A variety of approaches can be used to target EGFR family members, and the most popular two have been explored for extensive cancer chemotherapy against cancers that overexpress EGFR family receptors: blocking ligand binding to the extracellular domain with humanized monoclonal antibodies and using small-molecule inhibitors that interact at the ATP-binding site.[170] The most promising small-molecule inhibitors of the EGFR kinase are currently several scaffolds, which include quinazolines,[170−175] pyridopyrimidines,[176,177] benzamides,[178,179] indolinones,[180] pyrrolotriazines,[181] and others. Of these, the 4-anilinoquinazoline derivatives exhibit IC_{50} values up to the subnanomolar range in enzymatic assays.[182,183] Figure 12.5 includes some examples in the 4-anilinoquinazoline series that are currently approved drugs or in clinical trials.

Despite the high potency and prolonged inhibition of EGFR functions reported for some of the reversible inhibitors, the high intracellular concentrations of ATP make it difficult for inhibitors to reach sufficiently high concentrations *in vivo* to
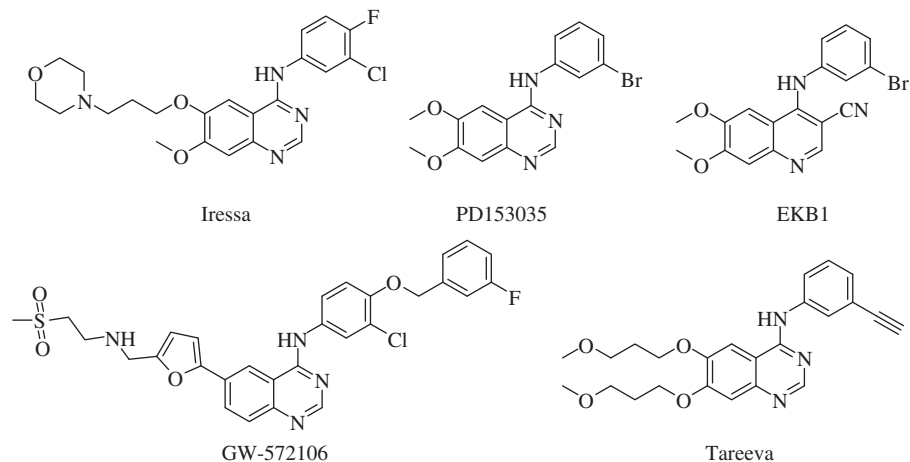
Figure 12.5 Representative small-molecule inhibitors of the EGFR kinase.

fully shut down EGF-stimulated signal transduction for long periods.[184] Some research groups[185] have therefore developed irreversible inhibitors based on the 4-(phenylamino)quinazolines, which can form a covalent bond with cysteines at the active site of the receptor by the Michael addition reaction. Furthermore, although EGFR inhibitors have exhibited curative effects in non-small cell lung cancer patients, some side effects with these agents have been sequentially found, such as cutaneous effects.[186] These thereby prompted researchers to discover novel EGFR inhibitors without serious side effects. Recently, a novel framework, which was identified by using a structure-based virtual screening approach[187,188] based on the crystal structure of OSI-774/EGFR-TK[189] in conjunction with chemical synthesis and bioassay, was presented by Li and coworkers.[190]

The crystal structure of EGFR-TK in complex with OSI-774 (PDB entry 1M17)[189] recovered from the Brookhaven Protein Data Bank was used as a target for virtual screening on the SPECS_1 database.[191] The DOCK 4.0 program (Kuntz group, San Francisco, CA, USA)[192] was employed for the primary screening. Conformational flexibility of the compounds from the database was considered during the docking search. Three thousand molecules with the highest score obtained by DOCK search were rescored by using the Consensus Score method (CScore).[193] Molecules with a CScore of ≥4 were reevaluated by the pharmacophore[194] model of EGFR inhibitors. Finally, 82 compounds were distinguished and purchased for bioassay on the basis of the above virtual screening flow. Among the 82 compounds, the biosensors RU of 26 compounds were concentration dependent. The collected data indicated that these 26 compounds (including compounds **1** and **2**) can bind to EGFR *in vitro* and the binding affinities to EGFR are in the submicro- or micromolar range (K_D = 97.7−0.39 mmol/l). Compounds **1** and **2** (Figure 12.2), bearing the higher binding affinities to EGFR as determined by the surface plasmon resonance (SPR) technology, were used as lead compounds to design new EGFR inhibitors.
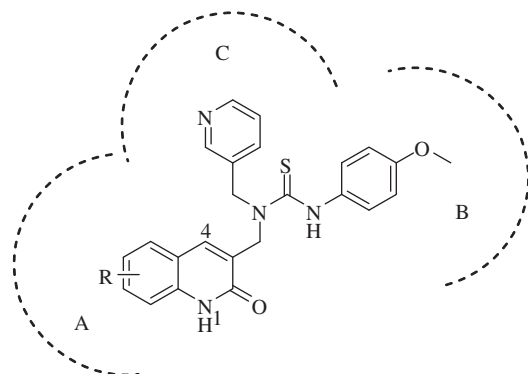
Figure 12.6 Structure of compounds 1 and 2.

1: $R = 6\text{-Me}$, $K_D = 1.46\,\mu\text{mol/l}$

2: $R = 7\text{-Me}$, $K_D = 80.7\,\mu\text{mol/l}$

Chemical structures and binding affinities of compounds **1** and **2** are shown in Figure 12.6. Having kept the common moiety of compounds **1** and **2** of the N-(2-oxo-1,2-dihydro-quinolin-3-ylmethyl)-thiourea framework, three regions of these two molecules were selected to perform chemical modifications suitable to provide expedient and significant SAR information and improve inhibitory activity: (A) 6- or 7-Me substituent; (B) the N′-phenyl ring; and (C) the N-pyridinylmethyl side chain (Figure 12.6).[190]

First, various steric electronic groups were used as substitute at positions 6, 7, and 8 of the 2-oxo-1,2-dihydro-quinoline ring in region A and obtained 7 analogs. Second, 23 compounds were designed by maintaining region A (6-Me or Br substituent) and replacing the 4-methoxyphenyl group in region B with other electronic and hydrophobic substituted aryl groups. Finally, eight compounds were achieved by replacing the pyridinylmethyl moiety in region C with electronic and hydrophobic groups.

N-aryl acetamides reacted as the starting material by refluxing with phosphoryl chloride in N,N-dimethylformamide (DMF), which afforded 2-chloro-quinoline-3-carbaldehydes. They were converted to the corresponding 2-oxo-1,2-dihydro-quinoline-3-carbaldehydes by refluxing with hydrochloric acid. Afterward, the aldehydes were condensed with various primary amines in ethanol and then were reduced with $NaBH_4$ giving the secondary amines. The target compounds were obtained by N-acylation of secondary amines using substituted isothiocyanates at room temperature.

12.5.1.2 Antitumor Evaluation

The EGFR kinase inhibitory activity of designed compounds was then evaluated using kinase autophosphorylation assay by ELISA. Disappointingly, most compounds just displayed low inhibition against the autophosphorylation of EGFR kinase at a concentration of 10 μmol/l. The enzymatic activities of the compounds **1** and **2**

do not consistently correlate with the SPR-binding affinities. The reason may be that the protein was immobilized to a sensor chip in the SPR assay, which affects the conformational flexibility of the protein. Compounds **1** and **2** are good binders and moderate inhibitors to EGFR. Accordingly, the effects of compounds on tumor cell activity and cell growth inhibitory activities on SPCA1 cell of the 40 inhibitors were determined. The results indicated that six compounds showed potent inhibitory influence on the viability of the SPCA1. It is remarkable that the inhibitory activity of compound **4a** increases approximately 10 times more than that of compound **1**. These encouraging results prove the validity of chemical modification.

According to the above results, some noteworthy conclusions could be drawn as follows: (1) 6-substitutions on the quinoline ring in region A is favorable for maintaining activity, especially within small groups; (2) 2-monosubstitutions on the phenyl ring in region B can substantially improve potency. Replacement of the phenyl ring with the large naphthanyl ring cannot be tolerated, nearly leading to a loss of activity; and (3) the introduction of the cycloalkyl moiety in region C improves activity distinctly compared with the (hetero) aromatic moiety. The inconsistency between enzyme activity and cellular efficiency was explained tentatively based on the docking simulation. Moreover, the 3D-binding models of OSI-774 to EGFR obtained from the crystal structure were compared with that of compound **4a** to EGFR generated based on the docking simulation (Figure 12.7).

Figure 12.7A shows that the nitrogen (N1) of the quinazoline of OSI-774 accepts a hydrogen bond (H-bond) from the amide nitrogen of Met769; simultaneously, the other quinazoline nitrogen (N3) forms a strong H-bond interaction with the Thr766 side chain through a water molecule bridge, which is important for maintaining inhibitory activity.[189,195] Whereas the N1 of the quinoline moiety of **4a** forms an H-bond with the backbone carbonyl group of Met769, the phenyl ring in the B region, and the piperidine ring in C region form weak hydrophobic interactions with residues Leu694, Val702, Leu820, and Thr830 (Figure 12.7B). The
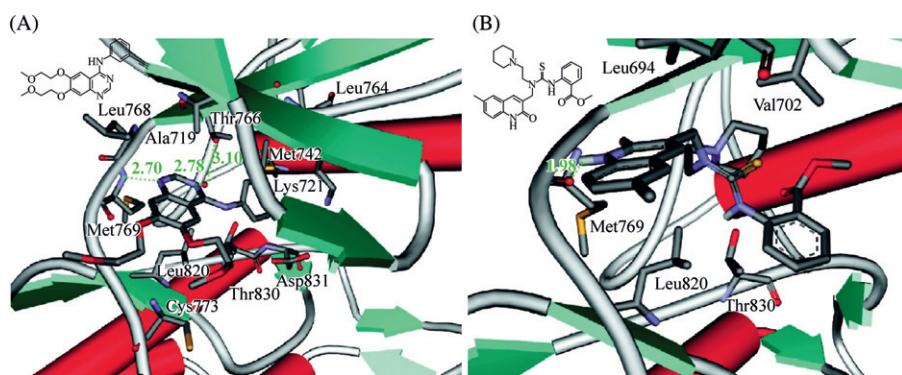
Figure 12.7 Binding models of OSI-774 (A) and **4a** (B) at the ligand-binding site of EGFR. The H-bond is represented by the green dotted line.

above difference in the formation of H-bonds between the inhibitors and the kinase domain may affect the inhibition of EGFR kinase activity. This indicates that the designed compounds might interact with multiple proteins involved in the EGFR signaling pathways and not only target the TK, thus leading to the promising anti-proliferation effect against the SPAC1 cell line.

12.5.2   3,5-Substituted Indolin-2-One Derivatives

12.5.2.1   Design and Synthesis

The indolin-2-one core is a well-known pharmacophore for developing PTK inhibitors (Figure 12.8). Sun et al. developed an extensive SAR for the indolin-2-one analogs suggesting that the inhibitory activity and selectivity of these compounds against particular PTK depends on the substituents of the indolin-2-one core, especially on the C-3 position.[196,197]

3-Substituted indolin-2-ones adopting the Z configuration (R1 is substituted by pyrrol-2-yls or thien-2-yls) are potent and selective inhibitors of the FGF TK.[196] However, compounds with the E configuration (R1 is substituted by benzylidenyl) show fairly good potency in inhibiting EGFR-TK.[197] The X-ray crystal structures of the FGFR TK in complex with 3-{[3-(2-carboxyethyl)-4-methylpyrrol-2-yl] methylene}-2-indolinone (SU5402)[198] revealed that the indolin-2-one core of SU5402 occupied the adenine pocket of the ATP-binding site of the TK, and the substituted groups at the R1 position bound to the hydrophobic pocket of the ATP-binding site (Figure 12.8). SU5402 with the Z configuration is a selective inhibitor of FGFR and VEGFR. What caught the researcher's attention in particular was the biological activity of E-3-substituted indolin-2-one derivatives. So it is very
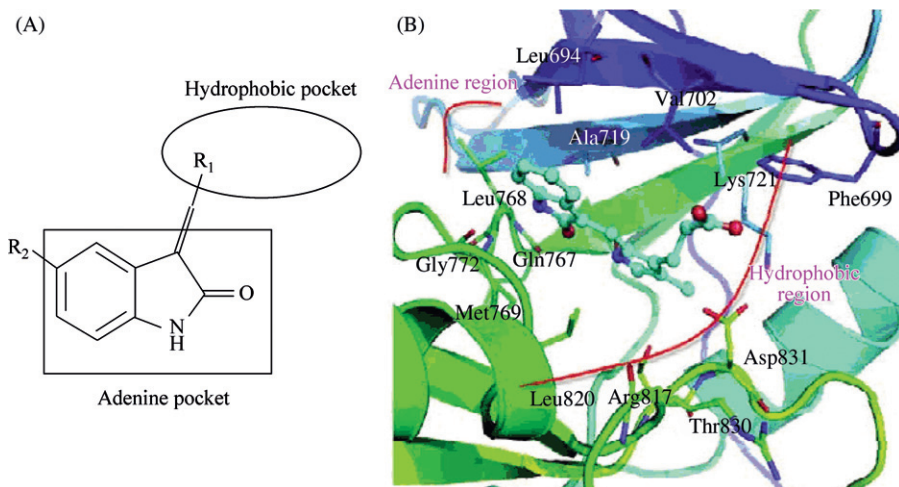
Figure 12.8 (A) Indolin-2-ones PTK inhibitors. (B) Three-dimensional structural model of SU5402 in the binding site of FGFR derived from the docking simulation.

interesting to explore whether it would enhance the interaction between indolin-2-one compounds and TK and increase antitumor activity by introducing a β-pyrrole group into the 3 position of indolin-2-ones. Accordingly, a novel class of 3-pyrrole, 5-substituted indolin-2-one derivatives (**5a−5t**) have been synthesized by Li and coworkers,[199] and their inhibitory activities against EGFR, FGFR, VEGFR, and PDGFR were determined.

Acetonedicarboxylates were used as the starting materials. In general, dimethyl-1,3-acetonedicarboxylate and diethyl-1,3-acetonedicarboxylate reacted with *t*-butyl acetoacetate by classic Knorr synthesis to produce substituted pyrroles, respectively. The pyrroles were hydrogenated, decarboxylated, and treated by Vilsmeier formylation condition, giving the pyrrole-3-carbaldehydes. Indolin-2-ones were commercially available. 5-Bromoindolin-2-one and 5-nitroindolin-2-one were prepared by bromination and nitration of indolin-2-one, respectively. 5-Carboxyindolin-2-one was afforded by hydrolysis of 5-chloroacetylindolin-2-one, which was prepared by chloroacetylation of indolin-2-one. 5-(Aminosulfonyl) and the other 5-(substituted aminosulfonyl)-indolin-2-ones were obtained by amidation of 5-(chlorosulfonyl) indolin-2-one, which was prepared by sulfonylation of indolin-2-one with chlorosulfonic acid. Target compounds were synthesized by condensing pyrrole aldehydes and 5-substituted indolin-2-ones in the presence of piperidine. In total, 20 new compounds were designed and synthesized.

The absolute configuration of **5a** was finally confirmed by X-ray structural analysis with a yellow needle single crystal obtained by slow evaporation of a dilute solution in EtOH/H_2O (40:1). The X-ray crystal structure of **5a** is shown in Figure 12.9A. The pyrrole ring and the carbonyl O of the indolin-2-one are at the opposite sides of the double bond, indicating that **5a** is E isomeric. The superposition of the AutoDock predicting conformation of **5a** with the X-ray structure is shown in Figure 12.9B. The root mean square deviation between these two conformations is ∼0.386 Å, and the major deviation is from the flexible moiety -$CH_2CO_2CH_2CH_3$, indicating that the bioactive conformation of **5a** is similar to its crystal structure.

12.5.2.2   Antitumor Evaluation

Disappointingly, most compounds only displayed low to moderate inhibition activity against EGFR, FGFR, VEGFR, and PDGFR at the concentration of 10 μmol/l. To some extent, compounds **5a**, **5b**, **5c**, **5g**, and **5h** exhibited a better ability to inhibit the EGFR and VEGFR kinase (percent inhibition at 10 μmol/l >20.0%) than the FGFR and PDGFR kinase. However, the cellular assay turned out more encouraging (Table 12.5). Four human carcinoma cell lines of A-431, A-549, MDA-MB-468, and ADPKD were chosen for the cell proliferation assay. The results indicate that compounds **5d**, **5g**, and **5h** show promising antiproliferation activities for A-431, A-549, and MDA-MB-468 (percent inhibition rates at 10 μmol/l >50%; Table 12.5). It is remarkable that **5d** and **5e** show fairly good activity against ADPKD (IC_{50} = 0.1 and 3.7 μmol/l, respectively). The bromination or nitration at the C-5 position of the indolin-2-one core increased the potency
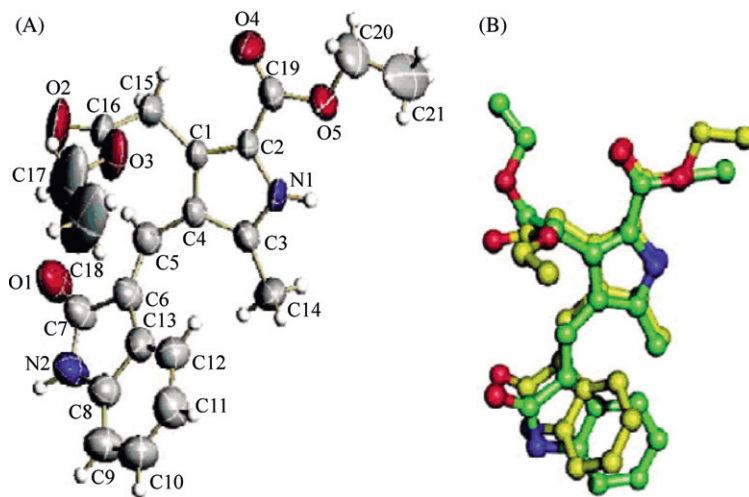
Figure 12.9 (A) X-ray crystal structure of compound **5a**. Crystal system, space group: Triclinic, P-1; Unit cell dimensions: a = 8.5204 (18) Å. b = 10.488(2) Å. c = 11.933(3) Å; Crystal size: 0.403 mm × 0.167 mm × 0.063 mm; Final R indices [I > 2 sigma (I)] R1 = 0.0749, wR2 = 0.1625; R indices (all data) R1 = 0.2466, wR2 = 0.2339. (B) Superposition of the X-ray crystal structure of **5a** (yellow) with the binding conformation derived from docking simulation (green).

Table 12.5 Inhibitory Effect of Selected Compounds on the Growth of Tumor Cell Lines

Compound	Tumor Cell Inhibition Rate[a] (%) or IC$_{50}$ (μmol/l)[b]			
	A-431	**A-549**	**MDA-MB-468**	**ADPKD[c]**
5c	7.8%	31.8%	1.9%	45.9
5d	61.5%	69.9%	69.7%	0.1
5e	NA	1.3%	NA	3.7
5f	6.0%	NA	13.6%	25.0
5g	79.4%	9.4[b]	8.9[b]	318.0
5h	71.2%	0.098[b]	0.065[b]	127.0
5l	11.6%	NA	4.6%	26.0
5o	12.7%	1.3%	9.7%	22.0

[a]The percent inhibition rate of tumor cell at 10 μmol/l inhibitor. Cell line A-431 (epidermoid carcinoma), A-549 (lung carcinoma), MDA-MB-468 (breast carcinoma), and ADPKD (kidney cyst) overexpresses EGFR-TK.
[b]Dose−response curves were determined at five concentrations. The IC$_{50}$ values are the concentrations in micromolar needed to inhibit cell growth by 50% as determined from these curves.
[c]The IC$_{50}$ values with dose−response curves were determined at seven concentrations.
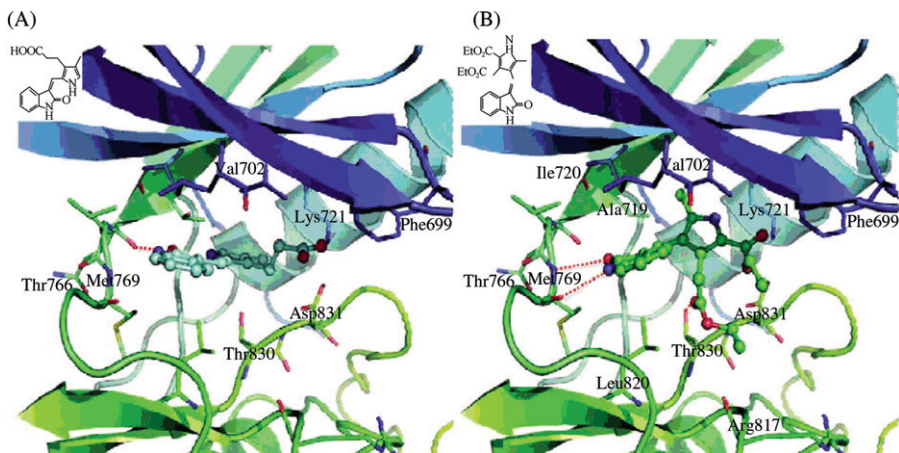
Figure 12.10 Three-dimensional structural models of SU5402 (A) and **5a** (B) in the active site of EGFR derived from the docking simulation. O atoms are shown as red, N atoms are shown as blue in SU5402 and compound **5a**.

(**5d** > **5c**, **5g** > **5e**). The inconsistency between the enzyme activity and the cellular efficiency could imply that the new type of indolin-2-one compounds might inhibit multiple key proteins involved in the EGFR and VEGFR signaling pathways, not only targeting the TK, thus leading to the significant antiproliferation effect against EGFR-dependent tumor cell lines, which is highly relevant to the overexpression of EGFR or VEGFR kinase. The definite mechanism is still under study.

SU5402 and compound **5a** was docked into the active site of EGFR-TK by using AutoDock 3.0.3. The predicted bioactive conformations are shown in Figure 12.10. The ATP-binding pocket of EGFR consists of Thr766, Gln767, Leu768, Met769, Gly772, Thr830, Asp831, Val702, Lys721, Ala719, Glu738, and Met742.[200] This binding pocket can be divided into three regions, including two hydrophobic regions and an adenine region. The adenine region mainly comprises Gln767 and Met769. Hydrophobic region I shaped by Ala719−Lys721, Leu764−Thr766, Thr830, and Asp831 is located deep inside the ATP-binding pocket. Hydrophobic region II mainly comprises Leu694 and Gly772. SU5402 formed one hydrogen bond with Thr766, and six hydrophobic interactions with Leu694, Val702, Ala719, Lys721, Leu830, and Asp831 (Figure 12.10A). The intramolecular hydrogen bonding between the N atom of the pyrrole ring and the carboxyl O atom of the indolin-2-one core in SU5402 is responsible for the Z isomeric form (Figure 12.10A). When changing the α-pyrrole ring to a β-pyrrole ring, the distance between these two atoms is lengthened, and the introduction of a methyl group in the 5′-position of the pyrrole ring avoids the intramolecular hydrogen bond formation. Figure 12.10B shows the interaction model of compound **5a** and EGFR. The indolin-2-one core of compound **5a** occupies the adenine pocket of EGFR, and the

pyrrole moiety lies in the hydrophobic region. Compound **5a** forms three hydrogen bonds with Met769 and Thr830, and eight hydrophobic interactions with Leu694, Phe699, Val702, Ala719, Ile720, Lys721, Arg817, and Leu820. This binding model is similar to that of Tarceva and EGFR.[201] The quinazoline and anilino moieties of Tarceva also occupied the adenine pocket and the hydrophobic region, respectively. There are more hydrogen bonds and hydrophobic interaction pairs between **5a** and EGFR than with SU5402 and EGFR. It provides a promising new template for further development of antitumor agents.

12.5.3   3-Nitroquinolines

12.5.3.1   Design and Synthesis

The crystal structure of OSI-774/EGFR-TK indicates that the nitrogen atom located at the 3 position of these quinazoline inhibitors is an important feature needed for good activity.[202] This nitrogen atom could be interacting with a water molecule and that this water molecule could then serve as a bridge between the drug and enzyme. Replacing this atom with a carbon leads to a significant loss in the ability of the compound to inhibit the enzyme. According to this, Wissner et al.[203] removed and replaced this nitrogen atom with a carbon atom that had an attached cyano group. A series of 4-anilinoquinoline-3-carbonitriles was then synthesized by Li and coworkers,[161] and some of them exhibited significant ability to inhibit EGFR kinase.

As pointed by Chen et al.,[204] when there is an indirect, water-mediated hydrogen bond from an inhibitor to the protein, there is good reason to attempt to build into the space occupied by the water molecule. The success of Wissner et al.[203] indicated that the space due to removal of the water molecule bound to Thr830 could accommodate a small group. On the basis of these considerations, a series of novel 3-nitroquinoline derivatives were designed, in which the cyano group at the 3 position is replaced by nitro group.[161]

An efficient and facile synthesis approach was developed to prepare a variety of 3-nitroquinoline derivatives with various C-4, C-6, and C-7 substituents. Beginning with the commercially available isovanillin or vanillin, the benzylation with benzyl bromide gave the aldehydes in good yield. Treatment of the aldehydes with the fuming nitric acid furnished the selective nitration products. They were converted to the corresponding *o*-nitrobenzoic acids by refluxing with 10% KMnO$_4$. Afterward, the nitro group was reduced and condensed with nitromethane followed by thermal cyclization in refluxing aceticanhydride giving the quinolines. The quinolines were then converted, in good yield, to the corresponding chloroquinolines by refluxing in an excess of POCl$_3$. Then, refluxing a solution of a chloroquinolines and a substituted aniline derivative in DMF generated the desired final products in good yields.

12.5.3.2   Antitumor Evaluation

The inhibition of the EGFR activity by the compound synthesized was evaluated and analyzed by the sulforhodamine B (SRB, Sigma) assay for their inhibitory

Table 12.6 Inhibitory Effect of Selected Compounds on the Growth of Tumor Cell Lines

		Tumor Cell Inhibition Rate (10 μM)	
Compound	**R**	**A-431**	**MDA-MB-468**
6a	3′-C≡CH	87.3	0
6b	3′-Br	47.6	0
6c	3′-F	0	44.3
6d	3′-F, 4′-Cl	82.8	17.9
6e	3′-OCH$_2$CH$_3$	14.6	50.0
6f	2′-OCF$_3$	4.8	41.2
6g	4′-OCH$_2$CH$_3$	89.0	88.8
6h	4′-OCH$_2$CH$_3$	61.0	0

activities toward human epidermoid carcinoma (A-431) and breast cancer (MDA-MB-468) cells. These cells are known to overexpress EGFR, which leads to continuous activation of the EGFR pathway involved in the cell proliferation. For the primary assay, the percent inhibitions of the compounds at the concentration of 10 μM against A-431 and MDA-MB-468 were measured. The biological results for the 3-nitro-4-anilino-6,7-dialkoxyquinolines inhibitors are shown in Table 12.6.

Due to earlier work by some research groups with the 4-anilinoquinazoline-based inhibitors of EGFR established that a meta-substituted electron-withdraw group in the aniline moiety is compatible with good activity, this feature was retained in initial compounds. The initial compound **6a** with an attached 3′-ethynyl group exhibited potential inhibition activities toward A-431 cell line, with 87.3% inhibition at the concentration of 10 μM; replacing the ethynyl group with bromo-atom did not improve the inhibition activities. Substitution of the bromo-atom with chloro-atom or fluoro-atom resulted in a sharp loss of inhibitory activities to A-431 cell line. The 3-fluoro-4-chlorobenzenamine substituted nitroquinoline derivative **6d** presented potent inhibitory effects against A-431 cell line. But changing the substituted groups at position 6 resulted in a clear decrease in the ability to inhibit EGFR. These findings indicate that both the aniline moiety and the 6,7-dialkoxy substitution play important roles in the inhibition activities. Thus, compound **6d** was chosen as the benchmark compound for subsequent optimization studies. The compounds which retained the 6,7-dialkoxy substitution of **6d** were first investigated. Among them, compound **6e** was a little more active than the initial
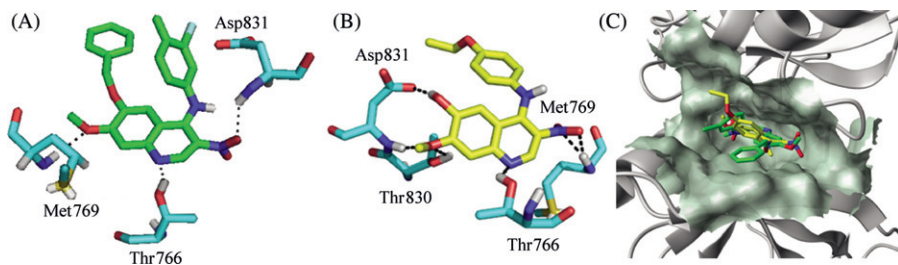
Figure 12.11 Binding models of compounds **6d** and **6g** in the binding cleft of EGFR. The left part (A, **6d**) and middle part (B, **6g**) describe the detailed interactions. The right part (C) shows the docking models of **6d** (green) and **6g** (yellow) into the active sites of EGFR.

compounds, with 50% inhibition against MDA-MB-468 at 10 μM. However, their inhibition activities toward A-431 were decreased. Surprisingly, the compound **6g** exhibited high inhibition activity toward both the A-431 and MDA-MB-468, with 89.0% and 88.8% inhibition at 10 μM, respectively. Subsequently, three derivatives were synthesized, which were designed based on the potent inhibitor **6g**. Disappointingly, all these compounds showed decreased inhibitory activities toward both A-431 and MDA-MB-468, and a few of them proved to have completely lost inhibitory activity. To some extent, **6h** exhibited a better ability to inhibit A-431 than the other compounds, whereas all of them are poor inhibitors of MDA-MB-468.

To determine the potency of the compounds that exhibited significant inhibition toward A-431 or MDA-MB-468 at 10 μM, three compounds (**6a**, **6d**, and **6g**) were further investigated in concentration-response studies. Compound **6a** displayed good activity for the cell line A-431 ($IC_{50} = 0.49$ μM) but was much less effective to inhibit the MDA-MB-468 cell line. Encouragingly, compound **6g** showed remarkable positive response on both the two cell lines ($IC_{50} = 0.40$ μM and $= 0.22$ μM, for A-431 and MDA-MB-468, respectively). More remarkable is that compound **6d** showed prominent inhibitory activities against A-431 cell line with the IC_{50} values up to nanomolar range. It exhibits inhibitory activity as high as 56.9% against A-431 even at 10 nM.

Molecular modeling experiments were carried out to investigate the binding interactions between this series of compounds and the active site of EGFR. The conformation with the lowest predicted binding free energy of the most occurring binding modes in EGFR active pocket was selected. In the final model with compound **6d** (Figure 12.11), the N1 atom of the quinoline forms a hydrogen bond with the hydroxyl group of Thr 766, and the 3-nitro group extending deep into the cleft forms a hydrogen bond interaction with the backbone NH of Asp831. As for the 6,7-dialkoxy moiety, the 6-benzyloxy group point to the entrance of the active pocket. Interestingly, the oxygen atom at 7-methoxy group forms a hydrogen bond with the NH of Met769. While for the orientation of **6g**, its interactions with the

protein are not similar to that observed in the **6d** model. The hydrogen bond of the N1 atom to the hydroxyl group of Thr 766 is retained. In this model, the 3-nitro group displaces the 7-methoxy group of **6d** that was previously hydrogen bonded to the protein and instead forms two hydrogen bonds with the backbone NH of Met769. Particularly, the significance of this interaction was reenforced by the performance of the 6-hydroxy group and the 7-methoxy group which form three hydrogen bonds with Asp831 and Thr830, respectively. The binding modes of **6d** and **6g** with EGFR showed that although different conformations were adopted for the two compounds in the EGFR active pocket, both of them formed favorable hydrogen bonds with the hydroxyl group of Thr 766 and the backbone NH of Met769. As reported previously, the interaction with the backbone NH of the Met769 is important for binding to the ATP site both for ATP and inhibitors, which can explain why **6d** and **6g** are potent with respect to their ability to inhibit the growth of EGFR-overexpressing cell lines. This model will be helpful for further structural elaboration of the novel nitroquinoline series to improve the kinase activity.

12.5.4   Quercetin-3-O-Amino Acid-Esters

12.5.4.1   Design and Synthesis

PTKs catalyze phosphoryl transfer of the γ-phosphoryl group of ATP to tyrosine residues of proteins playing a central role in signal transduction and cellular mechanisms.[205] Src, an NRTK which functions as an early upstream signal transduction protein, is activated in several human cancers, including carcinomas of the breast, lung, colon, esophagus, skin, parotid, cervix, as well as gastric tissues.[206,207] Therefore, it is an attractive target for the discovery of antitumor drugs.

Quercetin, a water-soluble flavanoid, and its derivatives have ameliorative effects on a host of disorders including cancer, renal, and cardiovascular diseases, and have inhibitory activity against SARS-CoV 3CL^{pro} or viral replication.[208−210] Particularly, quercetin is a well-known PTK inhibitor at micromolar level. For instance, its IC_{50} value is 0.9 μM against EGFR and 15 μM against Src TK respectively,[211,212] indicating that this natural product is more active against EGFR than Src TK. During the last decade, there has been considerable interest in synthesis, functional elucidation, and biological evaluation of quercetin and its derivatives.[213−215] Most of the studies were focused on the quercetin O-glycosides, the majority of which have a sugar linkage at the 3-OH. Up to now, the quercetin-3-O-amino acid-ester was neither discovered as a natural product nor reported on synthesis and bioactivity studies. Therefore, whether these types of compounds could be synthesized and whether they are still active against PTKs remain unknown. So a series of novel quercetin-3-O-amino acid-esters were synthesized by Huang and coworkers.[216] Remarkably, not only can these compounds be synthesized but also they show promising high selective inhibitory activity against Src TK.

To prepare a variety of quercetin derivatives with various O-3 substituents, an efficient and facile synthesis approach is developed. Beginning with the commercially available rutin, protection of the hydroxyl groups and subsequent deglycosylation led

to the selective protected quercetin which gave an entry in the series substituted on the 3 position. Indeed, it still exhibits two free hydroxyl groups. However, the higher reactivity of 3 position allows the selective esterification by protected amino acids in THF using 1.2 equiv. of *N*, *N*-dicyclohexylcarbodiimide (DCC) as condensing agent and a little 4-dimethylaminopyridine (DMAP) as catalyst. Cleavage of benzyl group was performed with hydrogenolysis catalyzed by 10% Pd/C. Then, the desired final products quercetin-3-*O*-amino acid-esters were obtained in good yields after purification by chromatography. Substitution at N atom of amino acid moiety is important. In the first attempt to get the compounds bearing deprotected amino group of amino acid moiety, trifluoroacetate acid solution of dichloromethane was used to remove the tertbutyloxycarbonyl group. However, no desired product was detected in the crude product. The target compound may be decomposed in the acidic medium. So an alternative approach performed under mild condition was then investigated. Unfortunately, attempts to debenzylate the compounds bearing benzylated N atom in amino acid moiety under H_2 atmosphere at ambient temperature were not successful. Based on the above study, it could be deduced that the compounds without protective groups of N atom are unstable.

12.5.4.2 Antitumor Evaluation

For the primary assay, the percent inhibitions of the compounds (**7a−7o**) at 10 μM were measured (data are listed in Table 12.7). Remarkably, the newly synthesized quercetin-3-*O*-amino acid-esters show low inhibition against EGFR kinase (<43%), whereas exhibit inhibitory activity as high as 76% against Src kinase (Table 12.7). This result suggests that the novel quercetin-3-*O*-amino acid-esters have higher inhibitory selectivity against Src kinase than EGFR kinase. Thus, the introduction of the amino acid group into quercetin leads to the reverse of the high inhibitory selectivity from EGFR to Src. To confirm the bioactivity, the IC_{50} values were further determined for the compounds with inhibition rate higher than 50% against Src kinase at 10 μM, namely, compounds **7a−7c**, **7g**, **7i−7k**, and **7m** (Table 12.8). The data show that all the eight compounds have prominent inhibitory activities with IC_{50} values ranging from 3.2 to 9.9 μM, indicating that some quercetin-3-*O*-amino acid-esters are moderately active inhibitors of Src kinase.

The binding free energies of quercetin to EGFR and Src kinase were calculated to be −6.6 and −5.7 kcal/mol, respectively, which is in good agreement with the experimental results that quercetin is more active against EGFR than Src kinase. Therefore, the docking approach and parameters are reasonably reliable. The predicted binding free energies (ΔG) of the eight new compounds are listed in Table 12.8. Noticeably, the predicted ΔG values of the new compounds to Src kinase (−8.1 kcal/mol in average) are stronger by 1.4 kcal/mol than that to EGFR (−6.7 kcal/mol in average), which is in agreement with experimental observation that the eight new compounds are stronger inhibitors against Src kinase than against EGFR kinase. Therefore, the selectivity of the newly synthesized quercetin-3-*O*-amino acid-esters should be attributed to the specific property of the substituted R groups, the amino acids (Table 12.7).

Table 12.7 Enzyme Inhibitory Activity of the Quercetin-3-*O*-Amino Acid-Esters
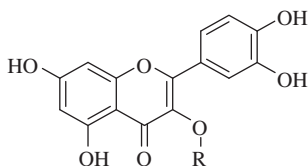
Compound	R-OH	Inhibition at 10 μM (%)	
		EGFR	**Src**
7a	*N*-Boc-(L)-Leucine	30.4	55.4
7b	*N*-Boc-(L)-Alanine	35.9	51.4
7c	*N*-Boc-(L)-Valine	21.0	52.5
7d	*N*-Boc-(D)- Leucine	29.2	44.5
7e	*N*-Boc-(D)- Valine	22.3	40.7
7f	*N*-Ac-(L)-Leucine	25.9	39.0
7g	*N*-Ac-(L)-Phenylalanine	20.9	60.5
7h	*N*-Boc-(L)-Phenylalanine	35.9	40.9
7i	*N*-Boc-(L)-Glycine	43.2	76.2
7j	*N*-Boc-(L)-Threonine	36.8	76.1
7k	*N*-Boc-(D)- Threonine	34.6	71.0
7l	*N*-Boc-(L)-Tryptophan	15.6	44.4
7m	*N*-Boc-(L)-Asparagine	23.8	50.8
7n	*N*-Boc-(L)-Proline	19.3	27.7
7o	*N*-Ac-(L)-Proline	21.0	24.3

Table 12.8 The IC_{50} Values (in μM) and Predicted Binding Free Energies (ΔG in kcal/mol) of Some Quercetin-3-*O*-Amino Acid-Esters

Compound	EGFR Kinase		Src Kinase		
	Inhibition at 10 μM (%)	Predicted ΔG	Inhibition at 10 μM (%)	IC_{50}	Predicted ΔG
7a	30.4	−6.20	55.4	4.2	−7.40
7b	35.9	−6.43	51.4	6.5	−8.03
7c	21.0	−7.40	52.5	7.4	−8.17
7g	20.9	−7.02	60.5	5.9	−7.89
7i	43.2	−7.45	76.2	3.3	−7.63
7j	36.8	−5.66	76.1	3.5	−7.07
7k	34.6	−7.82	71.0	4.9	−8.78
7m	23.8	−6.20	50.8	9.9	−10.14

Different conformations have been found for these compounds in the active pockets of both proteins. For the comparison of the difference in binding mechanism, the pairs of hydrophobic interaction (HI hereinafter) and the number of hydrogen bond (HB hereinafter) between the new compounds and the two targets are analyzed by the program LIGPLOT.[217] The result reveals that there are, in average, 14 HIs and 3.5 HBs between Src and each of the new compounds, while there are, in average, only 9 HIs and 2.5 HBs between EGFR and each compound. In other words, one-third more of these two kinds of interactions were observed between the compounds and Src kinase than EGFR kinase. As examples, the interaction details of the two most active compounds (**7i** and **7j**) are shown in Figure 12.12. There are 12 atoms of **7i** forming hydrophobic interactions with 7 residues of Src, of which 5 atoms are from the newly substituted group of **7i** (Figure 12.12B); while there are
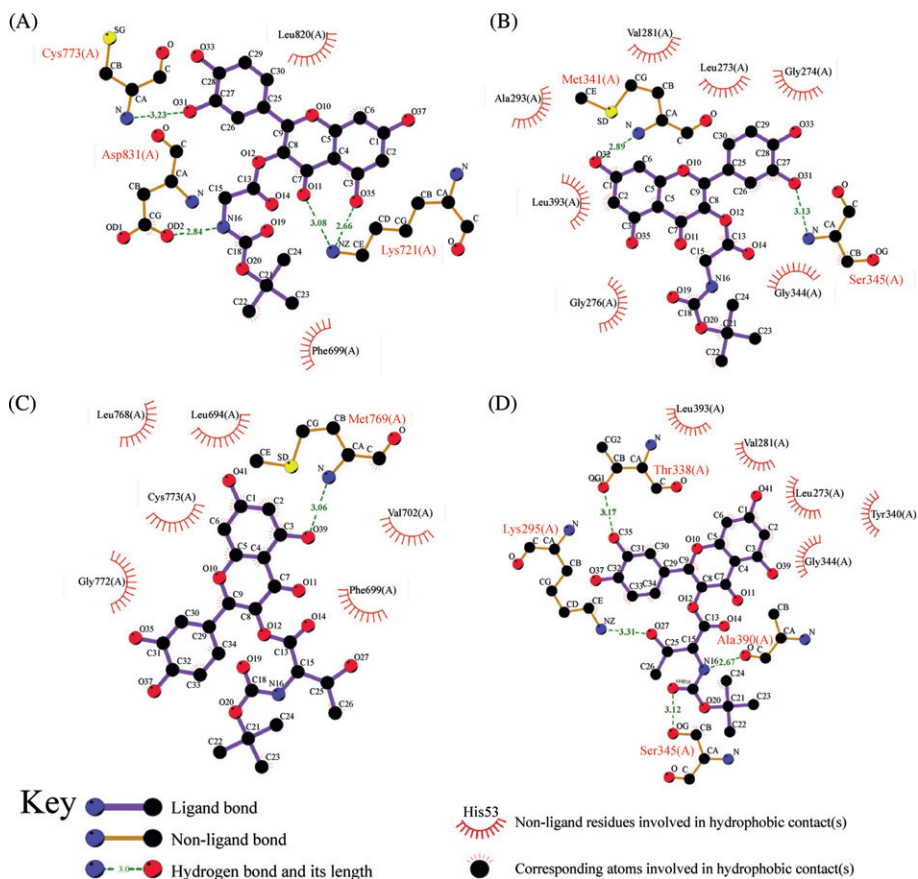
Figure 12.12 The interaction mechanism between the targets and the two most active ligands. A and B are for compound **7i** with EGFR and Src kinase, respectively; C and D are for the compound **7j** with EGFR and Src kinase, respectively.

only 5 atoms of **7i** forming hydrophobic interaction with two residues of EGFR, of which only two atoms are from the substituted group (Figure 12.12A). Two hydrogen bonds form between **7i** and Src, while four HBs between the compound and EGFR. Regarding the binding of **7j**, the hydrophobic interaction between **7j** and Src is similar to that between **7j** and EGFR, but there are four HBs between **7j** and Src while only one between **7j** and EGFR. Therefore, both hydrophobic and hydrogen bonding interactions are important to the high selectivity of the novel quercetin-3-*O*-amino acid-esters against Src kinase.

This study provides a new promising scaffold with moderate inhibitory activities (IC$_{50}$ values ranging from 3.2 to 9.9 μM) for further development of new anticancer drugs targeting Src TK.

### 12.5.5 Triaminotriazine Derivatives

12.5.5.1 Design and Synthesis

Triazines have been widely studied due to its broad range of biological activities, such as antimicrobial effects,[218] Erm (erythromycin-resistance methylase) methyltransferase inhibition,[219] anti-trypanosomal activity,[220] VLA-4 (integrin very late antigen-4) antagonism,[221] estrogen receptor modulation,[222] and cytotoxic activity.[223]

Based on the above findings and the availability of abundant tri-substituted 1,3,5-triazine derivatives, parts of compounds were screened on selected targets, especially some tumor cell lines. One of the exciting screening results is that compound (4,6-bis(*N*-morpholino)-[1,3,5]triazin-2-yl)-phenylamine (**8a**, Table 12.9) exhibited moderate inhibition activity toward HT-29 (one of the cell lines of CRC), with 80.5% inhibition at the concentration of 10 μM. Nowadays, CRC has become one of the major cancers that threaten people's lives. The American Cancer Society estimates that there will be about 106,680 new cases of colon cancer and 41,930 new cases of rectal cancer in 2006 in the US, and they will cause about 55,170 deaths. Though the death rate from CRC has been going down for the past 15 years, there is a continuing urgent need to develop new potent chemical agents.[224] The finding of compound **8a** prompted to undertake a study of the *in vitro* inhibition activities of triazine derivatives substituted by subunits of morpholino and arylamino toward CRC cell lines, HCT-116, and HT-29. Menicagli et al.[223] reported 2,4,6-tris(*N*-morpholino)-1,3,5-triazine and several 2-alkyl-4,6-bis(*N*-morpholino)-[1,3,5]triazines, with negligible cytotoxic activities against leukemia cell lines, L1210, and HL-60, and glioma cell line C6 but no (4,6-bis(*N*-morpholino)-[1,3,5]triazin-2-yl)-arylamines and 6-morpholino-*N*,*N*′-diaryl-[1,3,5]triazine-2,4-diamines have been considered. Consequently, a series of novel *N*-morpholino triaminotriazine derivatives were synthesized by Zheng and coworkers.[225]

Based on the structural feature of the screening hit **8a**, 12 compounds were designed and synthesized for the first round. Keeping the two morpholino groups of **8a**, compounds **8b−8e** were obtained by introducing different electronic substituents to the para position of the phenyl ring of **8a** or substituting the phenyl ring

Table 12.9 Chemical Structures of Compounds **8a**−**8f** and **9a**−**9g**, and Their Inhibitory Effects on the Growth of Tumor Cell Lines
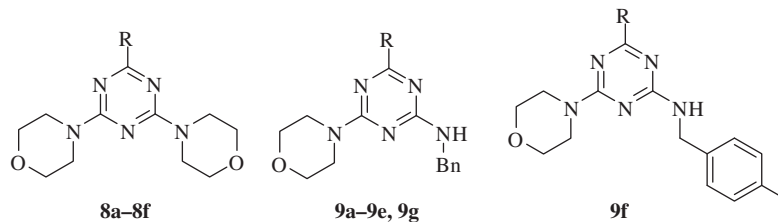
Compound	R-H	Inhibition[a]		Compound	R-H	Inhibition[a]	
		HT-29	HCT-116			HT-29	HCT-116
8a	Aniline	80.5%	44.9%	**9a**	Aniline	90.5%	48.7%
8b	4-Aminobenzene-sulfonamide	NI[b]	NI[b]	**9b**	4-Aminobenzene-sulfonamide	68.6%	NI[b]
8c	4-Methoxyaniline	74.2%	6.9%	**9c**	4-Methoxyaniline	88.4%	20.1%
8d	4-Fluoroaniline	80.2%	NI[b]	**9d**	4-Fluoroaniline	87.1%	10.1%
8e	Benzylamine	5.2%	NI[b]	**9e**	Benzylamine	50.0%	NI[b]
8f	Morpholine	NI[b]	NI[b]	**9f**	Aniline	75.3%	15.7%
				9g	Aniline	85.0%	81.3%

[a]Inhibition at 10 μM (%). Values are means of three determinations and deviation from the mean is <10% of the mean value.
[b]NI, no inhibition.

of **8a** with benzyl. Substitution of the anilino group of **8a** with morpholino gave tris-(*N*-morpholino)-1,3,5-triazine (**8f**). Replacing one of the morpholino units of compounds **8a−8e** with benzylamino, *p*-methylbenzylamino or anilino unit, the corresponding mono-N-morpholino substituted triazine derivatives (**9a−9g**) were obtained. According to the bioassay results of the first round, compounds **9h−9x** were further designed and synthesized (Table 12.10), using 6-morpholino-*N*, *N′*-diphenyl-[1,3,5]triazine-2,4-diamine (**9g**) as the benchmark compound. Compounds **9h−9r** were obtained by introducing various steric, electronic, and hydrophobic groups to one of the phenyl rings of **9g**. Compounds **9s−9x** were prepared by introducing various substituents to both of the phenyl rings of **9g**. Displacing the morpholino unit of the potent compounds 9 with the desired amines, compounds **10a−10g** were prepared (Table 12.11).

12.5.5.2   Antitumor Evaluation

Compounds **8a−8f**, **9a−9x**, and **10a−10g** were evaluated and analyzed by the sulforhodamine B (SRB, Sigma) assay[226] for their inhibitory activities toward CRC cell lines (HCT-116 and HT-29). For the primary assay, the percent inhibitions of the compounds at the concentration of 10 μM against HCT-116 and HT-29 were measured. The results are summarized in Tables 12.9−12.11. As shown in Table 12.9, the initial compound **8a** exhibited potential inhibition activities toward HT-29 and HCT-116, with 80.5% and 44.9% inhibition at the concentration of 10 μM, respectively. Introducing substituents to the para position of the phenyl ring of **8a** (compounds **8b−8d**) did not improve the inhibition activities. Substitution of the anilino unit of **8a** with benzylamino (**8e**) or morpholino (**8f**) resulted in a complete loss of inhibitory activities to both HCT-116 and HT-29. These findings indicate that the anilino group of compound **8a** plays an important role in the potent inhibition activities of the CRC cell lines. The mono-N-morpholino substituted triazine derivatives (**9a−9e**) presented more potent inhibitory effects against HT-29 than their corresponding 4,6-bis(*N*-morpholino)-[1,3,5]triazine derivatives (**8a−8e**). Among them, compounds **9a**, **9c**, and **9d** were a little more active than the initial compound **8a**, with 90.5%, 88.4%, and 87.1% inhibition against HT-29 at 10 μM, respectively. Dianilino derivative (**9g**) showed more potent activity against HT-29 and a large improved inhibitory activity against HCT-116 in comparison with **8a**, with 85% and 81.3% inhibition at 10 μM, respectively. Thus, compound **9g** was chosen as the benchmark compound for subsequent optimization studies. The inhibitory activities of the second round of compounds **9h−9x** against HCT-116 and HT-29 were tested and the results are summarized in Table 12.10. Compounds **9h−9r**, which retained the morpholino group and one of the anilino units of **9g**, were first investigated. Halogen (F, Cl, and Br) substituted derivatives (**9h−9j**) demonstrated improved activities toward HT-29. However, their inhibition activities toward HCT-116 were decreased. Electron-donating groups substituted on the phenyl ring of **9g** produced excellent to good anti-proliferatory potencies against HT-29. For instance, compounds **9l** (4-OCH$_3$), **9m** (2-OCH$_3$), **9n** (3-OCH$_3$), and **9p** (4-OCH$_2$CH$_3$) exhibited high inhibition activity toward

Table 12.10 Chemical Structures of Compounds **9h−9x** and Their Inhibitory Effects on the Growth of Tumor Cell Lines

9h–9r

9s–9t, 9x

9v–9w

9u
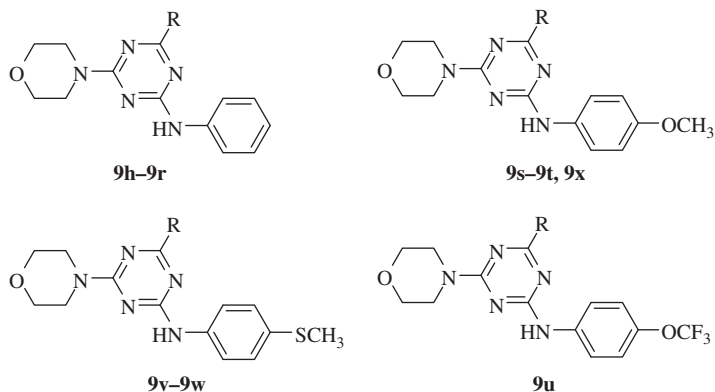
Compound	R-H	Inhibition[a]		Compound	R-H	Inhibition[a]	
		HT-29	HCT-116			HT-29	HCT-116
9h	4-Chloroaniline	87.1	51.7	**9q**	4-Aminobenzene-sulfonamide	80.4	96.2
9i	4-Bromoaniline	89.7	77.7	**9r**	4-Aminobenzamide	87.8	85.8
9j	4-Fluoroaniline	90.0	60.1	**9s**	4-Methoxyaniline	80.6	53.3
9k	4-(Trifluoromethyl)-aniline	87.3	63.4	**9t**	4-Fluoroaniline	82.3	NI[b]
9l	4-Methoxyaniline	100	81.4	**9u**	4-Fluoroaniline	85.5	62.0
9m	2-Methoxyaniline	89.3	78.3	**9v**	4-Methylaniline	54.1	71.1
9n	3-Methoxyaniline	87.4	82.6	**9w**	3,4-Dimethoxyaniline	81.4	79.2
9o	3,4-Dimethoxyaniline	76.4	84.7	**9x**	4-Aminobenzene-sulfonamide	83.2	69.0
9p	4-Ethoxyaniline	87.5	64.1				

[a]Values are means of three determinations and deviation from the mean is <10% of the mean value.
[b]NI, no inhibition.

Table 12.11 Chemical Structures of Compounds **10a−10g** and Their Inhibitory Effects on
the Growth of Tumor Cell Lines

10a, 10c, 10e   **10b, 10d, 10f**

10g
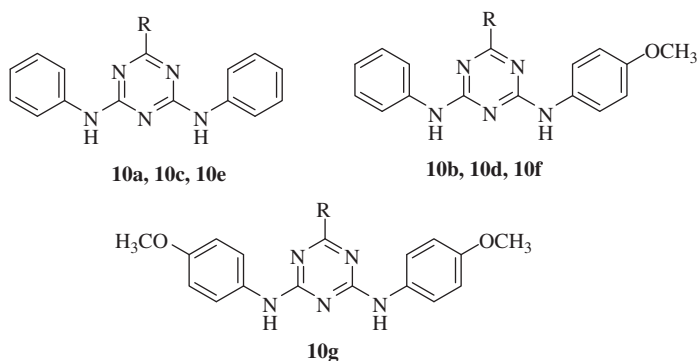
Compound	R	Inhibition[a]		Compound	R	Inhibition[a]	
		HT-29	HCT-116			HT-29	HCT-116
10a		23.4	30.1	**10e**		76.1	22.0
10b		74.6	NI[b]	**10f**		77.3	19.0
10c		33.9	NI[b]	**10g**		49.3	NI[b]
10d		13.7	NI[b]				

[a]Values are means of three determinations and deviation from the mean is <10% of the mean value.
[b]NI, no inhibition.

HT-29, with 100%, 89.3%, 87.4%, and 87.5% inhibition at 10 μM, respectively.
The similar potency of compounds **9l−9n** indicates that a methoxy substituent
walking on the phenyl ring had little impact on the inhibition potency to both
HCT-116 and HT-29. Synergistic increase in activity was not found for the two
methoxysubstituted derivative (**9o**) toward HT-29 but was found in its activity
against HCT-116 with 84.7% inhibition at 10 μM. The sulfanilamide derivative
(**9q**) and p-aminobenzamide derivative (**9r**) exhibited the high inhibitory activi-
ties against HCT-116, with 96.2% and 85.8% inhibition at 10 μM, respectively.
Among the second round of compounds, four compounds (**9l**, **9n**, **9q**, and **9r**)
exhibited significant inhibitory potency against both HT-29 and HCT-116.
Compounds (**9s−9x**), with substituents on both of the two phenyl rings of **9g**,
showed similar inhibition potency with the benchmark compound **9g**. However,
they were all less active than the p-methoxy analog (**9l**). Decreased potency
toward HCT-116 was observed throughout this subseries of compounds, and the

Table 12.12 Determination of IC_{50} Values of Selected Compounds of **9** on the Growth of CRC Cell Line

Compound		9g	9h	9i	9l	9n	9o	9q	9r	9s	9v
IC_{50}[a] (μM)	HT-29	31	37	25	30	39	ND[b]	ND[b]	8.1	100	ND[b]
	HCT-116	4.7	ND[b]	ND[b]	0.76	ND[b]	0.92	2.0	9.6	ND[b]	120

[a]Values are means of three determinations and deviation from the mean is <10% of the mean value.
[b]ND, not determined.

p-fluoro derivative (**9t**) proved to be virtually inactive against HCT-116 at 10 μM. Table 12.11 lists the biological results of derivatives **10a−10g**, which were designed based on the potent inhibitors of **9g** and **9l**. All these compounds showed decreased inhibitory activities toward both HCT-116 and HT-29, and a few of them proved to have completely lost inhibitory activity. These results suggest the morpholino subunit directly introduced to the 1,3,5-triazine nuclear is an important determinant of inhibitory activity toward both HCT-116 and HT-29. To determine the exact potency of the compounds that exhibited significant inhibition toward HCT-116 or HT-29 at 10 μM, 10 compounds (**9g−9i**, **9l**, **9n**, **9o**, **9q−9s**, and **9v**) were further investigated in concentration-response studies, and the results are summarized in Table 12.12.

Compounds **9g−9i**, **9l**, **9n**, and **9r−9s** were tested for their IC_{50}s (the compound concentration required for 50% growth inhibition of tumor cells) against HT-29. Most of these compounds showed moderate growth inhibition activities with IC_{50}s ranging from 8.1 to 39 μM. Compound **9r** ($IC_{50} = 8.1$ μM), which was most prominent in this series of compounds against HT-29, was nearly 4 times more active than the benchmark compound **9g** ($IC_{50} = 31$ μM). Compounds **9g**, **9l**, **9o**, **9q−9r**, and **9v** were tested for their IC_{50}s against HCT-116. Most of them proved to be potent inhibitors with IC_{50} values below 5 μM, except compounds **9r** and **9v**. Among them, compounds **9l** ($IC_{50} = 0.76$ μM) and **9o** ($IC_{50} = 0.92$ μM) were the optimal ones, which were 5 times more active relative to the benchmark compound **9g** ($IC_{50} = 4.7$ μM). Compound **9l**, which was the most potent one *in vitro* against HCT-116, was chosen as the representative one of this class of triaminotriazines to undertake a study of its pharmacokinetic properties and *in vivo* antitumor activities. The pharmacokinetic (PK) properties of **9l** were assessed in Sprague-Dawley rats. The orally administered **9l** was found to be rapidly absorbed from the gastrointestinal tract. The mean peak concentration (C_{max}) was 1.29 $\mu g/ml$ achieved at 15 min after oral administration. By comparing with intravenous data, the oral bioavailability (F) of **9l** was 30.9%. The elimination half-lives (T1/2) of **9l** by oral and intravenous administration were 1.16 and 1.09 h, respectively, while the mean resident times (MRT) were 1.88 and 1.06 h, respectively. The distribution volume (V_d) and clearance (CL) of intravenous **9l** were 2.11 and 1.36 l/h/kg, respectively. When evaluated for antitumor efficacy in a sarcoma 180 mice model, compound **9l** demonstrated modest tumor-inhibitory activity with 40.7% inhibition at a dose of 200 mg/kg/day. The detailed experimental data are shown in Table 12.13.

Table 12.13 *In Vivo* Experimental Data of Compound **9l**[a]

Group	Dose (mg/kg)	Mice (n) Initial/End	Body Weight (g) Initial/End	Tumor Weight (g) X ± SD	Inhibition Rate (%)	P
Control	/	20/20	18.6/29.4	1.50 ± 0.52	/	/
9l	100	10/10	18.5/29.3	1.65 ± 0.42	nc[b]	nc[b]
9l	200	10/10	18.6/27.3	0.89 ± 0.44	40.7	<0.05

[a]The *in vivo* experiment was carried out in the mice sarcoma 180 model, using intraperitoneal (ip) treatment. For other detailed procedures, see section 5.1.2.
[b]nc, not calculated.

The preliminary *in vivo* antitumor studies and pharmacokinetics studies on compound **9l** showed that it might be promising for the development of new antitumor agents.

12.6   Discussion and Perspectives

In the past 60 and more years cancer chemotherapy as a new discipline was developed very rapidly. From the discovery of nitrogen mustard in the 1940s up to now more than 10 classes of new chemical drugs have been discovered that bring great benefits to cancer patients. At the early stage, chemical drugs employed to treat tumors were only expected like chemical knife to replace surgical knife, its usefulness was quite limited. Afterward, a variety of chemical agents not only can cure more than 10 kinds of malignant tumors but also can be used in combination with other modalities to treat different cancers with remarkable efficacy. In recent years, the molecular-targeting drugs were discovered and helped cancer chemotherapy enter a new era with more selective action and less toxicity. Based on molecular oncology, molecular pharmacology, and modern genetic engineering, as well as computer simulating technology by means of docking simulation, crystal structure, and virtual screening methods, some new chemical agents recently designed and synthesized can act on specific genes regulating cancer cell proliferation, division, differentiation and apoptosis, or on metastatic process. It is known that imantinib, gefitinib, erlotinib, and other small-molecule EGFR-tyrosine-kinase inhibitors can treat chronic myelocytic leukemia, gastrointestinal stromal tumor (GIST), non-small cell lung cancer, and other tumors very effectively; their action mechanisms do not kill the cancer cells as former cytotoxic agents did but have a definite influence on signal transduction or on gene expression of cancer cells. Such chemical substances with more selective activity can regulate or normalize the process of tumor growth and progression. The progress of newly developed molecular target therapy in combination with other effective methods is greatly inspiring researchers to control cancerous disease. It is hoped that in the future, chemical anticancer agents will be used not only for the purpose of curing cancers but also for preventing, repairing, or recovering malignant processes, going from the chemical knife to

the chemical regulator or modulator. It will be an important and difficult task for the investigators, and we are confident that it will be successful in the future.

From the above-mentioned data, it can be seen that the progress of cancer chemotherapeutic drug research in China is advancing very quickly. Many anticancer drugs including compounds of natural origin containing TCM products, synthetic agents, inhibitors of topoisomerases, and tumor angiogeneses, as well as other effective preparations were successfully investigated and developed for clinical use and production within a relatively short period of time. A lot of leading compounds or new drug candidates discovered by Chinese scientists are very helpful for future investigations. Undoubtedly, such valuable works have played a favorable role in the cause of cancer prevention and therapy. This chapter aims to help people to find the original work in this area and more achievements and contributions by many organizations or pharmaceutical factories in China may be found in other publications. It is hoped that greater successes and more creative works in the field of discovering new effective drugs will appear in the next decades to control neoplastic diseases.

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