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MINI-REVIEW

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Medicinal mushroom modulators of molecular targets as cancer therapeutics

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Abstract Empirical approaches to discover anticancer drugs and cancer treatments have made limited progress in the past several decades in finding a cure for cancer. The expanded knowledge of the molecular basis of tumorigenesis and metastasis, together with the inherently vast structural diversity of natural compounds found in mushrooms, provided unique opportunities for discovering new drugs that rationally target the abnormal molecular and biochemical signals leading to cancer. This review focuses on mushroom low-molecular-weight secondary metabolites targeting processes such as apoptosis, angiogenesis, metastasis, cell cycle regulation, and signal transduction cascades. Also discussed in this review are high-molecular-weight polysaccharides or polysaccharide–protein complexes from mushrooms that appear to enhance innate and cell-mediated immune responses, exhibit antitumor activities in animals and humans, and demonstrate the anticancer properties of selenium compounds accumulated in mushrooms.

Introduction

Medicinal mushrooms have an established history of use in traditional oriental therapies. Modern clinical practice in Japan, China, Korea, and other Asian countries continues to rely on mushroom-derived preparations. Medicinal effects have been demonstrated for many traditionally used mushrooms (Ooi and Liu 2000), including extracts of species from genera *Auricularia*, *Flammulina*, *Ganoderma*, *Grifola*, *Hericium*, *Lentinus* (*Lentinula*), *Pleurotus*, *Trametes* (*Coriolus*), *Schizophyllum*, and *Tremella* (Wasser 2002).

Over the past two to three decades, scientific and medical studies in Japan, China, Korea, and more recently the United States have increasingly demonstrated the potent and unique properties of mushroom-extracted compounds for the prevention and treatment of cancer.

It is well established that many mushroom-extracted compounds are commonly used as immunomodulators or as biological response modifiers (BRM). The basic strategy underlying immunomodulation is to identify aspects of the host response that can be enhanced or suppressed in such a way as to augment or complement a desired immune response. Whether certain compounds enhance or suppress immune responses depends on a number of factors, including dose, route of administration, timing of administration of the compound, mechanism of action, and site of activity. Knowledge of the specific components of cytokine networks and signaling pathways and their role in the regulation of immune responses is important in designing strategies to augment these responses.

Immunomodulators isolated from more than 30 mushroom species have shown anticancer action in animals (Wasser and Weis 1999). Only a few have been taken to the next step, that is, objective clinical assessment for anticancer potential in humans. Of this relative few, all are chemically α- or β-glucans in nature or peptide-bound polysaccharides. Numerous reports have documented the ability of β-glucans to nonspecifically activate cellular and humoral components of the host immune system (Tzianabos 2000), but failed to define their exact mechanism of action until CR3 was identified as the leukocyte membrane receptor for β-glucans (Xia et al. 1999).

New discoveries in molecular oncology along with rapid expansion of our knowledge concerning the processes that govern differentiation, apoptosis, immune surveillance, angiogenesis, metastasis, cell cycle, and signal transduction control have unveiled an abundance of specific molecular targets for cancer therapy, including a variety of small-molecule compounds that inhibit or stimulate these molecular targets.

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Cancer

A neoplasm is an abnormal mass or colony of cells produced by a relatively autonomous new growth of tissue. Most neoplasms arise from the clonal expansion of a single cell that has undergone neoplastic transformation. The transformation of a normal to a neoplastic cell can be caused by a chemical, physical, or biological agent (or event) that directly and irreversibly alters the cell genome. Neoplastic cells are characterized by the loss of some specialized functions and the acquisition of new biological properties: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Neoplastic cells pass on their heritable biological characteristics to progeny cells.

The biological behavior or clinical course of a neoplasm is further classified as benign or malignant. A malignant neoplasm manifests a greater degree of autonomy, is capable of invasion and metastatic spread, may be resistant to treatment, and may cause death. A benign neoplasm has a lesser degree of autonomy, is usually not invasive, does not metastasize, and generally produces no great harm if treated adequately.

Cancer is a generic term for malignant neoplasms. Anaplasia is a characteristic property of cancer cells and denotes a lack of normal structural and functional characteristics. A tumor is literally a swelling of any type, such as an inflammation or other swelling, but modern usage generally denotes a neoplasm.

Anticancer activity of mushrooms

It has been known for many years that selected mushrooms of higher Basidiomycetes origin are effective against cancer. The antitumor activity of the higher Basidiomycetes was first demonstrated by Lucas et al. (1957), who employed extracts of fruiting bodies of *Boletus edulis* Bull.:Fr. and other Homobasidiomycetes in tests against the Sarcoma 180 line in mice. In the 1960s, calvacin was the most commonly cited natural product isolated from the medicinal mushroom and was broadly used in many laboratories as an antitumor agent. Calvacin was isolated from the giant puffball [*Calvatia* (= *Langermannia*) *gigantea* (Batsclh: Pers.) Lloyd] by Lucas et al. (1958) and showed activity against many experimental tumors, including Sarcoma 180, mammary adenocarcinoma 755, leukemia L-1210, and HeLa cell lines (Wasser and Weis 1999). There are approximately 650 species of higher Basidiomycetes that have been found to possess antitumor activity (Mizuno 1995a,b; Wasser 2002). Searching for new antitumor and other medicinal substances from mushrooms and studying the medicinal value of these mushrooms has become a matter of great significance.

High-molecular-weight mushroom compounds

Five mushroom preparations have shown clinically significant efficacy against human cancers and are used as BRMs: lentinan from *Lentinus edodes*, D-fraction from *Grifola frondosa*, schizophyllan (also called SPG, sonifilan, sizofiran, sizofilan) from *Schizophyllum commune*, PSK (also called krestin) from *Trametes versicolor*, and PSP (polysaccharide peptide), also from *T. versicolor*. Extensive research has established that *Ganoderma lucidum* polysaccharide (GLPS) fractions have immunomodulating properties, although only a few clinical trials with GLPS preparations to treat cancer have been reported in international peer-reviewed journals (Gao and Zhou 2002). Recently, an eight-herb mixture, PC-SPES, that includes a GLPS fraction showed promising results in prostate cancer treatment (Lu et al. 2003), but, unfortunately, the mixture was found to be contaminated with a synthetic estrogen, diethylstilbestrol, which has been used for many years to treat hormone-dependent prostate cancer (Guns et al. 2002).

Lentinan (1985), schizophyllan (1986), and PSK (1977) are approved in Japan as prescription drugs for the treatment of cancer (Mizuno 1999).

All of these preparations are chemically β-D-glucans in nature or β-D-glucans linked to proteins. The biological activity of β-D-glucans is influenced by their solubility in water (Ishibashi et al. 2001), molecular weight (Okazaki et al. 1995; Cleary et al. 1999; Mueller et al. 2000), branching rate (Cleary et al. 1999; Kataoka et al. 2002), triple helical solution conformation (Mueller et al. 2000; Falch et al. 2000), and β-(1→6)-bonding system in the β-(1→3) major chain (Cleary et al. 1999).

There is evidence indicating that β-D-glucans induce biological responses by binding to a membrane receptor. The identity of the β-glucan receptor has been defined as a β-glucan inhibitable receptor for particulate activators of the alternative complement pathway (Czop and Austen 1985). More recently, another receptor, Dectin-1, was characterized as a β-glucan receptor that mediates this activity (Adachi et al. 2004; Brown et al. 2003).

The ability of complement receptor type 3 (CR3; known also as Mac-1, CD11b/CD18, or $\alpha_M\beta_2$-integrin, functioning as an adhesion molecule and a receptor for factor I-cleaved C3b, i.e., iC3b, on immune effector cells, such as macrophage) to recognize β-glucans led to the proposal that this receptor is the major β-glucan receptor on leukocytes and that it mediates all the immunomodulatory effects of these carbohydrates (Ross et al. 1999; Xia et al. 1999). When phagocyte CR3 binds to iC3b on bacteria or yeast, phagocytosis and cytotoxic degranulation are triggered because of simultaneous recognition of iC3b via a CD11b I-domain binding site and specific microbial polysaccharides via a lectin site located COOH-terminal to the I-domain. In contrast, when phagocyte or natural killer (NK) cell CR3 adheres to iC3b on erythrocytes or tumor cells that lack CR3-binding membrane polysaccharides, lysis and cytotoxicity are not stimulated. Cytotoxicity of neutrophils, macrophages, and NK cell CR3 receptor for neoplastic tissues can be achieved by priming it with β-glucans, overriding the normal resistance of iC3b-opsonized tumor cells (Vetvicka et al. 1997; Yan et al. 1999; Ross et al. 1999). Moreover, the cytotoxic activation of β-glucan-primed NK cell CR3 by iC3b-opsonized tumors

was accompanied by a tumor-localized secretion of the cytokine tumor necrosis factor α (TNF-α), interferon α (IFN-α), IFN-γ, and interleukin 6 (IL-6) (Ross et al. 1999). Furthermore, it has been shown that ligand binding to the β-glucan receptor stimulated nuclear factor κB (NF-κB) activation in U-937 human myelogenous leukemic cells (Battle et al. 1998).

Indeed, many mushroom and yeast β-glucans have been shown to stimulate the mononuclear phagocyte system (e.g., macrophages, monocytes) and certain lymphocytes (e.g., NK cells) to produce cytokines such as IFNs, ILs, and others. These are regarded as the first line in the host defense system, and may themselves successfully eliminate infected or transformed cells prior to the establishment of fully fledged humoral and cell-mediated immune responses (Borchers et al. 1999).

Treatment of human peripheral blood mononuclear cells (PBMC) with *Lentinus edodes* water-soluble extract increased levels of TNF-α, IL-1β, IL-10, and IL-12 (Jin et al. 2003). The β-glucan lentinan induced a marked increase in the mRNA levels of IL-1α, IL-1β, TNF-α, IFN-γ, and macrophage colony-stimulating factor in peritoneal exudate cells and splenocytes (Liu et al. 1999).

The ability of grifolan, a purified β-(1→3)-D-glucan from *Grifola frondosa*, to induce various cytokines from macrophages was examined in vitro. Grifolan enhanced IL-1, IL-6, and TNF-α production in the RAW 264.7 macrophage-like cell line (Adachi et al. 1994; Okazaki et al. 1995). The solubility and molecular mass of grifolan had an effect on TNF-α production (Ishibashi et al. 2001). The D-fraction, a β-glucan extracted from the fruiting body of *G. frondosa*, significantly increased TNF-α expressed in NK cells and macrophage-derived IL-12 (Kodama et al. 2002a), IFN-γ in CD4(+) T cells (Kodama et al. 2002b), and IFN-γ, IL-12 p70, and IL-18 in spleen and lymph node cells (Inoue et al. 2002).

Schizophyllan, a β-glucan isolated from the culture filtrate of *Schizophyllum commune* increased production of IFN-γ and IL-2 in PBMC cultures (Sakagami et al. 1988). A modified single helical conformer of schizophyllan induced TNF-α and IL-8 production in the U-937 monocyte-like human cell line and TNF-α only in PBMC cultures (Hirata et al. 1998).

Water extract of PSP extracted from *Trametes versicolor* significantly increased IL-1β and IL-6, while substantially lowering IL-8 in human promyelocytic leukemic HL-60 cells (Hsieh et al. 2002a). PSK extracted from *T. versicolor* stimulated the production of IL-1 by PBMC cultures more efficiently than the production of TNF. More IL-1α was accumulated in the cells than in the medium fraction, whereas IL-1β was distributed evenly into both fractions (Sakagami et al. 1993).

Mushroom trace elements

Mushrooms are a rich source of rare minerals and amino acids. Mushrooms are also a good source of trace elements such as copper, zinc, selenium, iron, and molybdenum, which are involved in many biochemical processes supporting life.

Many mushroom species accumulate trace elements to a considerably higher extent than plants. About 40 trace elements have been reported in the literature to date. A number of reviews have been published on trace element content in mushrooms (Michelot et al. 1998; Kalac and Svoboda 2000). Selenium is a very important factor in maintaining good health and for the function of a large number of physiological processes (Chariot and Bignani 2003; Rayman and Rayman 2002), including a number of enzymes (Burk 2002). The ability of mushrooms to accumulate selenium depends on their species, inhabitation, phases of mushroom growth, and precipitation quantity. Various techniques have been employed to determine the selenium concentration of mushrooms (Dernovics et al. 2002; Slejkovec et al. 2000), which is found to range over 0.012–20.0 mg/kg dry weight. The results of analyses indicate that selenium content varies considerably among different mushroom species. The highest selenium contents were found in *Boletus edulis*.

During the past two decades, selenium emerged with the most consistent anticancer effect among a number of micronutrients tested in animal experiments and clinical trials (Clark et al. 1996, 1998; Sinha and El-Bayoumy 2004). Several chemical forms of selenium have been used in laboratory studies. The prototype forms are sodium selenite, which causes single- and double-strand-break DNA damage, and selenomethionine, which is relatively nontoxic and non-DNA-damaging. Although several mechanisms including DNA cytosine methyltransferase inhibition (Fiala et al. 1998), antioxidant protection (De Silva et al. 2004), enhanced immune surveillance (Kiremidjian-Schumacher et al. 2000; Safir et al. 2003), and inhibition of angiogenesis (Lu and Jiang 2001; Jiang et al. 2000) have been proposed to account for the anticancer effect of selenium (Raich et al. 2001), selective induction of apoptosis of tumor cells may be of special significance (Ghosh 2004).

Suggested molecular mechanisms include inhibition of the anti-apoptotic proteins IκB kinase (IKK) and NF-κB (Gopee et al. 2004; Gasparian et al. 2002), up-regulation of pro-apoptotic genes, such as $p21^{CIP1/WAF1}$, $p27^{KIP1}$, *APO-1*, and *Caspase-3*, while down-regulating cell growth-regulatory genes, such as *c-myc*, *cyclin D1*, *cyclin D2*, and proliferating cell nuclear antigen (PCNA) (El-Bayoumy et al. 2003; Jiang et al. 2002; Venkateswaran et al. 2002; Dong et al. 2002; Zhong and Oberley 2001).

The recognition that selenium has anti-carcinogenic properties, with the ability of mushrooms to accumulate this element, created a market niche for selenium-enriched mushrooms (e.g., *Agaricus bisporus*, *Flammulina velutipes*, *Pleurotus ostreatus*) that could be used as a new selenium source in dietary supplements or as a value-added ingredient for the formulation of functional foods or nutraceuticals (Anonymous 2003; Werner and Beelman 2002; Stajic et al. 2002; Spolar et al. 1999).

Low-molecular-weight mushroom organic substances

Another source for substances of therapeutic interest is the pool of secondary metabolites produced by a variety of mushrooms. These substances have their origins as derivatives from many intermediates in primary metabolism, but most of them can be classified according to five main metabolic sources. These are: (1) amino acid-derived pathways, (2) the shikimic acid pathway for the biosynthesis of aromatic amino acids, (3) the acetate–malonate pathway from acetyl coenzyme A, (4) the mevalonic acid pathway from acetyl coenzyme A which functions in primary metabolism for the synthesis of sterols, and (5) polysaccharides and peptidopolysaccharides (discussed in the previous section). The polyketide and the mevalonic acid pathways are most often involved; and they produce a greater variety of compounds than the other pathways.

NF-κB inhibitors

Rel/NF-κB (NF-κB) transcription factors are a family of structurally related eukaryotic transcription factors that are involved in the control of a large number of normal cellular and whole-organism processes such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. In addition, these factors are active in a number of disease states including cancer, arthritis, chronic inflammation, asthma, neurodegenerative diseases, and heart disease (Kumar et al. 2004).

The activity of NF-κB is tightly regulated by interaction with inhibitory IκB proteins. As with the Rel/NF-κB proteins, there are several IκB proteins that have different affinities for individual Rel/NF-κB complexes, are regulated slightly differently, and are expressed in a tissue-specific manner.

In most cells, NF-κB is present as a latent, inactive, IκB-bound complex in the cytoplasm. When a cell receives any of a multitude of extracellular signals, NF-κB rapidly enters the nucleus and activates gene expression. Therefore, a key step for controlling NF-κB activity is the regulation of the IκB/NF-κB interaction. Almost all signals that lead to activation of NF-κB converge on the activation of a high-molecular-weight complex that contains a serine-specific IKK. Activation of the IKK complex leads to the phosphorylation by IKK β of two specific serines near the N terminus of IκB α, which targets IκB α for ubiquitination and degradation by the proteasome. The unmasked Rel/NF-κB complex can then enter the nucleus to activate target gene expression. One of the target genes activated by NF-κB is that which encodes IκB α. Newly synthesized IκB α can enter the nucleus, remove NF-κB from DNA, and export the complex back to the cytoplasm to restore the original latent state (Lipniacki et al. 2004; Yamamoto and Gaynor 2004).

Supernormal activation of NF-κB has been shown to be associated with cancer. This is thought to be associated with enhanced transactivation of the RelA subunit of NF-κB via activation by the hypoxic environment of many tumors, or through the action of inflammatory cytokines released either by the tumor cells themselves or by infiltrating non-tumor cell types. Active NF-κB promotes tumor growth by increasing transcription of genes that induce cell proliferation, are anti-apoptotic, pro-angiogenic, pro-metastatic, and are responsible for other cellular mechanisms required for tumor growth. An important component of tumor prevention, therefore, involves the inhibition of aberrant NF-κB activity (Ravi and Bedi 2004).

Two epoxy compounds are involved in modulating the activity of NF-κB: panepoxydone (isolated from *Panus conchatus*, *P. rudis*, and later from *Lentinus crinitus* (Erkel et al. 1996) and cycloepoxydon (isolated from *Xylaria* strain 45-93; Gehrt et al. 1998); together with the lipid-soluble thiol compound gliotoxin (an epipolythiodioxopiperazine class of fungal toxin), originally isolated from the microscopic fungus *Gliocladium fimbriatum*, but also found in various species of *Aspergillus* (Watanabe et al. 2004), *Penicillium* (Waring et al. 1987), and *Candida* (Shah and Larsen 1991).

Panepoxydone inhibited TNF-α-induced activation of NF-κB in a promoter–reporter assay using COS-7 cells and was suggested as inhibiting TNF-α- or 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced phosphorylation and degradation of IκB (Erkel et al. 1996).

Recently, a 5-dehydroxymethyl derivative of epoxyquinomicin C (a weak antibiotic isolated from *Amycolatopsis* having a 4-hydroxy-5,6-epoxycyclohexenone structure like panepoxydone from species of the genera *Panus* and *Lentinus*) designated as DHMEQ (formerly DHM2EQ) was tested as a NF-κB inhibitor (Matsumoto et al. 2000). DHMEQ inhibited TNF-α- and TPA-induced transcriptional activity of NF-κB in human T cell leukemia Jurkat cells. It also inhibited the TNF-α-induced DNA-binding of nuclear NF-κB, but not the phosphorylation and degradation of IκB. Moreover, DHMEQ inhibited the TNF-α-induced nuclear accumulation of p65, a component of NF-κB. It also inhibited TNF-α-induced nuclear transport of green fluorescent protein-tagged p65. In contrast, DHMEQ did not inhibit the nuclear transport of Smad2 and large T antigen. Also, it did not inhibit TNF-α-induced activation of c-Jun N-terminal kinase, but synergistically induced apoptosis with TNF-α in Jurkat cells. These data indicate that DHMEQ is a unique inhibitor of NF-κB, acting at the level of nuclear translocation (Ariga et al. 2002).

The activity of NF-κB was inhibited by DHMEQ in DU145, JCA-1, and PC-3 hormone-refractory prostate cancer cell lines. Statistically, significant growth inhibition was achieved by 20 μg/ml DHMEQ; and marked levels of apoptosis were induced 48 h after DHMEQ administration in vitro (Kikuchi et al. 2003).

The effectiveness of DHMEQ against the advanced KU-19-19 human bladder cancer cell line, in which NF-κB is constitutively activated, was investigated. The DNA-binding activity of NF-κB was completely inhibited following 2–6 h exposure to 10 μg/ml DHMEQ. Marked levels of apoptosis were observed 48 h after DHMEQ administration. These results confirmed that DHMEQ inhibited constitutively activated NF-κB and, consequently, apoptosis was

induced. However, it was still possible that DHMEQ caused apoptotic cell death through some other mechanism, which has not yet been fully investigated (Horiguchi et al. 2003).

Cycloepoxydon inhibited TPA-induced NF-κB- and activator protein-1 (AP-1)-mediated secreted alkaline phosphatase (SEAP) expression with 50% inhibition concentration (IC_{50}) values of 1–2 μg/ml and 3–5 μg/ml, respectively, in COS-7 and HeLa S3 cells. Cycloepoxydon strongly reduced TPA- and TNF-α-mediated binding of NF-κB to a high-affinity consensus sequence, which was due to the inhibition of phosphorylation of the protein IκB (Gehrt et al. 1998).

Pahl et al. (1996) reported that nanomolar concentrations of gliotoxin inhibit the activation of NF-κB in response to a variety of stimuli in T and B cells. The effect of gliotoxin was specific because, at the same concentrations, gliotoxin did not affect activation of the transcription factor NF-AT or interferon-responsive signal transducers and activators of transcription. Likewise, gliotoxin did not alter the activity of the constitutively DNA-binding transcription factors Oct-1 and cyclic AMP response element-binding protein (CREB) and the activation of the protein tyrosine kinases p56lck and p59fyn. Very high concentrations of gliotoxin prevented NF-κB DNA-binding in vitro. However, in intact cells, inhibition of NF-κB did not occur at the level of DNA binding. Rather, gliotoxin appeared to prevent degradation of IκB α, NF-κB's inhibitory subunit.

Recently, Vigushin et al. (2004) demonstrated that gliotoxin inhibited proliferation of six breast cancer cell lines in culture with a mean IC_{50} value of 328±289 μM. Intracellular farnesylation of lamin B and geranylgeranylation of Rap1A were inhibited in a dose-dependent manner. In randomized controlled studies using the *N*-methyl-*N*-nitrosourea rat mammary carcinoma model, gliotoxin had pronounced antitumor activity in vitro and little systemic toxicity when administered to ten animals at 10 mg/kg by subcutaneous injection weekly for 4 weeks, compared with ten controls. Single doses up to 25 mg/kg were well tolerated.

Spores or dried fruiting bodies of *Ganoderma lucidum* inhibit constitutively active transcription factors AP-1 and NF-κB in breast MDA-MB-231 and prostate PC-3 cancer cells. Furthermore, *G. lucidum* inhibits both the expression of uPA and uPA receptor and the secretion of uPA, which results in the suppression of the migration of MDA-MB-231 and PC-3 cells. These data suggest that spores and unpurified fruiting bodies of *G. lucidum* inhibit invasion of breast and prostate cancer cells by a common mechanism and could have potential therapeutic use for cancer treatment (Sliva et al. 2002, 2003; Sliva 2003).

Five novel antibiotics described as irpexans (1, 2, 3a, 3b, 4) were isolated from fermentations of an *Irpex* species in the course of screening for new inhibitors of AP-1- and NF-κB-mediated signal transduction pathways in COS-7 cells, using SEAP as a reporter gene. The expression of an AP-1- and NF-κB-driven SEAP reporter gene was inhibited in a dose-dependent manner with 14-acetoxy-15-hydroxyirpexan (3b) being the most potent compound, followed by 14,15-irpexanoxide (2), 14,15-dihydroxyirpexan (3a), and 14-acetoxy-22,23-dihydro-15,23-dihydroxyirpexan (4). Irpexan (1) exhibited no activity. All five compounds were terpenoids with a mannose moiety; and their structures were elucidated by spectroscopic methods (Silberborth et al. 2000).

Protein kinase inhibitors

Protein kinases have emerged as key regulators of all aspects of neoplasia including proliferation, invasion, angiogenesis, and metastasis. Not surprisingly, sequencing of the human genome has revealed at least 500 distinct kinases, which can be grouped into some 20 known families on the basis of structural relatedness. Compounds that possess inhibitory activity for protein tyrosine kinases were initially isolated from natural sources in the early to mid-1980s. These compounds include the flavonoid quercetin, the isoflavonoid genistein (detected initially in culture media of *Pseudomonas* spp in the search for quercetin-like flavonoids), the benzoquinoid ansamycin antibiotic herbimycin A, and erbstatin. Genistein's capacity to inhibit the mitogen-stimulated growth of mammalian cells in culture was presumed by most investigators to be due to the inhibition of tyrosine kinase activities associated with critical growth factor receptors. However, experimental data from human breast cells (Peterson and Barnes 1996) and prostate cancer cells (Peterson and Barnes 1993) strongly suggested that genistein affects these cells (and therefore other systems) by mechanisms other than inhibition of protein tyrosine kinase activity.

Using a screening approach based on a comparative evaluation of antiproliferative effects in a panel of tumor cells with differential expression of protein tyrosine kinases, three benzoquinoid macrolidic fungal metabolites produced by *Clitocybe clavipes*, clavilactones A, B, and D (respectively CA, CB, CD) and two semisynthetic derivatives of these products, diacetyl-CA and dimethyl-CA, were identified as inhibitors of protein tyrosine kinases. Naturally occurring CA, CB, and CD showed inhibitory activity in kinase assays against the Ret/ptc1 and epidermal growth factor receptor (EGF-R) tyrosine kinases, while being less effective against the v-Abl tyrosine kinase and p34 (Cdc2) serine/threonine kinase (IC_{50} values of 2.8, 5.5, 81.3, 128.0 μM, respectively, for the most potent compound CD). CB was shown to be a non-competitive inhibitor of EGF-R with respect to ATP or poly(Glu(6)Ala(3)Tyr). CD also preferentially inhibited the growth of A431 cells, which overexpress a constitutively active EGF-R, as opposed to IGROV-1 and SKOV-3 cells, which express low levels of the receptor. Furthermore, EGF-R was shown to be a target for clavilactones in A431 cells, since EGF-induced receptor autophosphorylation was inhibited in the presence of CB, CD, and diacetyl-CA. Both CD and diacetyl-CA displayed weak activity when administered daily (intraperitoneally) to mice bearing the ascitic A431 tumor (Cassinelli et al. 2000).

Protein- and DNA-alkylating agents

The sesquiterpenes illudin S and illudin M are toxic compounds obtained from the mushrooms *Omphalotus illudens* and *Lampteromyces japonicus*. They possess antibacterial and antitumor properties, but have an unfavorable toxicity profile when tested in animals (Kelner et al. 1987). Derivatives of illudins have been prepared and display increased histiospecific toxicity toward malignant cells versus normal cells. Among these are dehydroilludin M and acylfulvene formed by the treatment of illudin S with dilute sulfuric acid (McMorris et al. 1996a). The most efficacious derivative is irofulven (MGI 114, or hydroxymethylacylfulvene) prepared by the reaction of acylfulvene with paraformaldehyde in dilute sulfuric acid (McMorris et al. 1996b). Studies of the mechanism of toxicity of illudins indicate that they may behave as alkylating agents of protein and DNA (McMorris et al. 2004).

Illudin S reacts spontaneously at room temperature with thiols, such as cysteine or glutathione, at an optimum pH of about 6. Toxicity to HL-60 myeloid leukemia cells can be modulated by altering glutathione levels in cells (McMorris et al. 1990). Michael-type addition to the α,β-unsaturated ketone gives a cyclohexadiene intermediate, an extremely reactive alkylating agent, which is rapidly converted to a stable aromatic product (McMorris et al. 1999).

Cytotoxicity of MGI 114, like that of illudin S, is believed to also involve enzymatic reduction by a cytosolic NADPH-dependent enzyme. Illudin S undergoes bioreductive activation with NADPH in a rat liver cytosol preparation. The addition of hydride to the α,β-unsaturated ketone produces a highly unstable intermediate, as in the reaction with thiols. This intermediate is a potent alkylating agent (McMorris et al. 1999). Aromatic products were isolated from the enzyme-catalyzed reaction.

Irofulven demonstrated antitumor activity against human pancreatic carcinoma cell lines in vitro and in vivo (van Laar et al. 2004), HT-29 and HCT-116 colorectal and A2780 ovarian carcinoma cells (Poindessous et al. 2003a), head and neck, non-small-cell lung, malignant glioma, colon, ovary, poorly differentiated androgen-independent prostate cancer carcinoma cells, and to a lesser extent, sarcoma and leukemia cell lines (Poindessous et al. 2003b), and human pancreatic cancer cells (Wang et al. 2002).

Modulators of G1/S and G2/M checkpoints

When normal mammalian cells are subjected to stress signals (e.g., hypoxia, radiation, DNA damage, etc.) *p53* is activated. In addition to its activation, ubiquitin-dependent degradation of the p53 protein is blocked, and consequently, the p53 half-life increases significantly (Soussi 2000), leading to the accumulation of p53 and transcription of target genes involved in cell cycle control, such as $p21^{WAF1/CIP1}$ (encoding a E-Cdk2 inhibitor; Fei et al. 2002), and target genes involved in apoptosis-like *BAX* (Chipuk et al. 2004) and *Apaf-1* (Hickman and Helin 2002). The resulting increase in p53-dependent gene transcription leads to the p53-mediated induction of apoptosis and/or cell cycle arrest.

Cancer cells, through a variety of strategies, can acquire resistance to apoptosis. The most commonly occurring loss of a pro-apoptotic regulator involves the *p53* tumor suppressor gene. The resulting functional inactivation of its product, the p53 protein, is seen in more than 50% of human cancers and results in the removal of a key component of the DNA-damage sensor that can induce the apoptotic effector cascade (Oren 1999).

Genistein is the aglycone of genistin. The isoflavone is found naturally as the glycoside genistin and as the glycosides 6″-*O*-malonylgenistin and 6″-*O*-acetylgenistin. Genistein and its glycosides are mainly found in legumes, such as soybeans and chickpeas, and recently have been discovered in the mushroom *F. velutipes* (Kang et al. 2003).

Rao et al. (2004) demonstrated that 1,25(OH)(2)D(3) and genistein cooperate to up-regulate the vitamin D receptor protein by increasing the stability of the vitamin D receptor. Genistein and 1,25(OH)(2)D(3) also cooperate to up-regulate the levels of $p21^{WAF1/CIP1}$. Small interfering RNA-mediated knockdown of $p21^{WAF1/CIP1}$ expression showed that $p21^{WAF1/CIP1}$ is essential for significant growth inhibition of LNCaP cells in response to either compound or their combination.

In human hepatocellular carcinoma (HepG2) cells, genistein modulates Cdc2 kinase activity and leads to G2/M arrest. It has been shown that genistein caused an increase in both Cdc2 phosphorylation and expression of the Cdc2-active kinase, Wee1. It also enhanced the expression of the cell cycle inhibitor, $p21^{WAF1/CIP1}$, which interacts with Cdc2. Furthermore, phosphorylation/inactivation of Cdc25C phosphatase, which dephosphorylates/activates Cdc2, was increased. Genistein enhanced the activity of the checkpoint kinase, Chk2, which phosphorylates/inactivates Cdc25C, induced accumulation of *p53*, and activated the ataxia-telangiectasia mutated (ATM) gene. Caffeine, an ATM kinase inhibitor, inhibited these effects of genistein on Chk2, *p53*, and $p21^{WAF1/CIP1}$ (Chang et al. 2004).

Genistein inhibited in a time- and dose-dependent proliferation of the SKOV-3 human ovarian carcinoma cell line. The characteristic morphological changes of apoptosis were observed by staining and electron microscopy in SKOV-3 exposed to genistein. Its inhibitory effect appears to be due to the up-regulation of $p21^{WAF1/CIP1}$ mRNA and protein expression and the down-regulation of PCNA and cyclin B1 protein. The onset of apoptosis in ovarian carcinoma cell is related to the up-regulation of Bax and down-regulation of Bcl-2 in mRNA and protein level induced by genistein (Li and Mi 2003).

Genistein suppressed the proliferation of p53-null human prostate carcinoma cells. Genistein significantly inhibited the cell growth (this effect being reversible) and induced a dendrite-like structure. The inhibitory effects of genistein on cell growth proliferation were associated with a G2/M arrest in cell cycle progression concomitant with a marked inhibition of cyclin B1 and an induction of Cdk inhibitor $p21^{WAF1/CIP1}$ in a p53-independent manner. Following genistein-treatment of cells, an increased binding

of p21 with Cdk2 and Cdc2 paralleled a significant decrease in Cdc2 and Cdk2 kinase activity with no change in Cdk2 and Cdc2 expression (Choi et al. 2000).

The antitumor effect of an alcohol extract of *G. lucidum* was investigated using MCF-7 breast cancer cells. Alcohol extract of *G. lucidum* inhibited cell proliferation in a dose- and time-dependent manner, which might be mediated through up-regulation of $p21^{WAF1/CIP1}$ and down-regulation of cyclin D1. Furthermore, this compound could directly induce apoptosis in MCF-7 cells, which might be mediated through up-regulation of a pro-apoptotic Bax protein (Hu et al. 2002).

PSP extracted from *Trametes versicolor* significantly reduced proliferation of MDA-MB-231 breast cancer cells, as compared with controls. Immunostaining showed that PSP increased $p21^{WAF1/CIP1}$ expression and decreased cyclin D_1 expression (Chow et al. 2003).

Inhibitors of MAPK protein kinase signaling pathways

The mitogen activated protein kinases (MAPKs) are an evolutionary conserved family of proline-directed serine/threonine kinases. Three major MAPK cascades have been identified so far in mammalian cells: the extracellular signal-regulated kinase (ERK1/2) cascade, the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK1/JNK) cascade, and the p38 MAPK. Following stimulation by an upstream component, a serine/threonine kinase (a MAPK kinase kinase; MEKK) phosphorylates a MEK, which in turn phosphorylates a MAPK on threonine and tyrosine residues, resulting in its activation. Activated MAPKs phosphorylate a wide gamut of substrates including other kinases, phosphatases, hormone and growth factor receptors, cytoskeletal proteins, and transcription factors. These kinases serve as the integrators of signals emanating from various receptor tyrosine kinases (RTKs), nonreceptor tyrosine kinases, G protein-coupled receptors, cytokine receptors and TGF-β family receptors. Nearly all cell-surface receptors utilize one or more MAPK cascades, mediating cell proliferation, differentiation, and transformation (ERKs), or stress responses, apoptosis, and inflammation (JNKs/RKs; Fu et al. 2004; Wada and Penninger 2004). The biological effects of MAPKs are modulated by cellular context and by cross-talk with other signaling pathways. For example, one of the signals that regulate the activation of MAPKs is cAMP, which operates by activation of PKA, which in turn activates B-Raf and inhibits Ras and c-Raf-1 (Liebmann 2001). The concept that activation of the MAPK pathway plays a pivotal role in oncogenesis is supported by the fact that several oncoproteins are mutationally activated forms of enzymes that act upstream of the MAPK cascade, including ErbB-2 and Scr RTKs, Ras and Raf (Hilger et al. 2002).

The pathways that mediate intracellular signal transduction and cell-to-cell signaling interactions are replete with a plethora of potential therapeutic targets, rendering cell signaling-directed cancer therapy a very promising therapeutic approach. The positioning of multiple onco-proteins and tumor-suppressive proteins in the same pathways underscores the importance of some of these signaling routes in malignant transformation. Therefore, identification of the critical components in these pathways provides tremendous opportunities for the creation of anticancer agents that target the fundamental molecular processes responsible for cancer development, bypassing the obstacle of killing cancer cells resistant to conventional chemotherapy and radiation.

The triterpene-enriched fraction, WEES-G6, was prepared from mycelia of *G. lucidum* by sequential hot water extraction, removal of ethanol-insoluble polysaccharides, and then gel-filtration chromatography. WEES-G6 selectively inhibited growth of Huh-7 human hepatoma cells, but not Chang liver cells, a normal human liver cell line. Treatment with WEES-G6 caused a rapid decrease in the activity of cell growth regulative protein PKC and the activation of JNK and p38 MAPKs. The changes in these molecules resulted in a prolonged G2 cell cycle phase and strong growth inhibition. None of these effects were seen in normal liver cells (Lin et al. 2003).

Modulation of gap junctional intercellular communication (GJIC) is a known cellular event associated with tumor promotion. The mushroom *Phellinus linteus* extract (PL; at 5 μg/ml, 25 μg/ml) prevented the inhibition of GJIC and blocked the hyper-phosphorylation of connexin 43 by H_2O_2. Furthermore, PL was able to inactivate both ERK1/2 and p38 MAPKs. However, PL did not affect the JNK pathway (Cho et al. 2002).

Ganoderma lucidum extract induced the neuronal differentiation of PC12 cells and prevented nerve growth factor-dependent PC12 neurons from apoptosis. Moreover, these effects of *G. lucidum* might be mediated via the Ras/ERK and CREB signaling pathways, as demonstrated by the phosphorylation of ERK1, ERK2, and CREB (Cheung et al. 2000).

Ethanolic extracts (at 70%) of YZ, a proprietary dietary supplement prepared from extracts of *Trametes versicolor*, significantly reduced androgen-dependent LNCaP cell growth and down-regulated the levels of secreted PSA, but had less effect on the expression of intracellular PSA and did not affect levels of the androgen receptor (Hsieh and Wu 2001). YZ had a much less pronounced suppressive effect on the proliferation of PC-3 and DU-145 androgen-independent prostate cancer cells, compared with LNCaP. Western blot analyses showed that the expression of Rb and PCNA, integrally involved in mammalian cell DNA replication, were significantly reduced by treatment with YZ in PC-3 and DU-145 cells, respectively. Further analysis showed that YZ increased the levels of signal transducers and the activator family of transcription factors STAT 1 and STAT 3 in JCA-1 androgen-independent prostate cancer cells and not LNCaP cells (Hsieh and Wu 2001; Hsieh et al. 2002b).

Aromatase and sulfatase inhibitors

Sulfation and desulfation are important reactions in the metabolism of many steroid hormones. Estrone, estradiol, and dehydro-epiandrosterone circulate predominantly in the sulfated form and, as such, are not biologically active (i.e., do not bind target receptors). Furthermore, the sulfated forms of many steroid hormones exhibit half-lives up to 10-fold higher than the desulfated form. Estrogen metabolism is an important factor in the proliferation of estrogen receptor (ER)-positive tumors. Anti-estrogens represent first line therapy against breast cancer. Sulfation and desulfation of estrone and 17 β-estradiol clearly occur in both normal and malignant breast tissues. In benign breast tumors, estrogens are biosynthesized from steroid precursors via the aromatization of androstenedione to estrone by aromatase in adipose tissue or via the hydrolysis of estrone sulfate by arylsulfatase C (ARSC) in epithelial tissue. Aromatase inhibitors are used widely as second-line therapy in breast cancer; and there is now evidence for a chemopreventive role for these agents (Masamura et al. 1995). However, in many ER-positive breast tumors, ARSC activity is 30- to 150-fold higher than aromatase activity, and, therefore, the ARSC pathway represents the major route for intra-tumoral estrogen synthesis. The realization of the importance of the ARSC pathway for intra-tumoral estrogen production has made the inhibition of this enzyme a target for the therapeutic control of breast tumors. Potent third-generation steroid- and non-steroid-derived sulfatase inhibitors are now being developed and targeted for breast cancer therapy (Ahmed et al. 2002).

Aromatase and sulfatase inhibitors were isolated from an edible mushroom, *Lepiota americana*. 2-Aminophenoxazin-3-one inhibited aromatase at IC_{50}=5.7 μM, and 3β-hydroxy-5,8-epidioxyergosta-6,22-diene inhibited sulfatase at IC_{50}=0.9 μM. It was found that 2-aminophenoxazin-3-one was not active against sulfatase, and 3β-hydroxy-5,8-epidioxyergosta-6,22-diene was not active against aromatase (Kim et al. 2000).

Interestingly, actinomycin D is one of the most exhaustively studied DNA-binding ligands; and its widespread interest stems from its function as a model sequence-specific antitumor antibiotic. It consists of a planar 2-aminophenoxazin-3-one chromophore with two bulky cyclic pentapeptide lactones. This drug is used clinically for the treatment of malignant tumors, such as pancreatic cancer (Kleeff et al. 2000), and is also used in combination with other antitumor agents to treat high-risk tumors (Matsui et al. 2002).

Agaricus bisporus extracts suppress aromatase activity dose-dependently. Enzyme kinetics demonstrated a mixed inhibition, suggesting the presence of multiple inhibitors or more than one inhibitory mechanism. "In cell" aromatase activity and cell proliferation were measured using MCF-7aro, an aromatase-transfected breast cancer cell line. Phytochemicals in the mushroom aqueous extract inhibited aromatase activity and the proliferation of MCF-7aro cells (Grube et al. 2001).

Matrix metalloproteinases inhibitors

The matrix metalloproteinases (MMPs) are a family of at least 15 secreted and membrane-bound zinc-endopeptidases. Collectively, these enzymes can degrade all of the components of the extracellular matrix, including fibrillar and non-fibrillar collagens, fibronectin, laminin, and basement membrane glycoproteins. MMPs are thought to be essential for the diverse invasive processes of angiogenesis and tumor metastasis. Numerous studies have shown that there is a close association between expression of various members of the MMP family by tumors and their proliferative and invasive behavior and metastatic potential. In some human cancers, a positive correlation has also been demonstrated between the intensity of new blood vessel growth (angiogenesis) and the likelihood of developing metastases (Klein et al. 2004). Thus, control of MMP activity in these two different contexts has generated considerable interest as a possible therapeutic target.

The novel hydroquinone, (E)-2-(4-hydroxy-3-methyl-2-butenyl)-hydroquinone, and a known compound, polyporenic acid C, were isolated from *Piptoporus betulinus* (Kawagishi et al. 2002). Polyporenic acid C, a lanostane-type triterpene, was also isolated from *Daedalea dickinsii* (Kawagishi et al. 1997). Both compounds have been shown to inhibit collagenase (MMP-1), which cleaves all three α-chains of native interstitial collagens (Kawagishi et al. 1997). The hydroquinone derivative has been shown to inhibit stromelysin (MMP-3), which degrades a wide variety of protein substrates, including gelatin, fibronectin, and laminin and the core protein of cartilage proteoglycans. It also inhibits a 92-kDa gelatinase (MMP-9), which cleaves gelatin and the basement membrane (Kawagishi et al. 2002).

Cyclooxygenase inhibitors

It is now well established that the inducible isoform of cyclooxygenase, COX-2, is commonly over-expressed in many solid tumors (Taketo 1998a,b). Epidemiological studies and clinical trials employing selective and nonselective COX-2 inhibitors indicate that COX-2 is mechanistically involved in colorectal carcinogenesis, gastric carcinoma, breast cancer, and prostate cancer.

One chemopreventive approach that has received attention is the regular use of nonsteroidal anti-inflammatory drugs (NSAIDs). It is now generally accepted that NSAIDs prevent the development of colorectal cancer, and there is some evidence for a protective effect against breast cancer and esophageal cancer. Proposed mechanisms for these effects include the induction of apoptosis, inhibition of angiogenesis, and direct inhibition of cellular growth, all occurring, at least partly, through the inhibition of the COX enzymes involved in prostaglandin synthesis. Over-expression of COX-2 has been observed in human prostate cancer cells (Hussain et al. 2003), and higher levels of prostaglandins have been detected in malignant (as compared with benign) prostate tissues (Kirschenbaum et al. 2001). Furthermore, NSAIDs have been shown to inhibit prostate

cancer cell proliferation and induce apoptosis in vitro (Hsu et al. 2000). Thus, the COX enzymes and the synthesis of prostaglandins may represent new targets for both chemoprevention and antitumor therapy (Lieberman 2002).

The bioassay-guided isolation and purification of the hexane extract of cultured mycelia of *Grifola frondosa* led to the characterization of a fatty acid fraction and three compounds: (1) ergosterol, (2) ergostra-4,6,8(14),22-tetraen-3-one, and (3) 1-oleoyl-2-linoleoyl-3-palmitoylglycerol. The fatty acid fraction and compounds 1–3 showed COX enzyme inhibition. The inhibition of COX-1 enzyme by the fatty acid fraction and compounds 1–3 at 250 μg/ml were 98, 37, 55, and 67%, respectively. Similarly, the fatty acid fraction and compounds 1–3 at 250 μg/ml reduced COX-2 enzyme activity by 99, 37, 70, and 4%, respectively (Zhang et al. 2002).

A methanol extract of *Cordyceps pruinosa* inhibited inflammatory mediators in a LPS-stimulated RAW264.7 murine macrophage cell line and primary macrophages, by suppressing the gene expression of IL-1β, TNF-α, inducible nitric oxide synthase (iNOS), and COX-2. Moreover, the extract suppressed NF-κB activation in LPS-stimulated RAW264.7 cells. Administration of the extract significantly decreased the plasma levels of these inflammatory mediators in LPS-injected mice (Kim et al. 2003).

The gerronemins A-F (1–6) were isolated as cytotoxic components of an extract of a *Gerronema* species. Their structures were elucidated by spectroscopic techniques; and they were composed of a C12–C16 alkane or alkene substituted at both ends by 2,3-dihydroxyphenyl groups. The gerronemins blocked the inducible expression of a human cyclo-oxygenase 2 (hCOX-2) and iNOS promoter-driven reporter gene with IC_{50} values of 1–5 μg/ml (Silberborth et al. 2002).

DNA topoisomerases and DNA polymerase inhibitors

The DNA topoisomerases and DNA polymerases recently emerged as important cellular targets for chemical intervention to treat cancer. DNA topoisomerases are a group of enzymes that alter the topological structure of DNA by briefly creating and resealing DNA strand breaks to facilitate the passage of other DNA strands. Type I topoisomerases (topo I) introduce transient single-strand breaks into DNA. Topo II is required to relieve torsional strain in the DNA helix during replication and to mediate the breakage of double-stranded DNA and re-ligation of the breaks. Both topo I and II can resolve positively or negatively supercoiled DNA domains during DNA replication. Topo II is increased in rapidly proliferating cells, reaching a peak during the G_2/M phase. Topo II is found in fairly constant levels throughout the cell cycle. Some anti-topo drugs have the inhibition of enzymatic activity as their primary mode of action. Other drugs targeting the topos interfere with the enzyme's cleaving and rejoining activities and, by doing so, they increase the half-life of the transient topo-catalyzed DNA break. Cancer chemotherapeutic drugs that interfere with topo function, such as doxorubicin, daunorubicin, mitoxanthrone, camptothecin, amsacrine, etoposide, and teniposide are used to treat leukemias, lymphomas, breast, lung, and testicular cancers (Topcu 2001; Holden 2001).

DNA polymerases are eukaryotic enzymes with different interactive roles in nuclear DNA synthesis. There are several subtypes of mammalian DNA polymerases and their localization and function have been clarified. DNA polymerases α, δ, and ε have been implicated as being responsible for DNA replication, whereas DNA polymerases β, δ, and ε have been suggested as working in DNA repair. DNA polymerase γ is encoded in the nucleus, but localizes in the mitochondria and is responsible for mitochondrial DNA replication. DNA polymerase is one of the critical mammalian S-phase enzymes, necessary for the initiation of DNA synthesis in cells traversing the G_1/S phase replication block associated with Rb and p53 and other cellular oncoproteins. After the initiation of DNA synthesis, nucleotides are added to the 3′ OH primer end generated by DNA polymerase. DNA polymerase is one of the most important target molecules of antitumor agents, especially for antimetabolite nucleosides that include 1-β-D-arabinofuranosyl cytosine, 2′-deoxy-2′,2′-difluorocytidine, and 1-(2-deoxy-2-fluoro-4-thio-β-D-arabinofuranosyl) cytosine (Miura and Izuta 2004).

Nine lanostane-type triterpene acids were found in sclerotia of *Poria cocos*. Among the nine compounds, only dehydroebriconic acid could potently inhibit DNA topo II activity (IC_{50}=4.6 μM), while the compound moderately inhibited the activities of DNA polymerases α, β, γ, δ, ε, η, ι, κ, and λ only from mammals, to similar extents. Another compound, dehydrotrametenonic acid, also showed moderate inhibitory effects against topo II (IC_{50}=37.5 μM) and weak effects against all the polymerases tested. Both compounds showed no inhibitory effect against topo I, higher plant (cauliflower) DNA polymerase I (α-like polymerase) or II (β-like polymerase), calf thymus terminal deoxynucleotidyl transferase, human immunodeficiency virus type-1 reverse transcriptase, prokaryotic DNA polymerases such as the Klenow fragment of *Escherichia coli* DNA polymerase I, Taq DNA polymerase, and T4 DNA polymerase, or DNA metabolic enzymes such as T 7 RNA polymerase, T4 polynucleotide kinase, and bovine deoxyribonuclease I. These findings suggest that dehydroebriconic acid and dehydrotrametenonic acid should be designated as topo II-preferential inhibitors although they also moderately inhibited all the mammalian DNA polymerases tested. Both dehydrotrametenonic acid and dehydroebriconic acid could prevent the growth of human gastric cancer cells and their LD_{50} values were 63.6 μM and 38.4 μM, respectively. The cells were halted at the G1 phase in the cell cycle (Mizushina et al. 2004; Akihisa et al. 2004).

Mizushina et al. (1998b) and Tanaka et al. (1998) isolated new triterpenoid compounds, designated fomitellic acids A and B from the basidiomycete *Fomitella fraxinea*, which selectively inhibit the activities of mammalian DNA polymerases α and β. Recently, Mizushina et al. (2000) reported that these triterpenoids were potent inhibitors of calf DNA polymerase α, rat DNA polymerase β, and human DNA topo I and II, and showed moderate inhibitory effects on

plant DNA polymerase II and human immunodeficiency virus reverse transcriptase. However, these compounds did not influence the activities of prokaryotic DNA polymerases, such as *E. coli* DNA polymerase I, or other DNA metabolic enzymes, such as human telomerase, T7 RNA polymerase, and bovine deoxyribonuclease I. These triterpenoids were not only mammalian DNA polymerase inhibitors, but also inhibitors of DNA topo I and II, even though the enzymic characteristics of DNA polymerases and DNA topos differed markedly, including their modes of action, amino acid sequences, and three-dimensional structures. These triterpenoids did not bind to DNA, suggesting that they act directly on these enzymes. Fomitellic acid A prevented the growth of NUGC gastric cancer cells, with LD_{50} values of 38 μM and 30 μM, respectively (Mizushina et al. 2000).

Terpenoids 1, 2 and 3, which selectively inhibit eukaryotic DNA polymerase activities, were isolated from the fruiting body of the basidiomycete *Ganoderma lucidum*, and their structures were determined by spectroscopic analysis. New terpenes, lucidenic acid O and lucidenic lactone, prevented not only the activities of calf DNA polymerase α and rat DNA polymerase β, but also those of human immunodeficiency virus type 1 reverse transcriptase. A third new terpene, cerevisterol, which was reported to be a cytotoxic steroid, inhibited only the activity of DNA polymerase α (Mizushina et al. 1999).

Two other compounds from *G. lucidum* that inhibit eukaryotic DNA polymerase were identified as cerebrosides: (4E,8E)-*N*-D-2′-hydroxypalmitoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine and (4E,8E)-*N*-D-2′-hydroxystearoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine. These cerebrosides selectively inhibited the activities of replicative DNA polymerases, especially the α-type, from phylogenetically broad eukaryotic species, whereas they hardly influenced the activities of DNA polymerase β, prokaryotic DNA polymerases, terminal deoxynucleotidyl transferase, HIV reverse transcriptase, RNA polymerase, deoxyribonuclease I, and ATPase. The inhibition of another replicative polymerase, the δ-type, was moderate. The inhibitions of the replicative polymerases were dose-dependent, and the IC_{50} for animal or mushroom DNA polymerase α was achieved at approximately 12 μg/ml (16.2 μM) and that for animal DNA polymerase δ at 57 μg/ml (77.2 μM; Mizushina et al. 1998a).

The 490 quinone, a natural sulfhydrylarylating reagent from the mushroom *Agaricus bisporus*, markedly inhibited L1210 murine leukemia DNA polymerase α while giving little inhibition of DNA polymerase β from this source. This quinone was more strongly inhibitory than *p*-chloromercuribenzoate or *N*-ethylmaleimide and was less readily neutralized by sulfhydryl-containing molecules, such as dithioerythritol. Preliminary experiments indicated that DNA protects DNA polymerase α from inhibition by the 490 quinone. The inhibition of DNA synthesis by the 490 quinone may contribute significantly to the cytotoxicity of this compound and to the potential of γ-L-glutaminyl-4-hydroxybenzene as an antitumor agent (Graham et al. 1977).

Anti-angiogenic substances

Tumor growth and metastasis depend upon the development of a new vasculature in and around the tumor. Angiogenesis is the process of new blood vessel formation from preexisting ones. Angiogenesis facilitates progressive tumor growth by providing adequate oxygenation to the tumor through a series of interrelated steps, including endothelial cell proliferation, motility of endothelial cells through the extracellular matrix toward angiogenic stimuli, and capillary differentiation. The balance between stimulatory and inhibitory factors released by the tumor and its microenvironment regulates the process (Beecken et al. 2000). Some important mediators that have been studied include acidic and basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), transforming growth factors (TGFs) α and β, platelet-derived growth factor (PDGF), angiogenins, IL-8, and TNF-α. Because tumor growth and metastasis depend on angiogenesis, a great deal of attention has been focused on therapy that can interrupt this process. Anti-angiogenic therapy can target endothelial cells directly and inhibit the production or action, or both, of pro-angiogenic peptides by the tumor cells or host, or enhance the expression of angiogenesis inhibitors within the tumor (Hayes et al. 1999). Anti-angiogenic therapy that targets endothelial cells, rather than tumor cells directly, has been evaluated as a novel therapeutic strategy for malignant diseases. The theoretical advantage of this therapy is that endothelial cells are unlikely to acquire mutations that lead to drug resistance. Endothelial cells are also readily exposed to blood-borne agents, circumventing the problem of drug delivery (Scappaticci 2002). Although it was widely assumed that anti-angiogenic therapy was antiproliferative, it was recently found that anti-angiogenic therapy can induce apoptosis and tumor regression (Gingras et al. 2003).

An antitumor substance was isolated from the lipid fraction of *Agaricus brasiliensis*. The substance was identified as ergosterol. Tumor growth was retarded by the oral administration of the lipid fraction extracted from *A. brasiliensis*. Oral administration of ergosterol to Sarcoma 180-bearing mice at doses of 400 mg/kg and 800 mg/kg administered for 20 days significantly reduced tumor growth without side-effects. Ergosterol had no cytotoxicity against tumor cells. Two in vivo models have been used to clarify the antitumor activity of ergosterol. Intraperitoneal administration of ergosterol at doses of 5, 10, and 20 mg/kg for five consecutive days inhibited the neovascularization induced by Lewis lung carcinoma cell-packed chambers, suggesting that either ergosterol or its metabolites may be involved in the inhibition of tumor-induced neovascularization. In the second model, the inhibitory effects of ergosterol were tested on Matrigel-induced neovascularization. Female C57BL/6 mice were subcutaneously inoculated with Matrigel containing acidic FGF and heparin with or without ergosterol. Ergosterol inhibited the Matrigel-induced neovascularization. From these results, it seems likely that the antitumor activity of ergosterol might be due to direct

inhibition of angiogenesis induced by solid tumors (Takaku et al. 2001; Didukh et al. 2004).

Human umbilical vein endothelial cells (HUVECs), HT1080, and B16-F10 cells were used to evaluate the effects of *Cordyceps militaris* extract (CME) on angiogenesis and tumor growth. DNA fragments, angiogenic related gene expressions, capillary tube formation, wound healing in vitro, and tumor growth in vivo were measured. CME inhibited growth of HUVECs and HT1080. CME at 100 mg/l and 200 mg/l reduced 72-kDa gelatinase (MMP-2) gene expression in HT1080 cells by 6.0% and 22.9% after 3 h and by 14.9% and 32.8% after 6 h treatment. CME did not affect 92-kDa gelatinase (MMP-9) gene expression in B16-F10 melanoma cells. CME at 100 mg/l and 200 mg/l also reduced bFGF gene expression in HUVECs by 22.2% and 41.3%. CME inhibited tube formation of endothelial cells in vitro and in vivo. CME repressed the growth of B16-F10 melanoma cells in mice, compared with the control group (Yoo et al. 2004).

Ethanol extract (70%) of *Phellinus linteus* (PL) showed strong anti-angiogenic activity, which was detected using the chick embryo chorioallantoic membrane assay. PL was also active as an antioxidant, and its activity was comparable with vitamin C in scavenging the stable free radical 1,1-diphenyl-2-picrylhyrazyl. It also inhibited lipid peroxidation in a concentration-dependent manner, arguing that antioxidant and anti-angiogenic activities of *P. linteus* could be partly responsible for its antitumor effect (Song et al. 2003).

The protein kinase C β (PKC_β) isoform plays an important role in angiogenesis (Berns et al. 2000). Yoshiji et al. (1999) demonstrated that PKC_β lies on the signal transduction pathway by which VEGF augments development and angiogenesis, not only at the initial stage, but also after the tumor is fully established. Naturally occurring 6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone (hispidin) was isolated from the culture broth of *P. linteus* (Park et al. 2004), *Gymnopilus marginatus*, *G. patriae*, *G. parvisporus* (Rees and Ye 1999), and *Inonotus hispidus* (Awadh Ali et al. 2003). Hispidin is a potent inhibitor of PKC_β (IC_{50}=2 μM). Hispidin synthesized by Gonindard et al. (1997) was shown to be cytotoxic (between 1 mmol/l and 0.1 μmol/l) toward normal MRC-5 human fibroblasts, the SCL-1 human cancerous keratinocytes cell line, and the Capan-1 human cancerous pancreatic duct cell line. Interestingly, the addition of hispidin in three successive doses (between 10 μmol/l and 0.1 μmol/l) led to a 100-fold increase in activity with an enhanced activity on cancer cells compared with normal cells (Gonindard et al. 1997).

The triterpenoid fraction (100 mg/kg, 200 mg/kg) of the fruiting bodies of *Ganoderma lucidum* inhibited primary solid-tumor growth in the spleen, liver metastasis, and secondary metastatic tumor growth in the liver in intrasplenic Lewis lung carcinoma-implanted mice. In addition, the triterpenoid fraction (800 μg/ml) inhibited angiogenesis induced by Matrigel (a soluble basement membrane extract of the Engelbreth–Holm–Swam tumor) supplemented with VEGF and heparin in an in vivo model. This suggested that the antitumor and antimetastatic activities of the triterpenoid fraction of *G. lucidum* might be due to the inhibition of tumor-induced angiogenesis. The acidic fraction of the triterpenoid fraction inhibited the Matrigel-induced angiogenesis. Compound I was isolated from the acidic fraction as an active substance that inhibited Martigel-induced angiogenesis. Compound I was identified as ganoderic acid F, based on the data of IR, 1H-NMR, 13C-NMR, and MS analyses (Kimura et al. 2002).

Human clinical evaluations

A number of mushroom high-molecular-weight polysaccharides have proceeded through phase I, II, and III clinical trials. Lentinan (*Lentinus edodes*), PSK, and PSP (*T. versicolor*) have been used in clinical trials with hundreds of cancer patients. However, other compounds have only been assessed with small numbers of patients.

There have been numerous clinical trials of lentinan in Japan although none have been placebo-controlled and double-blinded. However, lentinan has been approved for clinical use in Japan for many years and is manufactured by several pharmaceutical companies. Wasser and Weis (1999) reviewed the accumulated information on antitumor activity, prevention of metastasis, and suppression of chemical and viral oncogenesis in animal models by lentinan. Lentinan has proved successful in prolonging the overall survival of cancer patients, especially those with gastric and colorectal carcinoma (Furue and Kitoh 1981; Taguchi et al. 1985; Hobbs 2001).

There have been several decades of successful clinical trials using PSK to treat head and neck, upper gastro-intestinal, colorectal, and lung cancers with some reported success in treating breast cancer as well. Clinical trials with PSK were recently extensively reviewed by Kidd (2000). Remarkably, by 1987, PSK accounted for more than 25% of total national expenditure for anticancer agents in Japan.

While PSK has been almost exclusively developed and tested in Japan, PSP, in contrast, is a product of China and continues to be assessed for efficacy safety by their scientists and oncologists. Many Phase III clinical trials of PSP combined with conventional therapies have demonstrated significant benefits against cancers of the stomach and lung (Jong and Yang 1999; Yang 1999; Yao 1999).

The maitake D-fraction is a relatively new compound, and there are a number of clinical trials in breast, prostate, lung, liver, and gastric cancers underway in the United States and Japan. Most of these are at an early clinical stage (phase I/II). In early 1998, for example, Maitake Products received FDA approval for an Investigational new drug application to conduct a phase II pilot study on the efficacy of a maitake D-fraction in treating advanced breast and prostate cancer patients (Zhuang and Wasser 2004).

Despite the fact that schizophyllan has been approved for clinical use in Japan, not many clinical trials have been carried out; and many are not blinded. Examples include clinical trials evaluating schizophyllan as an assistant immunotherapy in the treatment of head and neck (Kimura et al. 1994) and gastric cancer (Fujimoto et al. 1991).

As for low-molecular-weight mushroom compounds, only a minute fraction of the many newly discovered compounds have proceeded to a higher level of clinical evaluation. To our knowledge, irofulven is the most extensively studied compound in this group. Phase II clinical trials were performed in advanced melanoma (Pierson et al. 2002), advanced renal cell carcinoma (Berg et al. 2001), relapsed or refractory non-small cell lung cancer (Sherman et al. 2004), metastatic colorectal cancer (Nasta et al. 2003), and recurrent or persistent endometrial carcinoma (Schilder et al. 2004). Unfortunately, irofulven demonstrated minimal to no significant antitumor activity in these trials. Despite evidence of irofulven activity in pancreatic cancer, MGI PHARMA (April 2002) stopped a phase III irofulven clinical trial for refractory pancreatic cancer, based on preliminary data analysis. There are still ongoing phase II clinical trials in ovarian, prostate, and hepatocellular cancers.

Conclusions

Natural products have been major molecular structural resources for drug discovery. Antibiotics, penicillin, the analgesic and antipyretic drug aspirin, the anticancer drug taxol, the anti-malarial drug artemisinin, and the anti-Alzheimer's disease drug huperzine A are typical, successful examples. The reservoir of natural products contains an abundance of chemical novelty and diversity: about 40% of the chemical scaffolds of the published natural products are unique and have not been made by synthetic chemistry. Another advantage is their chiral center. Most natural products are chiral and occur as single enantiomers; and many modern drugs are chiral and have their biological activity associated with only one of the enantiomers. Therefore, using natural products as enantiomerically pure starting materials is a good solution to this problem. In addition, statistical data indicate the advantage and potential of natural products for discovering new drugs or leads. Among the 520 new drugs approved in the United States between 1983 and 1994, 157 were the natural products or derived from natural products and more than 60% of antibacterials and anticancer drugs originated from natural products.

Drug discovery in the past 100 years has involved approximately 500 targets, while in the same period about 200,000 natural products have been isolated from different natural species. In addition, the completion of the human genome suggests there are 30,000–40,000 genes and at least as many proteins. Many of these proteins are potential targets for drug intervention to control human diseases.

Today, it becomes increasingly difficult to synthesize or discover new and interesting lead compounds. But, with the aid of techniques like high-throughput screening assays, 3-D protein–ligand models, virtual screening, and computer-assisted rational drug design and the expanding knowledge of the molecular basis of tumorigenesis and metastasis, it seems feasible to harness the natural pool and discover novel compounds that rationally target the abnormal molecular and biochemical signals leading to cancer.

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