The role of targeting kinase activity by natural products in cancer chemoprevention and chemotherapy (Review)

XIANGJIAN LUO¹-³*, XINFANG YU¹-³*, SUFANG LIU¹-³*, QIPAN DENG¹-³, XIAOLAN LIU¹-³, SONGLING PENG¹-³, HONGDE LI¹-³, JIKAI LIU⁴ and YA CAO¹-³

¹Cancer Research Institute, Xiangya School of Medicine, ²Key Laboratory of the Chinese Ministry of Education and ³Key Laboratory of Carcinogenesis of the Chinese Ministry of Public Health, Central South University, Changsha, Hunan; ⁴State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, P.R. China

Received December 10, 2014; Accepted May 18, 2015

DOI: 10.3892/or.2015.4029

Abstract. The WHO clearly identifies tumors as a curable or a chronic disease. The use of natural agents in cancer prevention and therapy is currently playing an important role. Our laboratory has been investigating various natural phenolic compounds, including grifolin, neoalbaconol and epigallocatechin-3-gallate (EGCG). In the present review, we focus on the anticancer activities and the molecular mechanisms of these compounds. Grifolin, a secondary metabolite isolated from the mushroom Albatrellus confluens, has been shown to inhibit cell growth and induce cell cycle arrest in multiple cancer cell lines by targeting extracellular signal-regulated kinase 1 or by upregulating death-associated protein kinase 1 (DAPK1) via p53. We also demonstrated that neoalbaconol, a novel small-molecular compound with a drimane-type sesquiterpenoid structure obtained from Albatrellus confluens, regulates cell metabolism by targeting 3-phosphoinositide-dependent protein kinase 1 (PDK1) and inhibits cancer cell growth. EGCG, a well known catechin found in tea, has gained much attention for its anticancer effects. Previously, we found that it regulates EBV lytic infection through the phosphoinositide-3 kinase/Akt (PI3K/Akt) and mitogen-activated protein kinase (MAPK) pathways in EBV-positive cancer cells. Therefore, these natural agents could be used as potential leading compounds in the prevention of tumor progression and/or EBV-related cancer.

Contents
1. Introduction
2. Grifolin, a promising kinase inhibitor candidate, regulates cancer growth via targeting ERK1/2 and DAPK1
3. Neoalbaconol, a potential inhibitor of PDK1, induces multiple types of cell death via the PI3K/AKT pathway
4. EGCG, a potential chemopreventive agent, controls EBV lytic infection by targeting the MEK/ERK1/2 and PI3K/Akt pathways
5. Conclusions and perspectives

1. Introduction

The rising burden of cancer places enormous strains on public health care systems and is one of the major causes of death in humans. To develop individualized cancer therapy and prevention, natural agents will become important strategies in the near future (1). Natural products, as an attractive source of new therapeutic candidate compounds for cancer prevention and therapy, have been playing an increasingly important role. The analysis concerning chemotherapeutic agents and their sources indicates that over 60% of approved drugs are derived from natural compounds (2). In the past few decades, through large-scale anticancer drug screening and discovery programs, various successfulanticancer agents in clinical use or in clinical trials have been identified that are derived from natural products and their synthetic analogues. Among these, vincristine, etoposide and paclitaxel are plant-derived compounds; and actinomycin D, rapamycin and doxorubicin are drugs originating from microbial sources (3). Through a variety of different mechanisms of action, such as DNA damage, inhibition of topoisomerases I or II, and disruption of cell signaling transduction, these agents inhibit cancer cell proliferation and progression (3). Carcinogenesis is a multistep process in which an accumulation of genetic mutations leads to progressive DNA repair, cell cycle, DNA methylation, cell death and cell growth deregulation, and eventually to carcinoma. By the inhibition of virus infection and the regulation of metabolism and the immune system,
cancer preventive agents are able to sustain cancer as a chronic disease.

A cellular signaling pathway, including a variety of proteins that consist of regulatory circles, is a complex signal communication network that controls basic biological activities of the cell and coordinates cell actions. Several cellular signaling pathways, including NF-κB, PI3K/Akt, MAPKs and p53, have been known to regulate cell proliferation and apoptosis (4). Due to the complexity and the abnormal alterations in cell signaling transduction, specific inhibitors that only have one target, most often fail in cancer therapy. It has been shown that different signaling pathways have crosstalk with each other. The simultaneous targeting of multiple cellular signaling pathways which control cell cycle and apoptosis to induce cell cycle arrest and cell death is an important strategy to control cancer cell proliferation and the formation of tumors (5).

Several excellent review have been published on the anticancer activity of natural compounds (2,3). In the present review, we summarize research conducted by our laboratory concerning various natural phenolic compounds by a screening platform established by our laboratory. These natural compounds include grifolin, neoalbaconol and epigallocatechin-3-gallate (EGCG). Grifolin, by targeting ERK or upregulating DAPK1 via p53, was found to induce the apoptosis and cell cycle arrest in multiple cancer cell lines (6-9). We also demonstrated that its analogue, neoalbaconol, regulated cell metabolism and ultimately induced multiple types of cell death in vitro and in vivo (10). Even though there are numerous studies concerning the anticancer activity of EGCG, our group found that it regulated EBV lytic infection through the PI3K/Akt and MAPK pathways in EBV-positive cancer cells (11). Therefore, we review the findings of three agents to be used as potential leading compounds in the prevention and inhibition of tumor progression and/or EBV-related cancer.

2. Grifolin, a promising kinase inhibitor candidate, regulates cancer growth via targeting ERK1/2 and DAPK1

Grifolin, a farnesyl phenolic compound (Fig. 1A), is a secondary metabolite isolated from the fresh fruiting bodies of the mushroom Albatrellus confluens. It has also been reported to originate from the edible mushroom Boletus pseudocalopus (12). Grifolin displays various pharmacological and microbiological effects. Recent evidence indicates that grifolin also possesses antioxidant and antitumor activities (6-8,13-15). It has been shown to inhibit the growth of various cancer cell lines in vitro by induction of cell cycle arrest and apoptosis in previous studies by our group (6,7).

Our research indicates that grifolin exhibits certain selective antitumor effects following the comparison of the respective IC_{50} values of a broad spectrum of tumor cell lines to normal or non-tumor cell lines (6,7). The apoptotic effect of grifolin on nasopharyngeal carcinoma (NPC) cell line CNE1 was found to be mediated by successive cascade responses, including a decrease in the Bcl-2 level, an increase in Bax, release of cytochrome c from mitochondria, and activation of caspase-8, -9 and -3.

To identify the molecular targets in the signal transduction pathways, apoptosis-related cDNA microarray analysis was employed to investigate the mechanism of grifolin-induced cell death at the gene expression level. Our studies identified that the dapk1 gene was significantly upregulated. DAPK1 is an apoptotic-positive mediator (16). It acts as a tumor suppressor largely due to its ability to sensitize cells to many apoptotic signals including those generated by death receptors, cytokines, matrix detachment and oncogene-induced hyperproliferation that are encountered as a cell undergoes tumorigenesis. Extensive data in human primary tumors demonstrate a significant loss of DAPK1 expression in a large variety of tumor types (17,18). Grifolin was found to upregulate DAPK1 mRNA and protein expression in a dose-dependent manner in NPC cells. DAPK1 mRNA levels were found to increase in a p53-dependent manner in various cellular settings (19,20). We observed that grifolin promoted the phosphorylation of p53 at Ser392 and Ser20, while it had little effect on the phosphorylation of Ser15 or Thr81 as well as the total p53 protein level. General consensus remains that the phosphorylation of N-terminal regulatory sites of p53 occurs rapidly in response to various stress stimuli to activate p53. Reduced levels of Ser20 phosphorylation were found to attenuate p53 activity as a transcription factor (19,21). It is currently believed that both the core DNA-binding and the C-terminal domains of p53 possess DNA-binding activities, in that the former primarily provides sequence specificity whereas the latter recognizes structural features of target DNA. Using EMSA, we further demonstrated that grifolin significantly increased the binding activity of p53 to the dapk1 gene in vitro. ChiP assays of the p53-dapk1 promoter complex confirmed that endogenous p53 interacts with this region in vivo (8). We introduced an siRNA targeting DAPK1 and a scrambled siRNA as a control into CNE1 cells. Depletion of DAPK1 reduced the apoptotic effect as well as the activation of caspase-3 induced by grifolin. Grifolin-induced upregulation of DAPK1 was also observed in tumor cells derived from human breast cancer and human colon cancer. In SW480 and MCF7 cells, grifolin also upregulated DAPK1 via the p53 pathway, and DAPK1 was found to mediate the grifolin-induced apoptotic effect (8). Reintroduction of p53 and DAPK1 into p53-null H1299 cells markedly enhanced the apoptotic rate, which further confirmed the function of the p53-DAPK1 pathway in grifolin-induced apoptosis.

Collectively, upregulation of DAPK1 expression by grifolin may be an important mechanism contributing to its ability to induce an apoptotic effect in tumor cells. Due to the high frequency of loss of DAPK1 expression in a large variety of tumor types, grifolin may rescue the pro-apoptotic function of DAPK1 via the p53 pathway.

At high doses, both the ERK1/2 and the ERK5 pathways may be involved in grifolin-induced cell cycle arrest. ERK has a well-established role in regulating G1 to S phase progression in response to mitogenic stimulation. ERK mediates activation of multiple transcription factors including Elk1, c-Jun, c-Myc and c-Fos. These transcription factors control the expression of genes important for cell cycle progression, including cyclin D1 and p21^{WAF1/CIP1} (22). The results obtained from evaluating the effects of grifolin on the expression of G1-related protein suggested that cell cycle arrest was associated with the downregulation of cyclin D1, CDK4, cyclin E as well as the phosphorylation of pRB induced by grifolin. In contrast, INK4 family member p19, which mediates the inhibition of CDK4,
was obviously upregulated by grifolin in a dose-dependent manner (7).

In our previous study, we demonstrated that DAPK1 also mediated grifolin-induced G1 phase arrest effects in NPC cells (7). DAPK1 can be activated by several mechanisms, including dephosphorylation of Ser308 by an unknown phosphatase, which can be activated by several death signals (23,24). We found that grifolin induced dephosphorylation of DAPK1 (Ser308) and subsequent phosphorylation of p21 (Thr145). Inhibition of DAPK1 by introducing siDAPK1 reversed grifolin-induced phosphorylation of p21. Furthermore, we confirmed that grifolin increased the half-life of p21 and promoted its stability by increased ubiquitination of p21. Normal p21 turnover is suppressed by inhibitors of the proteasome pathway, yet degradation is independent of p21 ubiquitination of lysine residues despite amino terminal addition (25,26). Therefore, a different mechanism-driven ubiquitin-linkage may lead to a different fate for the p21 protein. The ubiquitination of p21 enhances its stability and promotes its function as a cell-cycle inhibitor. During G1-phase progression, cyclin D1/CDK4/6 complexes are activated in mid-G1 phase and are essential for G1/S transition. The enhanced stability of p21 induced by grifolin may effectively inhibit the activity of the cyclin D1/CDK4/6 complex, leading to G1 phase arrest in NPC. In addition, the cytoplasmic retention of ERK1/2 synergistically enhances the G1 phase arrest induced by grifolin (9).

Most importantly, we observed that grifolin inhibited the kinase activity of ERK1/2 protein in vivo and in vitro. We next used molecular modeling with the crystal structure of ERK2 to analyze whether grifolin binds to ERK2. We found that grifolin formed a hydrogen bond with Ile-29 at the backbone of ERK2, and also formed hydrophobic interactions with ERK2 at Val-37, Leu-154 and Cys-164. We further identified that grifolin physically binds to ERK2 using affinity chromatography and fluorescence quenching analysis (unpublished data). Grifolin, a natural compound from mushroom *Albatrellus confluens*, represents a promising kinase inhibitor candidate in the intervention of cancer via targeting ERK1/2 and DAPK1 (Fig. 2).

3. Neoalbaconol, a potential inhibitor of PDK1, induces multiple types of cell death via the PI3K/AKT pathway

Neoalbaconol (NA), a novel small-molecular compound with a drimane-type sesquiterpenoid structure (Fig. 1B), was also isolated from the fruiting bodies of the mushroom *Albatrellus confluens*. *Albatrellus confluens*, mainly distributed in southeast China, is a member of the Polyporaceae family. Several compounds, such as grifolin and albaconol, with anticancer potential or anti-inflammatory action have been isolated from this fungus (28,29).

Cell death plays a key role in regulating tissue homeostasis and physiologic processes; abnormal regulation of
this process is associated with a number of diseases, such as neurodegeneration, ischemia and cancer (30). According to the morphological appearance, cell death can be mainly classified into three distinct routes: apoptosis, autophagic cell death and necrosis (31). Traditionally, apoptosis and autophagic cell death are recognized as programmed cell death, and necrosis is considered as accidental and unregulated. Yet, a previous study demonstrated that necrosis is also regulated. This form of programmed necrosis termed necroptosis can be inhibited by small-molecular compounds such as necrostatin 1 (Nec-1) and its execution involves the receptor interacting protein kinase (RIPK) 1 and 3 (32). Our previous studies demonstrated that NA significantly inhibited the proliferation of many types of cancer cell lines in a dose-dependent manner. Yet, it did not affect the proliferation of normal immortalized cell lines even at high doses, indicating the selectivity of NA toward cancer cells. Using flow cytometric, protein, confocal and electron microscope (EM) assays, our results showed that NA induced cell apoptosis, autophagy and necroptosis (10). In our previous study, apoptosis and necroptosis were responsible for the death-inducing efficacy of NA, and necroptosis was found to contribute to the main effect of NA-induced cell death in NPC cells (10). The apoptosis inhibitor zVAD-fmk and necroptosis inhibitor Nec-1 rescued the viability of the NA-treated cells, which confirmed the role of apoptosis and necroptosis in NA-induced cell death (10).

As investigations have recently discovered that autophagic vesicles are commonly observed in necrotic cells, the role of autophagy in cell viability is believed to be dependent on the cell context yet remains controversial (32). It has been reported that the suppression of pro-survival autophagy leads to necroptosis induced by zVAD-fmk in L929 cells (33). Yet, in proliferative T cells, Fas-associated protein with death domain (FADD) in combination with caspase-8 limited autophagy and protected T cells from necroptosis; while Nec-1 reduced light chain levels in FADD−/− cells and rescued the cell cycling and proliferation of FADD−/− T cells simultaneously (34). We found that the NA-mediated apoptosis, necroptosis and autophagy occurred independent of each other (10). Inhibition of autophagy by 3-MA enhanced cell death in NA-treated cells, suggesting that autophagy provides a survival force in this model. Energy metabolic reprogramming is one of the significant features of cancer cells. The oncogenic PI3K/Akt/mTOR pathway plays a key role in reprogramming metabolic pathways in cancer cells (35). PDK1, an upstream regulator of Akt/mTOR signaling, activates a group of protein kinases belonging to the AGC kinase family including protein kinase A, G and C (36). In response to various cellular stimulations, PDK1 phosphorylates Akt at Ser308, resulting in the activation of Akt and regulates energy metabolism, cell proliferation, cell cycle progression and migration. Based on the important functions of PDK1 in tumor cells, researchers have recently shown that PDK1 serves as an effective therapeutic target for anticancer treatment and several PDK1 inhibitors, such as AR-12 and GSK233447, have been developed to kill cancer cells (37). Using the phase module of the Schrödinger molecular modeling software package, we identified PDK1 as a potential protein target of NA (10). More than three ligand-binding sites are located in the PDK1 kinase domain, including an ATP-binding pocket, a peptide substrate-binding site and a groove in the N-terminal that binds to the C-terminal hydrophobic motif of the kinase substrates (37). NA was able to dock into the ATP binding pocket of PDK1 and form three hydrogen bonds with the backbone of PDK1. By targeting PDK1 and inhibiting the downstream PI3-K/Akt pathway, NA inhibited the key energy metabolic enzyme HK2 in cancer cells (10). Thus, the final effect of NA treatment not only significantly decreased the glucose concentration in the medium, yet also blocked ATP generation in a time-dependent manner.

Moreover, the necroptosis inhibitor Nec-1 rescued the viability of the NA-treated cells. Akt overexpression decreased LC3 expression and inhibited NA-induced autophagy, implying that Akt inactivation and energy crisis are responsible for NA-induced autophagy (10). We also demonstrated the efficacy of NA in inhibiting tumor growth by suppressing the Akt signaling pathway in vivo (10). Taken together, NA may be involved in NA-induced apoptotic and necrotic cell death by remodeling cellular energy metabolism by targeting the PDK1/PI3K/Akt signaling pathway (Fig. 3). All these findings strongly suggest that the inhibition of tumor cell growth by NA makes it an ideal candidate as a leading anticancer agent.

4. EGCG, a potential chemopreventive agent, controls EBV lytic infection by targeting the MEK/ERK1/2 and PI3K/Akt pathways

The consumption of green tea has long been associated with a reduced risk of cancer development. Green tea is chemically characterized by the presence of high amounts of polyphenolic compounds known as catechins. The most abundant component is epigallocatechin-3-gallate (EGCG) (Fig. 1C), which appears to be the primary active ingredient responsible for the important biological and pharmacological properties of green tea.

Cancer chemoprevention by the natural compound, EGCG, has been studied by many investigators and has attracted much attention in recent years. Many mechanisms have been proposed for the antitumor activities of EGCG. These include antioxidant activities, cell cycle arrest, induction of apoptosis, induction or inhibition of drug metabolic enzymes, modulation of cell signaling, inhibition of DNA methylation, effect on miRNA expression, histone modifications, proteases and telomerases (38,39). Based on the study of the effects of EGCG in regulating different key proteins in various signaling pathways, the direct interaction of proteins with EGCG was found to be a key step in the process of EGCG-induced effects. The eight phenolic groups of EGCG can serve as hydrogen bond donors to many biomolecules. The identification of proteins interacting directly with EGCG is important in understanding the molecular mechanisms of the effects. Several proteins that can directly bind with EGCG have been identified and include 67-kDa laminin receptor, Bcl-2, GRP-78, insulin-like growth factor-I receptor, G3BP1, ZAP-70, Ptn1 and vimentin (40–43).

The inhibitory effects of EGCG against carcinogenesis at different organ sites have been demonstrated in many animal models (44,45). The cancer-preventive effects of EGCG are also widely supported by results from clinical studies (46). A prospective cohort study with over 8,000 individuals revealed that the daily consumption of green tea resulted in delayed

...
cancer onset and a follow-up study of breast cancer patients found that stage I and II breast cancer patients experienced a lower recurrence rate and a longer disease-free period (47). Moreover, EGCG delivered in the form of green tea extract...
and survival (52). Considering that EGCG can modulate ERKs, PI3K/Akt, JNKs and STAT3, to alter cell growth, it engages multiple signal transduction cascades, including MEK/ERK1/2, MAPK and PI3K/Akt pathways. Through the cytoplasmic carboxy terminus, LMP1 triggers multiple signal transduction pathways, including MEK/ERK1/2 and PI3K/Akt pathways, thereby imparting strong cancer chemopreventive as well as therapeutic effects (53,54). In our recent study, we investigated the effects of EGCG on EBV spontaneous lytic infection and the mechanism involved in EBV-positive cells. We found that EGCG effectively inhibited the constitutive lytic infection of EBV at DNA, gene transcription and protein levels by decreasing the phosphorylation and activation of ERK1/2 and Akt (11). Using signaling pathway-specific inhibitors, we also explored the signaling mechanisms underlying the inhibitory effects of EGCG on EBV spontaneous lytic infection in cell models. The results showed that specific inhibitors of MEK (PD98059) and PI3K (LY294002) markedly downregulated gene transcription and expression of BZLF1 and BMRF1, indicating that the MEK/ERK1/2 and PI3K/Akt pathways are involved in the EBV spontaneous lytic cycle cascade (11). Therefore, one of the mechanisms by which EGCG inhibits EBV spontaneous lytic infection appears to involve the suppression of the activation of MEK/ERK1/2 and PI3K/Akt signaling. Since EBV lytic infection plays a critical role in the development of EBV-associated malignancies, we described here the potential chemopreventive activity of EGCG in controlling EBV lytic infection. Our findings support the future investigation of EGCG as an anticancer and chemoprevention agent for EBV-associated malignancies (Fig. 4).

5. Conclusions and perspectives

In the present review, we summarize the anticancer effects of three compounds, grifolin, neoalbaconol and EGCG. These natural agents show high potential in cancer treatment and prevention.

EGCG also reportedly exhibits antibacterial, antifungal and antiviral effects (49). Epstein-Barr virus is a human herpes virus that infects 90% of the human population. EBV infection, as one of many environmental factors, has been reported to be strongly associated with the development of several human malignancies, including Burkitt's lymphoma, Hodgkin's disease and NPC. Like all other herpes viruses, EBV establishes a latent or lytic infection in host cells. Intriguingly, evidence indicates that EBV reactivation into the lytic cycle may play a role in the pathogenesis of malignancies. Evidence indicates that the ERK1/2, MAPK and PI3K/Akt signaling pathways play a critical role in EBV lytic infection (50,51). Latent membrane protein 1 (LMP1), which is the only viral gene product with oncogenic properties among the EBV-encoded gene products, are detected in 90% of NPC patients. Through the cytoplasmic carboxy terminus, LMP1 triggers multiple signal transduction cascades, including MEK/ERKs, PI3K/Akt, JNKs and STAT3, to alter cell growth and survival (52). Considering that EGCG can modulate signaling pathways induced by LMP1, other groups and ours have shown that EGCG modulates multiple signal transduction pathways including the MAPK and PI3K/Akt pathways, thereby imparting strong cancer chemopreventive as well as therapeutic effects (53,54). In our recent study, we investigated the effects of EGCG on EBV spontaneous lytic infection and the mechanism involved in EBV-positive cells. We found that EGCG effectively inhibited the constitutive lytic infection of EBV at DNA, gene transcription and protein levels by decreasing the phosphorylation and activation of ERK1/2 and Akt (11). Using signaling pathway-specific inhibitors, we also explored the signaling mechanisms underlying the inhibitory effects of EGCG on EBV spontaneous lytic infection in cell models. The results showed that specific inhibitors of MEK (PD98059) and PI3K (LY294002) markedly downregulated gene transcription and expression of BZLF1 and BMRF1, indicating that the MEK/ERK1/2 and PI3K/Akt pathways are involved in the EBV spontaneous lytic cycle cascade (11). Therefore, one of the mechanisms by which EGCG inhibits EBV spontaneous lytic infection appears to involve the suppression of the activation of MEK/ERK1/2 and PI3K/Akt signaling. Since EBV lytic infection plays a critical role in the development of EBV-associated malignancies, we described here the potential chemopreventive activity of EGCG in controlling EBV lytic infection. Our findings support the future investigation of EGCG as an anticancer and chemoprevention agent for EBV-associated malignancies (Fig. 4).
eling cellular energy metabolism. The mode of cell death induced by NA is important since multiple cell death pathway activation helps to overcome chemotherapy and radiotherapy resistance. Consequently, induction of apoptosis and alternative cell death–necroptosis by NA provides therapeutic benefits. Considering the role of necroptosis in viral infection, NA is a potential chemopreventive agent for virus–related cancer.

In regards to EGC, we will further assess its effect on the lytic replication of EBV in animals and humans. Infection with EBV is universal and associates with the pathogenesis of many types of tumors, providing a specific approach for chemoprevention. Our in vitro experiments showed that EGC induces EBV lytic replication, and in vivo experiments need to be conducted.

The above scientific research was performed on experimental systems of tumor cells and xenografts in nude mouse models, while suitable experimental systems of precancerous conditions are needed to confirm the chemopreventive effects of these compounds. Meanwhile, water solubility and biological availability of these compounds need to be improved. Currently, total synthesis for the two agents, grifolin and neoalbaconol, has not been achieved. Through the collaboration of chemists, computer experts and biologists, we hope to obtain the natural compounds and provide further evidence concerning the anticancer mechanisms in vitro and in vivo.

Acknowledgements

The authors would like to thank Professor Zigang Dong and Ann M. Bode for their kind advice. This study was supported by the National Key Basic Research Program of China (nos. 2009CB522300 and 2011CB504305), the National Natural Science Foundation of China (nos. 81430064 and 81372393), the Hunan Provincial Innovation Foundation for Postgraduates (CX2012B082), and the Open-End Fund for the Valuable and Precision Instruments of Central South University (CSUZC20154).

References

4. Sarkar FH, Li Y, Wang Z and Kong D: Cellular signaling perturbation by the compounds and provide further evidence concerning the anticancer mechanisms in vitro and in vivo.