

Apoptotic and Immune Restoration Effects of Ganoderic Acids Define a New Prospective for Complementary Treatment of Cancer

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Abstract

Considering the fact that a key factor in tumor development is the evasion of immune detection, the search for natural products, which have reduced toxicity towards normal tissues as well as immunostimulatory capabilities has received growing interest. One attractive source of antitumor products is the *Ganoderma lucidum* mushroom, which has been used for centuries as an herbal medicine for the prevention and treatment of a variety of diseases, including cancer, and has been shown to improve immune function. Interestingly, its methanol soluble triterpenoid extracts, namely Ganoderic Acids (GAs), have been the subject of several recent investigations on their chemotherapeutic effects. While current research has revealed GAs' role in inducing apoptosis of cancer cells with a much lower toxicity to healthy cells, little information is available on their *in vitro* and/or *in vivo* immune activities. In this review, we aim to discuss the current knowledge on GAs, and their potential as apoptosis inducing as well as immune activating molecules that could be a potential alternative approach for designing novel chemoimmunotherapeutics against malignant diseases. We also discuss other new approaches for exploiting the advantages of using a nanoparticle polymer-GA conjugate as a tool for a sustained and targeted delivery of drug *in vivo*.

Keywords: Ganoderma lucidum; Ganoderic acids; Cancer; Apoptosis; Cytokines; Immune regulation; Nanoparticles

Introduction

Cancer is one of the world's leading causes of death and arises when the homeostatic balance between cell growth and death is disturbed [1,2]. Recent reports estimate the total worldwide economic burden associated with cancer therapy to be several hundred billions of US\$ [3]. Consequently, advances in cancer therapy will have economic as well as public health implications. As more evidence highlights the toxic side effects of traditional chemotherapies, there is a growing interest for alternative medicines to improve the function of the immune system in order to target late stage metastatic tumors. Natural extracts from fungi have been the focus of recent investigation, particularly those with reduced cellular toxicity to healthy tissue. When considering any new therapy, both efficacy and cost-effectiveness of the cancer treatment strategy are the two main players that decide the healthcare costs associated with cancer management. Advances in technology have allowed for widespread screening of natural products to identify those with anticarcinogenic and immunostimulatory potential. This has led to a renewed investigation into the anecdotal evidence of health benefits associated with folk medicine, with the goal of identifying the active components which could be used as alternative or complementary treatment for cancer. This may be of particular importance to societies and countries where modern medicine is scarce, expensive to buy, or simply unavailable.

Ganoderic acids (GAs), the triterpenoid extracts of *Ganoderma lucidum* mushroom (Figure 1) are potential low-cost candidates with interesting therapeutic properties. The mushroom itself has long been used in Eastern herbal medicine and is thought to increase vitality, immune function and life expectancy. Several recent studies have shown them to possess antitumor and anti-metastatic properties in a range of cancer cell types [4-6]. More than 130 GAs and related derivatives have been isolated and identified from the spores, fruiting bodies and cultured mycelia of the *Ganoderma spp.* in the past two

decades [7-9]. GAs and derivatives have received interest due to their substantial pharmacological activities. Interestingly, a number of GAs including GA-A, GA-B, GA-H and GA-C1 showed anti-HIV-1 activity [10,11]. Other possible reported activities include: antihistamine [12], antinociceptive [13], antihypercholesterolemic [14], antiangiotensin [15] and antitumor effects [16]. This review aims to discuss the current knowledge of antitumor activities associated with *G. lucidum* crude extracts with particular emphasis on the anticancer apoptotic and

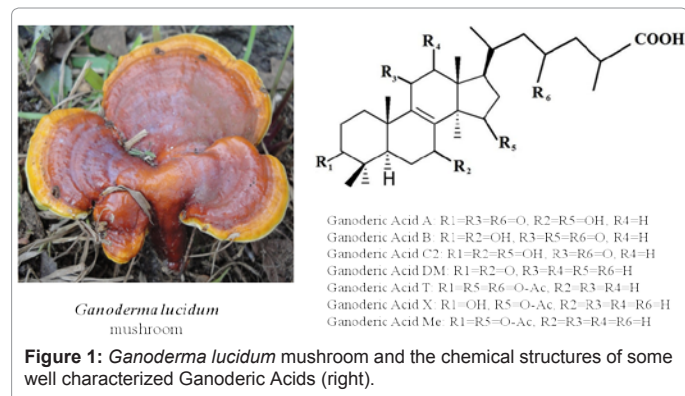


Figure 1: *Ganoderma lucidum* mushroom and the chemical structures of some well characterized Ganoderic Acids (right).

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immune restoration activities of purified GAs. Also, we will discuss our development of a novel treatment-delivery approach of GAs via nanoparticles, termed GAIN “Ganoderic Acid-Infused Nanoparticle” complex, that were particularly designed to target the malignant cells with reduced bystander effects. GAIN could also be enrobed with cancer-targeting ligands to allow more of the incorporated compound to reach the target tissue. This article will thus further highlight both chemo- and immunotherapeutic potentials of GAs in addition to the use of nanotechnology to develop antitumor treatment.

G. lucidum as a Natural Anticancer Drug Source

Ganoderma, a genus of polypore mushrooms that grows on wood, includes about 80 species, and is distributed in many tropical regions [17]. *Ganoderma*'s economic importance was gained as several species of the mushroom were extensively used in traditional Asian medicines due to its potential bioremediation. One species, however, *G. lucidum* (known as Lingzhi in China and Reishi in Japan) (Figure 1) has become one of the natural sources of numerous efficacious medicines, and is now being used to complement or sometimes substitute modern medicine in remote places of the world. In fact, the popularity of using alternative medicines has increased steadily as their prevention activity has been proven. The curative use of mushrooms has a very long tradition in the Asian countries, whereas their use in the Western hemisphere has considerably increased since the last decade [18]. Many agents derived from medicinal mushroom fruiting bodies, cultured mycelium, and/or culture filtrates exert a wide range of beneficial biological effects when tested *in vitro* or *in vivo* in animal models. Lingzhi has gained its popularity as a therapeutic mushroom and named king of herbs in China since ancient times for improving longevity, health promotion and its marked immune-modulating effects as demonstrated by an increase in T lymphocytes [19,20]. Many further investigations into the anticancer activity of *G. lucidum* have been carried out both *in vitro* and *in vivo*, which support its application for cancer treatment and prevention [21-23]. Although anticancer activities of *G. lucidum* extracts have prompted the usage of the mushroom by cancer patients, its medicinal/therapeutic value remains debatable. So far, there has been no report of human trials using *G. lucidum* or its extracts as anticancer agents. Thus studies focused on cytotoxic, apoptotic, antiangiogenic, and immunogenic mechanisms of crude preparation of *G. lucidum* and its purified GAs should be encouraged.

Recent clinical studies in cancer patients have shown that a crude *G. lucidum* water extract (i.e. Ganopoly) can enhance host immune function with increased activity of T cells, macrophages, and natural killer cells, although remarkable antitumor responses were not observed [24-27]. Other study, however, suggests that the cancer preventive and tumoricidal properties of *G. lucidum* could be ascribed to its ability to enhance the host's immune functions, antioxidative and radical-scavenging effects, inhibition of metabolic activation and enhanced detoxification of carcinogens, and their cytotoxicity to cancer cells [28]. The major active constituents from *G. lucidum* extract (including polysaccharides and triterpenoids) were also found to exert chemopreventive and tumoricidal effects through a possible modulation of signaling molecules and induction of cell-cycle arrest and apoptosis [29-32]. Other mechanisms, such as antiangiogenesis, antipromotion, and antiproliferation activities were also assumed to play a role, and will be discussed in this review.

G. lucidum Bioactive Extracts

Investigations on biologically active components of the carpophores and the cultured mycelia of *G. lucidum* have shown

that this mushroom is rich in various natural metabolites that have demonstrated both antitumor and immunomodulatory activities [33,34]. Among the most medically important constituents of the mushroom are its polysaccharides and alcohol soluble triterpenoids that also include GAs [22,35,36]. Polysaccharides represent the major water extract fraction of *G. lucidum* that exhibits significant antitumor effects in several tumor-bearing animals, mainly through their immuno-enhancing activity [34,37]. They are mostly high molecular weight glucans that vary in their water solubility and nature of their side chains. Researchers have found that polysaccharide-rich fractions exhibit antitumor properties in rodents mainly through the induction of cytokines such as IL-1, IL-6, IL-12, IFN- γ , TNF- α , GM-CSF, G-CSF, and M-CSF in monocytes/macrophages and T lymphocytes [34,37-39]. *G. lucidum* triterpenes are considered to be potential anticancer agents due to cytotoxicity against growing tumors, although much of the credit of enhancing the immune system was given to the polysaccharide-rich extract. The crude triterpenoid extracts of *Ganoderma* have been reported in numerous publications to significantly induce cytotoxicity and to reduce tumor growth [40-42]. Purified triterpenoids have also been shown to possess anti-invasive properties through inhibition of matrix metalloproteinase-9 (MMP-9), and reduction of NF- κ B/AP-1 DNA-binding activities in PMA-induced HepG2 cells [43]. Also the inhibition of AP-1 and NF- κ B was documented in highly invasive human breast cancer cells, which coincided with downregulation of Cdk4 expression and reduced secretion of uPA [44]. The anti-inflammatory and antitumor properties of purified triterpene acids and sterols were also reported by other groups [45,46], encouraging the potential use of GAs in combination therapy against inflammation and cancer.

The main focus of this review is the methanol soluble fraction of *G. lucidum* extract, predominantly GAs, a family of compounds that possesses a molecular structure similar to steroid hormones [46] (Figure 1). Studies suggest that GAs possess antitumor properties that seem to be related to a cytotoxic activity against cancer cells [5,47,48]. Both the antitumor and immuno-modulatory properties of *G. lucidum* extract, along with its low cytotoxicity, increase the possibility that it could be very effective in a complementary treatment for cancer patients receiving conventional chemotherapy and/or radiation treatment.

The potential clinical value and wide acceptability of *G. lucidum* extracts as alternative anticancer medication have attracted intense interest in the search for their molecular mechanisms. Studies have reported that *G. lucidum* extracts combine both polysaccharides and triterpenoids that collectively possess aspects of immuno-modulatory and antitumor effects [5,41] (Table 1). Yet, little work has been done on identifying the antitumor activity and immune regulation mediated by purified triterpenes *in vivo*. Understanding the mechanisms by which purified triterpenes exert their antitumor and immune stimulation effects would contribute to the design of alternative strategies for cancer treatment.

GAs in Apoptosis and Immune Activation

GAs have shown a wide range of medicinal benefits, most notably their potent toxicity to tumor cells with a comparatively limited toxicity to bystander cells (Table 1). It has been reported that GA-X inhibits topoisomerases and sensitizes the cancer cells toward apoptosis [49]. The *in vitro* treatment of human hepatoma HuH-7 cells causes immediate inhibition of DNA synthesis as well as activation of ERK and JNK, and induction of apoptosis. The observed molecular events include: degradation of chromosomal DNA, decreased levels of Bcl-

xL, disruption of mitochondrial membrane potential, cytosolic release of cytochrome c and activation of caspase-3. The involvement of mitochondrial dysfunction was also supported by a later work [50], in which GA-T induced apoptosis of metastatic lung tumor cells through the intrinsic pathway mediated via the mitochondria-dependent caspase pathway and p53 expression. This study also detected the exposure of p53 and Bax proteins in 95-D cells, while the expression of Bcl-2 was not significantly changed. *In vivo* study also showed that GA-T suppressed the growth of human solid tumors in athymic mice. In addition to its antiproliferative effects, recent studies have found that GA-T effectively inhibits cancer cell invasion and metastasis both *in vitro* and *in vivo* [51]. This study also showed that GA-T promoted aggregation and simultaneously inhibited adhesion to the extracellular matrix, thus impairing the migration of colon (HCT-116) and lung

(95-D) cancer cells in a dose- and time-dependent manner. Strikingly, GA-T treatment inhibits the nuclear translocation of NF-κB and the degradation of inhibitor of κB-α (IκB-α), which ultimately led to a decrease of MMP-9, inducible nitric oxide synthase (iNOS), and urokinase-type plasminogen activator (uPA). Furthermore, *in vivo* administration demonstrated that GA-T suppresses tumor growth and metastasis, and down-regulates MMP-2 and MMP-9 mRNA expression in a Lewis Lung Carcinoma (LLC) model *in vivo*.

Recently, it has been found that p53 plays an important role in the suppression and anti-invasion properties of GA-T in human colon cancer (HCT-116) cell lines [52]. Comparing the results in HCT-116 p53(+/+) and p53(-/-), it is suggested that p53 could modify GA-T-mediated inhibition of NF-κB translocation, degradation of IκBα,

I- Ganoderma extracts	Applications	Observed Activities	Suggested Mechanism(s)	Refs
Dried powder of <i>G. lucidum</i> (dissolved in boiled water)	Highly invasive breast cancer (MDA-MB-231) and prostate cancer (PC-3) cell lines.	Downregulates transcription factors AP-1 and NF-κB in breast and prostate cancer cells.	Inhibition of uPA and uPAR reduces cell motility.	[65]
Dried powder of <i>G. lucidum</i> (13.5% polysaccharides & 6% triterpenes)	Human prostate cancer cells (PC-3), and human aortic endothelial cells (HAECs)	Inhibits early events in angiogenesis & capillary morphogenesis of HAECs, and modulates the phosphorylation of Erk1/2 & Akt kinases in PC-3 cells, potentially decreasing the activity of AP-1.	Inhibition of AP-1 down-regulates the secretion of VEGF and TGF-β1 from PC-3 cells. Suppression of angiogenesis by modulating MAPK and Akt signaling.	[66]
Ethanol and water extract of <i>G. lucidum</i>	Human urothelial cells (HUC, bladder cancer) consisting of two cell lines (HUC-PC cells and MTC-11 cells).	Ethanol extracts show a stronger growth inhibition than those of water extracts. Induces growth arrest and reduces cell migration <i>in vitro</i> .	Increased actin polymerization inhibits carcinogen 4-aminobiphenyl-induced cellular migration.	[67]
Methanolic <i>G. lucidum</i> extract	26 types of human cancer cell lines including 16 hematological cell lines (lymphomas & multiple myelomas), and 10 other solid tumor cell lines.	Exhibits cytotoxicity to HL-60 (ED ₅₀ ≥26 μg/ml), U937 (ED ₅₀ ≥63μg/ml), K562 (ED ₅₀ ≥50μg/ml), Blin-1 (ED ₅₀ ≥38μg/ml), Nalm-6 (ED ₅₀ ≥30 μg/ml) and RPMI8226 (ED ₅₀ ≥40 μg/ml).	Induction of cell cycle arrest, mitochondrial dysfunction, and upregulation of p21/p27.	[68]
<i>G. lucidum</i> polysaccharide extract	<i>In vivo</i> treatment of ovarian cancer in rodents.	Reduces MDA adducts by increasing activity of serum antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px)].	Reduction of malignant growth by modulation of serum antioxidant enzymes.	[69]
Hot water <i>G. lucidum</i> extract	Drug-sensitive (H69) and multi-drug resistant (VPA) human SCLC cells. VPA was derived from H69 cells selected in etoposide.	Exhibits cytotoxicity and induces apoptosis in both drug-sensitive and drug-resistant cells.	Induction of apoptosis similar to the conventional chemotherapeutics (etoposide and doxorubicin) via DNA fragmentation and caspase activation.	[70]
Semi-purified <i>G. lucidum</i> (methanol extract)	Human leukemic cell line NB4.	Induces apoptosis in NB4 cells.	Reduction and modulation of Bcl2/Bax, p53, Akt, Erk; Inhibition of NF-κB.	[71]
Ethanol extract of <i>G. lucidum</i> (GLE)	Pre-cancerous human uroepithelial cell line (HUC-PC) .	Induces apoptosis and upregulates IL-2, IL-6, and IL-8 in HUC-PC cells in a dose-dependent manner.	Enhancement of cytokine expression by p50/p65 NF-κB activity. Migration of neutrophils via upregulation of IL-8.	[72]
II-Ganoderic acid subtypes	Applications	Observed Activities	Suggested Mechanism(s)	Refs
Ganoderic acid X (GA-X)	Hepatoma cells (HuH-7), colorectal carcinoma (HCT-116), Burkitt's lymphoma (Raji cells), acute promyelocyte leukemia (HL-60).	Inhibits topoisomerases I and IIa <i>in vitro</i> , resulting in immediate inhibition of DNA synthesis as well as activation of ERK and JNK mitogen-activated protein kinases.	Induction of apoptosis with degradation of chromosomal DNA; decreased levels of Bcl-xL, disruption of mitochondrial membrane, release of cytochrome c and activation of caspase-3.	[73]
Ganoderic acid T (GA-T)	Human metastatic lung tumor (95-D), liver tumor (SMMC7721), epidermal cancer (KB-A-1&KB-3-1), cervical cancer (HeLa), melanoma (A375), normal lung (HLF), embryonic liver (L-02), kidney (HEK293), and colon carcinoma (Ls174t) cell lines.	Induces cytotoxicity to cancer cells, but less toxic to normal cells. Induces cell cycle arrest at G1 phase. Suppresses MMP-2 and MMP-9 gene expression through the inhibition of NF-κB activation.	Reduction of mitochondrial membrane potential (Δψ _m), release of cytochrome c and apoptotic activity in lung cancer cells. Induction of p53 and Bax, which stimulates the activity of caspase-3 but not caspase-8.	[50, 74]
Ganoderic acid ME (GA-Me)	<i>In vivo</i> Lewis lung carcinoma in C57BL/6 mice, human colon carcinoma cells (HCT-116), MDR human colorectal carcinoma cell lines, and metastatic lung carcinoma (95-D), p53-null lung cancer (H1299), HCT-116 p53+/+ and HCT-116 p53 -/- colon cancer cells.	Inhibits tumor growth and lung metastasis in rodents (28 mg/kg i.p.), increases NK activity with upregulation of NF-κB. Kills cancer cells via p53 and mitochondria-mediated apoptosis. Reverses multidrug resistance of HCT-116 cells enhancing chemosensitivity to anticancer agents.	Induction of cell cycle arrest. Induction of apoptosis in MDR cells via upregulation of p-p53, p53, Bax, caspases-3/9 with downregulation of Bcl-2. Upregulation of IL-2 and IFN-γ <i>in vivo</i> .	[54, 55, 75, 76]

Table 1: Some recent applications, observed activities and suggested mechanisms of actions for *Ganoderma lucidum* raw extracts (I), and its purified Ganoderic acids (II) on several types of cancer cells.

and downregulation of uPA, MMP-2/9 and iNOS/NOS2 protein expression. A recent study has also demonstrated that GA-Mf as well as GA-S induces apoptosis of human HeLa cells through a mitochondria-mediated pathway [53]. Both GA isomers showed inhibition of cellular growth in various human carcinoma cell lines. GA-S was also shown to cause cell cycle arrest in the S phase, while GA-Mf caused cell cycle arrest in the G1 phase. Treatment of HeLa cells with these GAs resulted in a decreased mitochondrial membrane potential and the release of cytochrome c from mitochondria into the cytosol. Caspase-3 and caspase-9 activities were also observed with a sharp decrease in the Bcl-2/Bax ratio as recorded in GA-treated HeLa cells. Figure 2 depicts the possible anticancer activity of GAs.

The potential use of GAs as a complementary anticancer therapy also depends on their effectiveness against multidrug resistant cancer cells. In this direction, GA-Me has been shown to reverse the multidrug resistance (MDR) in multidrug resistant colon cancer cells by inducing apoptosis via upregulation of p-p53, p53, Bax, caspase-3, caspase-9 and downregulation of Bcl-2 [54]. In addition, GA-Me treatment can stimulate the decline in mitochondrial membrane potential and release of cytochrome c into the cytosol [54]. Beside its apoptotic effects, GA-Me has been reported to effectively inhibit tumor growth and lung metastasis of Lewis lung carcinoma in C57BL/6 mice through enhanced immune function [55]. Administration of GA-Me (28 mg/kg) resulted in a significant increase in NK cell activity and the production of IL-2 and IFN- γ , possibly through an upregulation of NF- κ B. Multiple roles of GA's antitumor activities are depicted in Figure 2. Our group has found that purified GA-A has a profound apoptotic effect on B-cell lymphoma cell lines with minimal toxicity on non-malignant B-cells. Treatments with GA-A caused mitochondrial dysfunction, identified by the release of cytochrome c into the cytosolic compartment, caspase 3, and 9 activities, and overexpression of Bax (unpublished data). We also found that GA-A-treated B-cell lymphoma express elevated levels of HLA class II proteins with an increased antigen presenting capacity to stimulate T cells *in vitro* (unpublished data). However, the mechanisms by which GA-A induces immune activation and tumor killing *in vivo* remains unclear. Table 1 summarizes some recent investigations on GAs antitumor activities, their application, and suggested mechanism(s) of actions.

Ganoderic Acid-Infused Nanoparticles (GAIN)

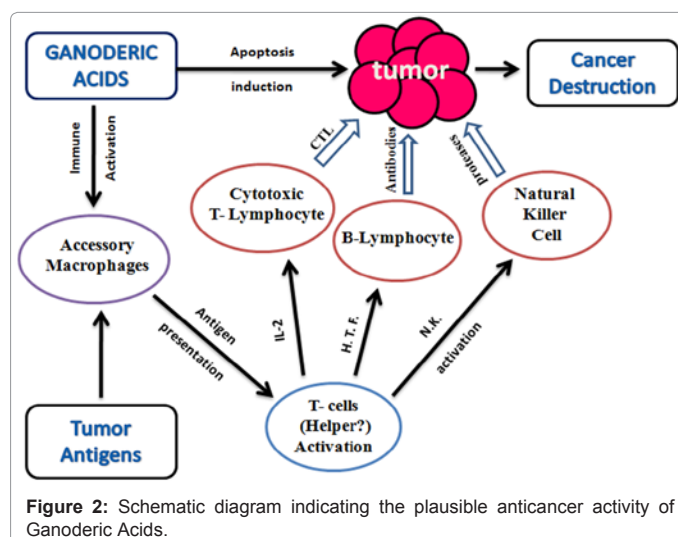
It has been reported that most anticancer drugs have limited selectivity [56], hence the levels of drug required to kill a sufficient number of tumor cells to achieve and maintain a state of complete remission in patients causes significant toxicity towards actively proliferating nonmalignant cells, such as normal cells of the gastrointestinal tract and bone marrow. Many of the curative properties of conventional treatments can be improved through the use of targeted drug delivery systems [57], which include the use of nontoxic, biodegradable particulate carriers, composed primarily of lipids and/or polymer nanoparticles, and their associated therapeutics [58-61]. This new delivery system is designed to alter the pharmacokinetics and biodistribution of the associated drug, or to function as drug reservoirs (i.e., sustained release), in addition to their advantage in stabilizing the drug and preventing its premature metabolic breakdown.

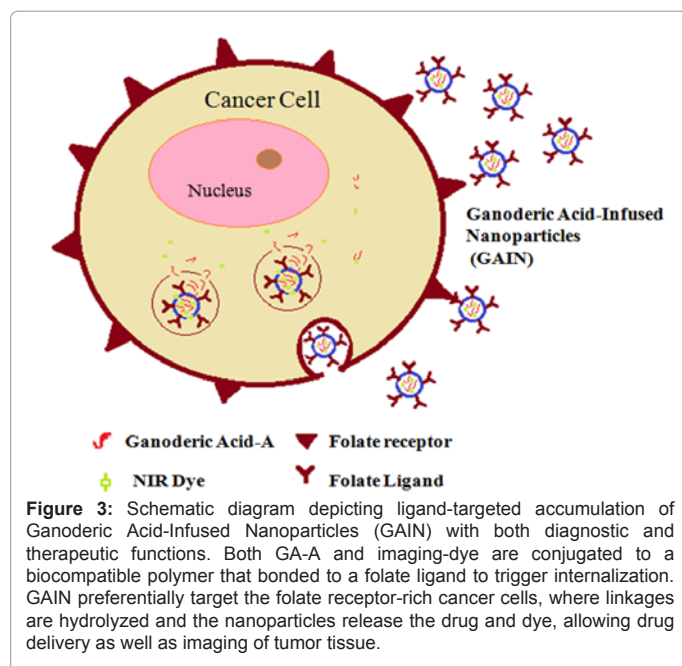
Nanoparticle approaches to targeted drug delivery for malignant tumors offer new opportunity to improve patient care and quality of life by reducing off-target toxicities. Development of targeted nanoparticles ensures delivery of chemotherapeutics directly to cancer cells, followed by their slow release in potentially sustainable levels that may provide superior efficacy and lower toxicity for treating primary or advanced metastatic tumors. Our recent studies suggest that a combinatorial approach using the co-encapsulation of a lipophilic near infrared (NIR) dye and an anticancer drug within hydrophobic pockets in the polymeric matrix of poly acrylic acid (PAA)-coated IONPs (PAA-IONPs) could be employed for combined optical imaging, magnetic resonance imaging (MRI) detection, and targeted cancer therapy [58]. Our study suggests that a natural triterpenoid such as GA-A can be readily entrapped with a high degree of latency within its hydrophobic pockets, remain associated for appropriate lengths of time and be released at an appropriate rate, with sustained levels of drug in the tumor cells [62].

GA-A-carrying nanoparticles could also be designed to deliver the drug to the cancer cells, allowing targeted cancer treatment without harming healthy cells. For example, receptor-mediated cellular uptake of nanoparticles could be achieved by attaching a vitamin derivative such as folic acid to these particles. Folic acid is an effective targeting agent for the folate receptor which is believed to be overexpressed in many human tumor cells [63,64]. In this scenario, cancer cells may consume high amounts of folic acids and ensure that nanoparticles preferentially accumulate at the tumor site before releasing their drugs (Figure 3). Studies performed by our group suggest that nanoparticles can also carry a fluorescent dye and an iron oxide magnetic core [58,59], thus their locations within the cells and the body could be seen by optical imaging and MRI, allowing a physician to see how the tumor is responding to the treatment. Our study also suggests that the nanoparticles could be engineered without the drug for their use as imaging (contrast) agents for detection of cancer [58]. If there is no cancer, the biodegradable nanoparticles may not bind to the tissue allowing their elimination by the liver cells. Ultimately, high concentration of GAIN accumulated at the tumor site may spare exposing the body to the side-effects of chemotherapy, while having larger doses of drugs to target cancer cells.

Conclusions

Exploring the scientific basis among known cures used in folk medicines has resulted in purifying potential natural products that





could combat many diseases, particularly cancer. Hence, further examination of the anticancer relevance of these products may relieve the economic burden associated with the management of cancer, especially in societies where modern medicine is scarce, or expensive to buy or even unavailable. One valuable resource of those medicinal products is the fruiting body of *Ganoderma lucidum*, a wild mushroom that grows on logs, decaying wood, and tree stumps in Asia and surrounding regions, and which has been used for thousands of years in herbal remedies to promote human immune function. Given that the development of tumor is aided by immune evasion, current literature suggests GAs possess both apoptotic and immune restoration properties. Our study suggests that GAs are unique natural candidates which could be used to enhance the cellular expression of immune components as well as antigen processing and presentation. The above mentioned properties of GAs make them ideal candidates for potential use in complementary chemo-immunotherapeutics. In this direction, we are currently developing a new GAs treatment-delivery system termed GAIN by utilizing nanotechnology to develop an antitumor treatment strategy that will allow cancer targeting. GAIN is embedded in cancer-targeting protein ligands to allow more of the incorporated compound to reach the target tissue and greatly diminish any toxic side effects associated with using higher doses. Collectively, this review suggests that enhanced targeting resulting from nanoparticle delivery, coupled with the reduced bystander toxicity of natural products such as GAs will pave the way for potential treatments of human malignancies.

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