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Cancer Biomarkers for Integrative Oncology

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Abstract

Purpose of Review There has been an increasing interest in using complementary and alternative medicine (CAM) approaches to treat cancer. It is therefore relevant and timely to determine if CAM biomarkers can be identified and developed to guide cancer diagnosis and treatment. Herein, we review the status of cancer biomarkers in CAM research and treatment to stimulate further research in this area.

Recent Findings Studies on promising anti-cancer natural products, such as PHY906, honokiol, bryostatin-1, and sulforaphane have demonstrated the existence of potential cancer biomarker(s). Additional studies are required to further develop and ultimately validate these biomarkers that can predict clinical activity of the anti-cancer natural products used alone or in combination with chemotherapeutic agents.

Summary A systematic approach is needed to identify and develop CAM treatment associated biomarkers and to define their role in facilitating clinical decision-making. The expectation is to use these biomarkers in determining potential options for CAM treatment, examining treatment effects and toxicity and/or clinical efficacy in patients with cancer.

Keywords Biomarker · Cancer complementary and alternative medicine (CAM) · Integrative oncology · Cancer diagnostics · Anti-cancer natural product · Anti-cancer herbal medicine

Introduction

In recent years, there has been increasing interest to use alternative approaches and therapies for treating cancer patients. Therefore, it is relevant to review promising anti-cancer natural products and to examine the identity of potential biomarkers/genetic signatures associated with herbal medicine treatment, since these biomarkers may play an important role in guiding diagnosis, evaluating treatment response, and identifying drug target(s) and biochemical pathway modulation.

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It has been well-established that natural products can have anti-cancer properties. However, exploiting these bioactive compounds for optimal therapeutic use has been a challenge primarily due to inadequate specificity of sources; biological variability and heterogeneity of starting material, the presence of mixtures of a variety of compounds, some of which are inert and others of which affect therapeutic activity; and variability in therapeutic use by practitioners. Given the fact that many of the most widely used and therapeutically effective anti-cancer drugs, including taxanes and camptothecin, were isolated from plants and microorganisms, it is clearly worth the effort to define the activity of anti-cancer natural products and identify clinically useful biomarkers.

Studies with promising natural products, such as PHY906 (a pharmaceutical grade of a Chinese herbal formulation), honokiol, bryostatin-1, sulforaphane, and curcumin, have shown anti-cancer properties. However, curcumin studies are briefly discussed and considered out of scope due to concerns. Research on some of these agents has revealed effects on cell survival, cell proliferation, invasion, and angiogenesis involving expression of an array of genetic/molecular signatures leading to activation/deactivation of signaling pathways at various levels [1–4, 5•]. Despite these research findings, there remains limited progress in the development and use of biomarkers in Complementary and Alternative Medicine (CAM) research and clinical applications.

Many herbal products are generally considered safe. However, there are concerns that their active components and molecular targets are not well defined, except for a very few agents. While many positive effects of CAM treatment have been revealed, to our knowledge, defined approaches to quality control and validation of anti-cancer natural products and to identify and validate biomarkers for use in CAM treated cancer patient management have not been adequately explored. Therefore, a systematic approach is needed to conduct preclinical and clinical studies to identify potential biomarkers emerging from cancer CAM research. The rationale is to use these cancer biomarkers/genetic signatures in patients considering or undergoing CAM treatment.

While there are limited publications on CAM biomarker research, reports particularly on some of the thoroughly studied natural products can help establish a basis for conducting research to identify and incorporate CAM biomarkers in research and clinical applications. This review is primarily focused on in vitro, preclinical, and clinical studies with PHY906, honokiol, bryostatin-1, and sulforaphane to provide a comprehensive understanding of the present status of cancer CAM biomarker research, acknowledge challenges, and address what needs to be done to identify, develop, validate, and incorporate clinically relevant cancer CAM biomarkers in patient-centered care in Integrative Oncology.

Preclinical and Clinical Studies With Natural Products and Identity of Possible Biomarkers

Studies With PHY906

PHY906 is one of the systematically studied natural products that is derived from *Huangqin Tang* (HQT), a classic Traditional Chinese Medicine (TCM) formula first described about 1800 years ago. This formula has been widely used in China and other Asian countries to treat GI disorders, including nausea and vomiting, abdominal cramps, and diarrhea [6, 7] and is composed of four main herbs, *Glycyrrhiza uralensis* Fisch (G), *Paeonia lactiflora* Pall (P), *Scutellaria baicalensis* Georgi (S), and *Ziziphus jujuba* Mill (Z). A proprietary protocol was specifically developed by Phytoceutica, a Yale-sponsored company, in collaboration with Sun Ten Pharmaceuticals in Taiwan, to prepare PHY906 packaged in capsules according to Current Good Manufacturing Practices (cGMP) under the United States Food and Drug Administration (FDA) guidance.

PHY906 Quality Control Quality control is a major issue with respect to the development of any herbal medicine including TCM. In general, herbal extracts may contain up to hundreds of individual phytochemical components. Investigators from Yale and Phytoceutica developed the Phytomics QC platform, a comprehensive set of methodologies that involved chemical analysis, bioresponse profiling, and in vivo animal pharmacology to assess PHY906 quality control and batch-to-batch reproducibility [8, 9]. Liquid chromatography/mass spectrometry (LC/MS) was used to determine the chemical fingerprint profile. For bioresponse fingerprinting, gene expression profiling was used, as this methodology provides a sensitive, unique, and comprehensive pattern in response to herbal medicine exposure. A Phytomics Similarity Index (PSI) was then developed for both the chemical and the bioresponse fingerprint analyses. The PSI value provides a quantitative measure of similarity that integrates peak patterns, peak ratios, and peak intensities from various batches. This focus on quality control has been critical for all the PHY906 pre-clinical and clinical studies.

A mechanism-based quality control (MBQC) approach has been used to examine five different cGMP batches of PHY906 prepared over a 15-year period using both chemical and bioresponse fingerprinting analyses. This unique methodology has demonstrated > 90% consistency of similarity index, which is markedly different from commercial preparations of HQT. It is important to note that the ability to control batch-to-batch consistency is a major step forward not only for PHY906 but other promising herbal medicines to make these products more reliable and useful for clinical applications.

Identification of PHY906 Chemicals and Metabolites A novel methodology was developed by Cheng and colleagues involving LC/MS along with enzymatic digestion and n-octanol/water partition coefficient to identify PHY906 chemicals and metabolites in plasma [10]. Using this approach, a total of 57 individual chemicals were identified in the parent formulation of PHY906. In addition, 27 new metabolites were identified in the plasma of a patient with metastatic colorectal cancer (mCRC) treated with PHY906. Introducing this analytical approach was an important first step to identify the active chemicals and/or metabolites of PHY906 that mediate its biological activity in in vivo model systems. Moreover, they represent potential pharmacodynamic biomarkers in patients treated with PHY906.

Pre-clinical Studies With PHY906 Preclinical studies with the murine MC38 model documented the ability of PHY906 to reduce animal weight loss associated with irinotecan treatment while enhancing irinotecan's antitumor effects. PHY906 was able to promote the recovery of damaged intestinal tissue in response to irinotecan therapy by promoting the regeneration of intestinal stem cells through stimulation of *Wnt* signaling [1]. Treatment with PHY906 resulted in an anti-inflammatory effect with a reduction in infiltration of neutrophils and macrophages, reduction in expression of TNF-α in the intestine, and decreased concentrations of various pro-inflammatory cytokines in peripheral blood. In addition, PHY906 and some of its individual components were found to be potent inhibitors of NF-κB, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase. Additional pre-clinical in vivo studies have provided further evidence that PHY906 caused an enhanced expression of two important stem cell markers Olfm4 and Lgr5, which may be associated with the recovery of damaged intestinal tissue. Moreover, PHY906 was able to inhibit irinotecan-associated inflammatory processes in the mouse intestine, which include infiltration of the intestine by neutrophils and macrophages and reduction of MCP1 and TNF-α expression in the intestine [11]. Treatment with PHY906 resulted in decreased levels of the pro-inflammatory cytokines G-CSF and MCP1 in the circulating plasma, which was consistent with the reduced expression of these inflammatory cytokines in the intestinal tissue [11].

Preclinical studies from the Cheng lab at Yale University have shown that PHY906 is able to enhance the antitumor activity of various cytotoxic chemotherapy agents, reduce their toxicities, and, in some cases, do both [12]. These agents are completely unrelated in terms of their own mechanism of action, and they include 5-fluorouracil (5-FU), capecitabine, etoposide, irinotecan, paclitaxel, oxaliplatin, gemcitabine, sorafenib, sunitinib, and thalidomide. As it relates to the interaction between PHY906 and irinotecan, Cheng and colleagues have performed a series of experiments to document that all four main herbs of PHY906 (G, P, S, H) are, in fact, required for optimal biological activity [12]. One potential mechanism by which PHY906 may be able to enhance the antitumor activity of completely unrelated cytotoxic agents is by triggering the inflammatory process in the tumor microenvironment [13].

Clinical Studies With PHY906 and Identity of Possible Biomarkers PHY906 has been investigated in six clinical trials in the USA and Taiwan, and these studies included two phase 1 trials in metastatic colorectal cancer (mCRC), one randomized phase 2 trial in mCRC, one phase 1/2 trial in advanced hepatocellular cancer (HCC), one phase 2 trial in advanced HCC, and one phase 1/2 trial in advanced/metastatic pancreatic cancer [14, 15•, 16, 17]. PHY906 has shown promising clinical activity when used in combination with sorafenib for advanced hepatocellular cancer (HCC) [18], and especially in patients who are hepatitis B (HBV) positive. To date, nearly 200 patients have received PHY906 using various dosing regimens. The general conclusion derived from these studies is that this herbal medicine is safe and well tolerated. Perhaps more importantly, in nearly every clinical trial conducted to date, PHY906, when combined with cytotoxic chemotherapy, appears to reduce the toxicities typically associated with chemotherapy and enhances the therapeutic index of the chemotherapy agent.

The first clinical study conducted with PHY906 was a double-blind, placebo-controlled, cross-over phase 1 study led by the Chu and Cheng groups at Yale University in collaboration with PhytoCeutica, the sponsor of PHY906, to evaluate the safety and tolerability of oral PHY906 in combination with the irinotecan-based IFL (concurrent treatment with irinotecan, leucovorin, 5-FU) regimen for patients with mCRC [14, 15•]. The initial dose level of PHY906 used in this study was 1.2 g/day, with the second dose level being 2.4 g/day. Of note, these dose levels were significantly lower than what is currently used in daily practice in Asian countries. This study showed that PHY906 therapy significantly reduced the incidence of grades 3–4 diarrhea, nausea/vomiting, and fatigue [13, 14]. Although the study was not specifically designed to test for the effect of PHY906 on the clinical efficacy of the IFL regimen, nearly all patients exhibited a partial response or stable disease after two treatment cycles. As part of this study, pharmacokinetic (PK) studies were conducted, which showed that PHY906 did not alter the metabolism of the two main cytotoxic agents used in the IFL regimen, 5-FU and irinotecan, suggesting no adverse herb-drug interactions.

A second phase 1 clinical study was subsequently conducted where PHY906 was combined with irinotecan monotherapy in the second-line treatment of patients with mCRC and other solid tumors [19]. This study used a traditional 3 + 3 design and identified the recommended phase 2 doses of irinotecan and PHY906 to be 215 mg/m^2 and a daily dose of 3.6 g/day, respectively. PK studies were also performed,

which confirmed that PHY906 did not alter the metabolism of irinotecan, SN-38, and their respective glucuronidated metabolites. PD biomarker studies were incorporated in this study to begin to correlate the effect of PHY906 on toxicity and/or clinical activity of irinotecan. For these translational studies, the effect of drug treatment on expression of cytokines, chemokines, and various growth factors, on metabolomic profiling, and on expression of key signaling proteins (total protein and phosphoprotein profiling) was also examined. In addition, a LC/MS/MS assay, as described earlier, was used to identify the individual chemical components and metabolites in the peripheral blood of patients treated with PHY906.

A randomized, double-blind, placebo-controlled phase 2 study was subsequently designed where the effect of PHY906 on the toxicity and clinical efficacy of single-agent irinotecan was investigated in the second-line treatment of mCRC. A series of translational studies were incorporated into this study, which included profiling of immunocytokines, chemokines, and growth factors, metabolomic profiling, and assessment of tumor mutational load as determined by the presence of mutations of K-Ras, B-Raf, and/or PI3K/Akt. The outcome of this study with biomarker analyses has not yet been reported.

With respect to the metabolomics analysis, 136 metabolites (typically MW < 1000) have been identified in plasma samples from patients treated on the phase 2 clinical trial with PHY906 plus irinotecan. A preliminary analysis has identified several distinct metabolites whose expression in plasma appears to be altered in response to PHY906 treatment. There were also individual differences in the circulating levels of flavones and their metabolites following treatment with PHY906. Results from these studies will be analyzed to determine if there is a potential correlation between the presence of these herbal metabolites and the biological activity of an individual patient's plasma sample against several key regulatory signaling pathways as well as immune signaling pathways.

It is hoped that these translational studies will begin to identify potential biomarker(s) that can be used to predict the effect of PHY906 on toxicity and/or clinical efficacy of irinotecan chemotherapy.

Studies with Honokiol and Bryostatin-1

The following two examples of natural compounds, honokiol and bryostatin-1, with potent biological activities serve to highlight the importance of biomarkers for predicting the response to novel anti-cancer natural products, particularly those associated with therapeutic properties from traditional use.

Studies With Honokiol Extracts of the Magnolia tree have long been used for their medicinal properties. While this has been particularly well described in East Asian traditional medicine, Magnolia species are also found in North America, and may have been used in Native American healing. When *Magnolia grandiflora* seed cones were ground and extracted with boiling water, the resultant aqueous extract showed a number of important biological properties, including inhibition of endothelial cell proliferation [20]. When separated by high-pressure liquid chromatography, the fractions that possess this activity contain the biphenol honokiol and the highly related compound magnolol. While traditional medicines routinely used mixtures of bioactive compounds, the biological findings with honokiol in these experiments employed analytically purified honokiol that was shown to be at least 99% pure by HPLC. Honokiol induces apoptosis of endothelial cells, and it inhibits several key signaling pathways, including *Akt*, Map kinase, and *Src*. Interestingly, this effect on endothelial cells is mediated, at least in part, by the autocrine production of TNF-related apoptosis-inducing ligand (TRAIL), since an antibody to TRAIL partially reverses this effect. The potential clinical relevance of this activity was demonstrated by the ability of honokiol to have a therapeutic effect in a mouse model of angiosarcoma.

The anti-cancer effect of honokiol is not restricted to angiogenesis or angiogenic tumors. Chronic lymphocytic leukemia (CLL), the most common form of leukemia in western countries, was also shown to be susceptible to inhibition by honokiol [5•]. Honokiol induces apoptosis in primary CLL cells, through downregulation of the anti-apoptotic protein MCL1 and increased expression of the pro-apoptotic protein BAX. Importantly, peripheral blood mononuclear cells from healthy donors were largely spared any cytotoxic effect of honokiol. The ability of honokiol to induce apoptosis of CLL cells can even overcome the pro-survival effects of the cytokine IL-4, which recapitulates the pro-survival environment of the lymph node. Furthermore, honokiol sensitizes cells to chemotherapeutic-based drugs used to treat CLL clinically, including fludarabine, chlorambucil, and cladribine. This effect of honokiol in lowering the apoptotic threshold may also explain the sensitization to radiation induced by honokiol in head and neck squamous cell carcinoma cells [21•]. In this case, honokiol mediates this effect through downregulation of the pro-survival protein survivin.

Honokiol clearly has a range of other anti-cancer mechanisms, as well. For example, honokiol has been reported to inhibit epithelial-mesenchymal transition (EMT) in breast cancer cells [22], which may be an important mechanism by which a primary tumor attains the ability to invade and spread. By targeting intracellular pathways, honokiol can overcome resistance to the antibody to the EGF receptor cetuximab [23]. Reflecting its pleiotropic effects, honokiol may also have distinct actions in mitochondria, thereby altering cellular bioenergetics [24]. In addition, honokiol may inhibit the oncogenic transcription factor STAT3 [25], which is activated inappropriately in a wide range of human cancers.

Despite the centuries of use of Magnolia extracts for traditional therapy and the extensive pre-clinical literature on the molecular effects of honokiol, the introduction of this compound into therapeutic cancer clinical trials has been slow.

Studies With Bryostatin-1 A major source of diverse and complex biologically active compounds is marine organisms, which also have been used in traditional medicine. Isolated from an aquatic invertebrate of the phylum Bryozoa, the macrocyclic lactone bryostatin-1 typifies the potential and difficulties of these natural products. While the medicinal value of sea water has been described for centuries [26], the concentration of bryostatin-1 is so low in organisms that it seems unlikely to exert medicinal effects without purification. On the other hand, the structure is so complex that total synthesis has only recently been achieved [27]. For most of the biological studies described, bryostatin-1 (also designated NSC 339555) was obtained from the Cancer Therapy Evaluation Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, to ensure that the findings had immediate translational relevance. Bryostatin-1 is highly potent, and its immediate target appears to be the modulation of protein kinase C (PKC) [28]. However, the biological effects of bryostatin-1 are not identical to other activators of PKC, such as phorbol esters [29], and this may relate to secondary effects of this compound. For example, bryostatin-1 can affect proteasome function [30] and modulate NF-κB [31], and these effects may underlie, at least in part, its unique therapeutic potential.

One example of this broader effect was identified in CLL cells, where bryostatin-1 induced therapeutic functional maturation [32•]. Although this action of bryostatin-1 was mediated by protein kinase C, bryostatin-1 also triggered the autocrine production of interferon-γ that led to the activation of the transcription factor STAT1, which was critical for the differentiation of these CLL cells. However, interferon-γ by itself is not sufficient to induce this differentiation program. It is the combination of the STAT1 activation driven by interferon-γ and other signals induced by bryostatin-1 that is necessary for this response.

Despite these intriguing in vitro anti-cancer properties, clinical trials of bryostatin-1, dating back to the 1990s, have not shown strong evidence of efficacy [33, 34]. The developmental stories of honokiol and bryostatin-1 show informative parallels, with impressive histories in traditional medicine and extensive pre-clinical data, but limited success with clinical translation. This raises important questions on what is holding back clinical transition, and how one might be able to move this field forward more effectively.

Molecular and Biomarker-Based Strategies The examples of honokiol and bryostatin-1 are emblematic of many natural products related to traditional medicines. A common theme is that a wide variety of distinct mechanisms have been proposed for their action, with supportive evidence coming from many tumor systems and models. However, it has been a challenge to move forward, in a rational and effective way, from natural products identified from traditional practices to reproducibly effective modern cancer therapeutics. In this regard, understanding the direct molecular targets of these agents is critical, particularly at concentrations likely to be achieved in humans. At high concentrations, many non-specific and off-target effects will undoubtedly occur. Thus, there should be skepticism about compounds that repeatedly affect the same targets, such as NF-κB, STAT3, and *Myc*, as these pathways are often modulated non-specifically by toxic compounds, in part due to their short half-lives and rapid turnover. It should be realized that "therapeutic" compounds made by plants and microorganisms are often produced to help relatively immobile organisms from being consumed by predators. Thus, non-specifically toxic effects, such as inhibiting microtubule function or damaging DNA, are not unexpected.

It is critical to understand what cellular or genetic background will make a cancer cell particularly sensitive to a specific compound. Testing a limited number of cell lines or cancer types one by one can often yield a skewed understanding of the optimal utility of a compound. Larger systems and databases, such as the NCI-60 screen or the Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle/) can rapidly provide even more useful information.

Once mechanisms and targets are defined, one can then consider biomarkers to identify potentially sensitive tumors, as well as to monitor treatment responses. Thus, if the therapeutic effect of bryostatin-1 in CLL is mediated by activation of PKC and autocrine production of interferon-γ, several testable hypotheses can be considered. For example, does the level or activation state of PKC correlate with biological response to bryostatin-1? Does the basal expression of interferon-γ, or the methylation state of the gene encoding this cytokine correlate with response? What other biological changes occur in cells treated with this agent? Does it alter cell cycle progression, directly induce apoptosis, lower the threshold for apoptosis, alter DNA repair pathways, or modulate epithelial-mesenchymal transition?

The answers from these laboratory-based investigations would then immediately translate into clinical questions that can be incorporated into clinical trials, such as will immunohistochemical staining (IHC) of tumors for PKC correlate with clinical response to bryostatin-1? Will circulating levels of interferon-γ, either prior to or on therapy, correlate with treatment response? If bryostatin-1 alters DNA repair, might it synergize with a PARP inhibitor? With this type of scientifically rigorous and iterative approach, one can build from anecdotal reports from the traditional medical literature to therapies that may be uniquely effective in cancer patients.

Studies With Sulforaphane

Sulforaphane (SFN), (−)-1-isothiocyanate-(4R)-(methylsulfinyl) butane CH3-SO-(CH2)4-NCS is an isothiocyanate found in high concentrations in broccoli sprouts. Early studies by Zhang and his colleagues isolated and demonstrated the potential anticarcinogenic properties of isothiocyanates [35]. A significant amount of broccoli isothiocyanates (ITCs) accumulates as the phytonutrient glucoraphanin (4-methylsulfinylbutyl glucosinolate) and ultimately metabolizes in vivo to the biologically active sulforaphane. This conversion requires myrosinase, which is present in the plant as well as in the gastrointestinal tract [36]. Several epidemiological, in vitro, preclinical, and early-phase trials have shown that the phytochemicals, isothiocyanates, specifically allyl isothiocyanate and sulforaphane (SFN) present in Brassicaceae or "cruciferous" vegetables as their precursor glucosinolates—sinigrin, glucotropaeolin, gluconasturtiin, and glucoraphanin [36–39], respectively, may have distinctive effects at various stages of bladder carcinogenesis and may play a critical role in reducing risk of bladder cancer [40–42].

Based on the evidence from population studies, in vitro, in vivo, and early-phase clinical trials, researchers have evaluated the molecular signatures of SFN to identify possible biomarkers of efficacy, relevant for use in the secondary chemoprevention of bladder cancer.

In Vitro Studies With Sulforaphane In vitro studies in bladder cancer (BC) [43–47], lung, [48] prostate, [49, 50], colorectal [51], and leukemia cell lines [52] have shown SFN to be a potent inhibitor of carcinogenesis through several molecular mechanisms [37]. SFNs have been shown to inhibit survival and proliferation of a wide array of animal and human BC cell lines [43]. Although multiple molecular targets in cellular and animal models have been identified, the most sensitive target for SFN is Keap1, a key sensor for the adaptive stress response system regulated through the transcription factor Nrf2. Interaction of SFN with Keap1 disrupts this function and allows for nuclear accumulation of Nrf2 and activation of its transcriptional program. Enhanced transcription of Nrf2 target genes has been shown to provoke a strong cytoprotective response that enhances resistance to carcinogenesis, potentially mediated by exposures to electrophiles and oxidants [53, 54].

Nrf2 transcription factor has been shown to be essential for the chemopreventive efficacy against urinary bladder carcinogenesis and for induction of phase II proteins [53, 55]. In addition, SFN has been shown to have antitumor effects against BC cells through a ROS-mediated intrinsic apoptotic pathway. These studies suggest that ER stress and Nrf2 may represent strategic targets for SFN-induced apoptosis [53].

Other mechanisms implicated include downregulation of NF-κB resulting in the induction of cell cycle arrest and apoptosis [56], while selectively targeting abnormal/malignant cells [57, 58] compared to normal bladder cells [43, 59]. Studies in BC cell lines [57] demonstrated downregulation of survivin, epidermal growth factor receptor (EGFR), and human epidermal growth factor receptor 2 (Her2/neu), G [2]/M cell cycle accumulation, and apoptosis [57]. SFN, in addition, has shown to inhibit inflammatory responses, including downregulation of cyclooxygenase-2 (Cox-2) and reduction of prostaglandin E2 level [60, 61].

SFN potently inhibits the growth of cells derived from both low-grade superficial and high-grade invasive human bladder cancers and drug-resistant BC cells, demonstrating antiproliferative mechanisms, such as causing the cleavage of the same set of caspases (caspase-3, caspase-8 and caspase-9) in apoptosis induction, and arresting cells in the same S and G2/M phases [37, 39, 46, 62]. The novel therapeutic combination of acetazolamide (AZ) and SFN in the HTB-9 and RT112(H) human bladder tumor cell lines produced a potent anti-proliferative and anti-clonogenic effect, and induced apoptosis through caspase-3 and PARP activation. The antiproliferative effect was corroborated by significant reductions in Ki-67 expression [63]. Evidence from in vitro studies demonstrates that SFN targets multiple molecular pathways (Fig. 1), preventing the initiation of carcinogenesis as well as preventing tumor progression [64].

Pre-clinical Studies With Sulforaphane The potential for systemic effects of SFN has been shown in animal models that demonstrated bioavailability of SFN with metabolites distributed to all tissues, including the bladder [57, 65, 66]. Rats with N-butyl-N-(4-hydroxybutyl) nitrosamine-induced BC were administered a freeze-dried aqueous extract of broccoli sprouts, showed a significant decrease in BC development, in a dose-dependent manner [66]. The extract significantly increased induction of phase II enzymes such as glutathione S-transferase and NAD(P)H:quinone oxidoreductase-1 in the bladder. Results of the preclinical studies indicate that the incidence, multiplicity, size, and progression of BC were all inhibited by the extract, while the extract itself caused no histological changes in the bladder, suggesting relevance in the chemoprevention of multiple stages of bladder carcinogenesis.

Preparation of Sulforaphane-Rich Broccoli Sprout Extract Formulation for Clinical Trials A few standardized formulations of sulforaphane have been developed adhering to guidelines developed by the United States Food and Drug Administration (FDA) for botanicals, utilizing meticulous quality control procedures. These include sulforaphane-rich broccoli sprout extracts formulations where selected broccoli seeds (*Brassica oleracea var. italica*) with adequate levels of

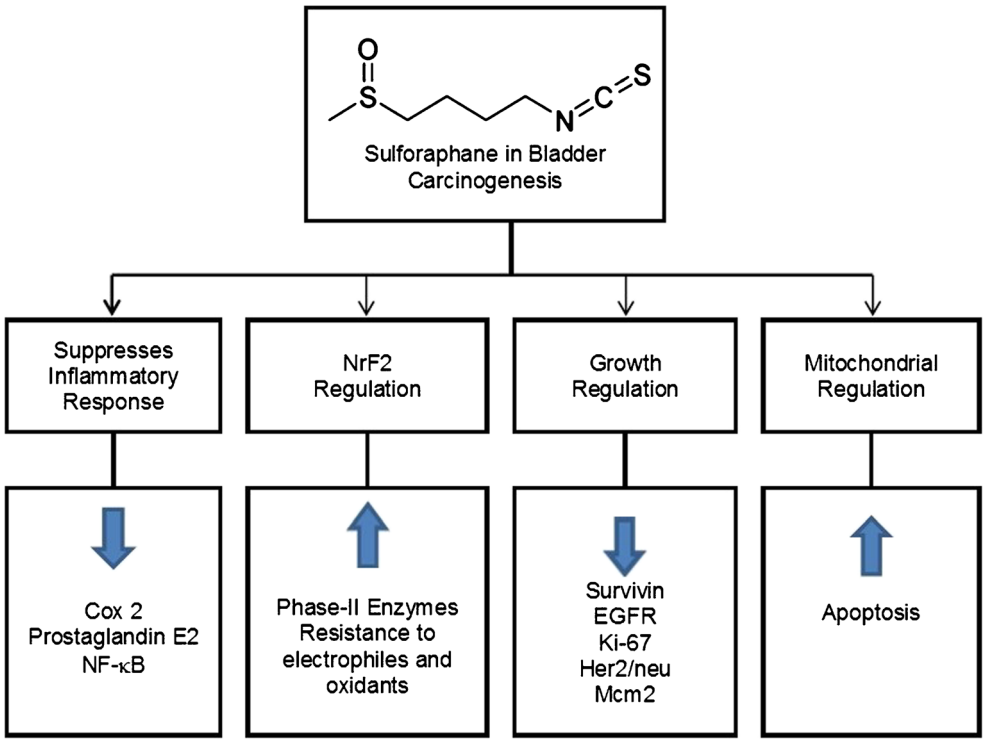

Fig. 1 Molecular mechanisms and potential biomarkers of sulforaphane

glucoraphanin (GR; the precursor of sulforaphane) have been cultivated to yield sprouts with levels of at least 6 μmol of GR per gram. An aqueous extract containing ~ 5 μmol of GR per milliliter was treated with the enzyme myrosinase to convert the GR to sulforaphane. The levels of total isothiocyanate, sulforaphane, and residual GR were then quantified by cyclocondensation and by direct HPLC, respectively. The hydrolyzed aqueous extract was frozen rapidly and bioassayed for potency and microbial contaminants. Before clinical use, the sulforaphane-rich broccoli sprout extracts were re-analyzed for sulforaphane content. Doses of the powder were aliquoted by a commercial pharmacy (ALFA Pharmacy, Columbia, MD) into opaque, purple gel caps delivering 218 mg of powder (containing 50 μmol sulforaphane) per gel cap ready for delivery to subjects. A similar methodology was used in the preparation of Prostaphane® capsules, containing 10 mg of free stabilized SFN extracted from broccoli seeds (Nutrinov Labs, France) and provides 200 μmol SFN. To improve the stability of SFN, a cold press process was developed by Nutrinov to produce immediate-release tablets of microencapsulated active component powder extract. The production of Prostaphane® complies with European regulations. The following clinical trials utilized these standardized formulations to maintain consistency.

Clinical Studies with Sulforaphane Clinical trials to date have been conducted to evaluate the effectiveness of SFN for chemoprevention focused on prostate cancer [67•, 68•, 69•, 70] and breast cancer [44]. In a clinical trial evaluating 60 mg SFN (340 μmol Prostaphane®) vs. placebo [69•] for 6 months in men with biochemical recurrence of cancer after radical prostatectomy, a reduction in serum PSA was observed in 8/20 (40%) of prostate cancer patients in the treatment arm compared to placebo. Targeting men with recurrent prostate cancer in a single-arm trial with 200 μmol SFN daily for 20 weeks, Alumkal et al. [67•] reported that 1 of 20 patients had a 50% decline in serum PSA at 5 months, with 7/20 men experiencing smaller PSA declines. A significant lengthening of the on-treatment PSA doubling time (PSADT) was observed compared with the pre-treatment PSADT (6.1 months pre-treatment vs. 9.6 months on-treatment ($p = 0.044$)). SFN was well tolerated with no grade 3 toxicities. Using 400 g broccoli/week vs. 400 g peas/week, targeting men with high-grade prostate intraepithelial neoplasia (HGPIN) for 6 months, Traka et al. [70] showed significant changes in TGFβ, insulin signaling, and EGF receptor pathways. In a randomized trial focused on women, 2–8 weeks prior to undergoing breast biopsy, Atwell et al. compared the effect of SFN (glucoraphanin, 30 mg GFN BroccoMax™) vs. placebo [44] on selective biomarkers in breast tissue in addition to bioavailability (urinary metabolites and plasma) and safety. Comparing pre- and post-treatment levels within each treatment group, Ki-67 ($P = 0.003$) and HDAC3 ($P = 0.044$) levels significantly decreased in benign tissues, but not in the invasive ductal carcinoma tissue [44]. These data provide early evidence of bioavailability and safety as well as potential chemopreventive effects in intermediate endpoint biomarkers implicated in breast and prostate carcinogenesis.

Potential Biomarkers of Sulforaphane in Bladder Carcinogenesis Evidence from in vitro, in vivo, and early-phase clinical trials have identified molecular signatures of SFN that can be examined in bladder tissue, as potential biomarkers of efficacy for the use in the secondary chemoprevention of bladder cancer. For example, the inflammatory biomarkers that can be examined include tissue Cox-2, prostaglandin E2, and NF-κB. Biomarkers relevant to NrF2 regulation include phase II enzymes in treated samples of plasma, urine, and tissue glutathione transferases, epoxide hydrolase, NAD(P)H: quinone reductase, and glucuronosyl transferases, as have been previously reported by Egner et al. [71] and Ye et al. [72].

For identification of inhibition of Keap1:Nrf2 binding, fluorescence polarization can be used. Activated NF-κB can be evaluated by western blot analysis for the presence of phospho-p65 subunit of NF-κB in BC cell lysates. As such, these studies provide evidence on the mechanistic pathway targeted by SFN, contributing to the reduction in proliferation and inflammation and increasing apoptosis and phase II enzymes in BC cells. Similarly, targets of growth regulation such as survivin, EGFR, Ki-67, and Her2/neu are ideal biomarker candidates. Candidate biomarkers of mitochondrial regulation include the change in apoptosis in bladder cells treated with SFN. The presence of apoptotic cells in BC cells and adjacent non-malignant cells induced by SFN can be measured using an IHC assay that detects activated caspase-3. Additionally, elevated expression of Ki67 has been shown with the progression of non-muscle invasive (NMIBC) BC tumor and muscle-invasive bladder cancer (MIBC) grade and stage, associated with advanced pathologic stage, higher grade BC, lymphovascular invasion, and metastases to lymph nodes [73, 74]. Ki67 labeling index is an independent predictor of high grade, multiple tumors and recurrence-free survival in Ta/T1 bladder cancer [75–78]. Ki67 is thus a validated and consistently identified independent prognostic factor in organ-confined BC and has been corroborated in NMIBC and MIBC as a negative prognostic factor [73, 79]. The creation of an index that takes both proliferation (Ki-67) and apoptosis (assessed by caspase-3) into account may accurately reflect the pharmacodynamic effect of SFN. Evaluation of the effectiveness of SFN in bladder carcinogenesis should be based on the change in biomarkers of proliferation (Ki-67) and apoptosis (caspase-3) from baseline to post-intervention in both NMIBC and MIBC cells and benign/adjacent cells. A summary of the possible molecular signatures/biomarkers is presented in Fig. 1.

Studies With Curcumin

Curcumin (an active ingredient of turmeric) is a potent anticancer agent, treatment of which leads to modulation of a wide array of proteins (e.g., inflammatory cytokines, transcription factors, gene products linked with cell survival, proliferation, apoptosis, invasion, and angiogenesis) and modifies their expression and activity for chemopreventive or therapeutic effects [2]. In Hodgkin's lymphoma cells, curcumin inhibits both NF-κB and STAT3 activation, leading to a decreased expression of proteins involved in cell proliferation (e.g., Bcl-2, Bcl-xl, survivin, *c-myc*, cyclin D1), and causes cell cycle arrest in G2-M and triggers apoptosis via caspase-9 and caspase-3 [80]. However, curcumin comes up as a "hit" in so many screening assays (perhaps because of its bright yellow color), that it is almost impossible to determine its true activity. This has raised the concern, "that there's no evidence it has any specific therapeutic benefits, despite thousands of research publications and more than 120 clinical trials" [81].

Novel Approaches to Identifying Biomarkers for Integrative Oncology

In addition to the development of mechanism-based biomarkers, it would be extremely useful to identify biomarkers that are predictive of response or can monitor the response to complementary and integrative therapies for which the precise mechanism of action is unknown. Since the number of circulating analytes, even excluding nucleic acids and those present in exosomes and microvesicles, are in the thousands, it is not feasible to approach this question on a candidate-by-candidate basis. Therefore, more integrative approaches to understanding the physiologic milieu of the body, and how it can change with both pharmacologic and non-pharmacologic therapies (including acupuncture, yoga, meditation, massage, and others) would be enormously helpful.

One strategy is to develop bioassays sensitive to stimuli that modify well-defined transcriptional pathways that reflect key physiological processes. For example, the acute-phase response is a well-defined constellation of changes induced by stresses including cancer, as well as infection, trauma, and inflammation. This process can be mediated by interleukin (IL)-6 and other related cytokines. In fact, for a variety of cancer types, IL-6 levels correlate with poor outcome. This finding may reflect both the direct burden of the cancer, as well as the body's ability to tolerate this stress. However, measuring only the levels of IL-6 or related cytokines may provide a partial reflection of the integrated level of the acute-phase response. In addition to the cytokines, there are also circulating receptors, such as the soluble IL-6 receptor, which can enhance signaling in this pathway. There are also decoy receptors that may attenuate this signaling. Thus, to get an accurate picture of the activation state of the acute-phase response pathway from a patient's sample, it would be important to integrate all these components.

It is possible to create cell-based bioassays, in which the net effect of a sample from a patient's serum or plasma is reflected in an integrated readout. The key intracellular mediator of IL-

6 and related cytokines, which regulates the expression of downstream gene expression, is the transcription factor STAT3. Systems have been developed that can quantitate STAT3 signaling through the production of a reporter gene such as luciferase. These systems have been extremely useful for identifying small molecules and other agents that can modulate STAT signaling for therapeutic purposes (Fig. 2). However, these cellular systems can also be used to determine the intrinsic effect of a biological sample on this pathway, as well as the effects of the sample in enhancing or inhibiting signaling induced by IL-6. This approach can integrate a pattern of expression of circulating factors, including cytokines, receptors, and endogenous inhibitors, and could comprise a unique biomarker to identify patients suitable for therapy. It would also allow the quantitation of therapeutic benefit in an objective and reproducible manner, which would be an important addition to cancer complementary and alternative medicine research.

While IL-6 and the acute phase response are of obvious importance to cancer and the effect of complementary and alternative therapies on both a tumor and a patient's symptoms, there are other pathways that can be interrogated in a similar way. For example, a key mediator of physiologic inflammation is tumor necrosis factor (TNF) and related cytokines. These signal largely through the transcription factor NF-κB. Analogous NF-κB-dependent luciferase reporter systems have been generated to measure the effects on this pathway. This could be useful as a biomarker to identify patients likely to respond to therapies with anti-inflammatory properties, as well as a pharmacodynamic biomarker to measure the response to therapy.

The activation of innate immune pathways may occur in cancer and as a result of systemic therapies. Among the cytokines central to this response are the interferons, which are known to induce fatigue, fever, anorexia, and other systemic symptoms. A key intracellular mediator of interferons is STAT1. Thus, a STAT1-dependent reporter system could be extremely useful in dissecting how complementary and alternative treatments might affect these pathways and may serve as a biomarker for responsiveness to these treatments.

Present Status of Biomarker Studies in Cancer CAM Clinical Trials

Evaluation of promising anti-cancer natural products discussed in this article, particularly, PHY906, honokiol, bryostatin-1, and sulforaphane registered in the www.clinicaltrials.gov showed incorporation of 16 laboratory biomarker studies in 54 cancer clinical trials but there were no follow-up published reports on these biomarker studies or biomarker applications (Table 1) in cancer CAM research and clinical applications. Cancer clinical trials that incorporated biomarker studies are 6 PHY906 clinical trials of which one

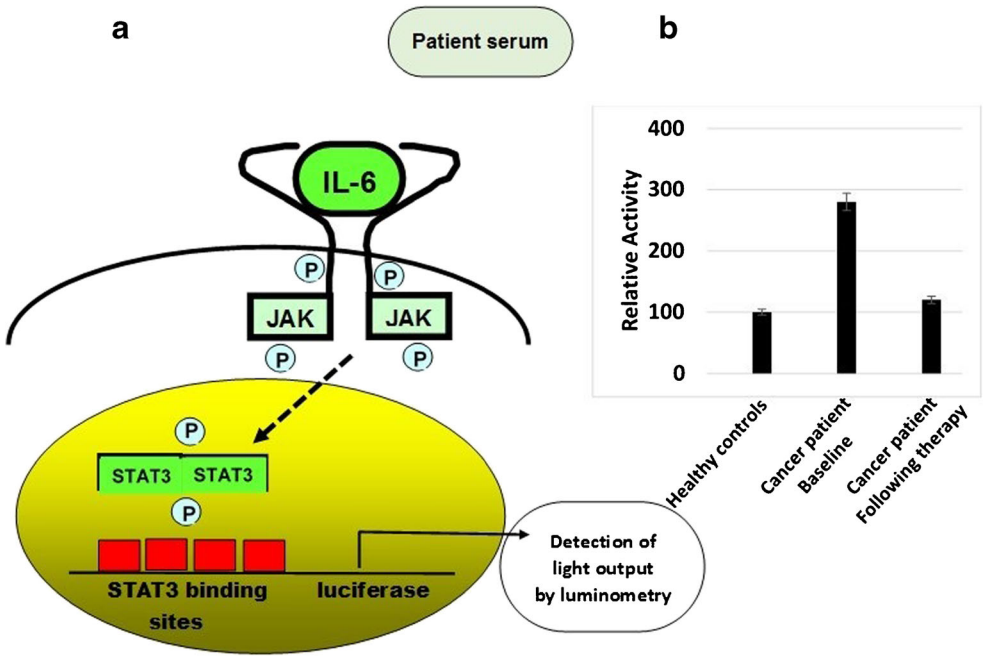

Fig. 2 A cell-based bioassay can provide integrated biomarkers to predict response and monitor the efficacy of complementary and alternative therapies. **a** A cell-based luciferase reporter assay can integrate signals from biological fluids like serum to determine how they modulate key pathways, like the acute-phase response mediator STAT3. **b** Representative data are presented to model how such a system can measure the activity of a physiologically relevant circulating biomarker in a cancer patient relative to a healthy control population. Elevation of STAT3-dependent signaling, in this example, might identify patients likely to respond to a particular complementary or alternative pathway. Longitudinal measurements could then be used to rigorously quantitate the physiologic response to the therapy

Table 1 Clinicaltrials.gov registered cancer clinical trials and biomarker studies in the USA on natural products emphasized in this article (search date: October 2, 2018)

Natural product	Number of clinical trials	Number of laboratory biomarker studies	Clinical application of CAM biomarkers reported
PHY906	6	1	None
Honokiol and Magnolia extract	None on cancer treatment; two trials for treating dental caries	0	N/A
Bryostatin-1	35	6	None
Sulforaphane	13	9	None
	54 (total)	16 (total)	

trial incorporated biomarker studies. Likewise, out of 35 bryostatin-1 clinical trials, 6 incorporated laboratory biomarker studies, while out of 13 sulforaphane clinical trials, 9 incorporated laboratory biomarker studies. For studies with honokiol/magnolia extract, despite centuries of use in traditional therapy and substantial literature on its molecular effects, there have been no registered cancer clinical trials (Table 1). While there are indications that a fraction of clinical trials with anti-cancer natural products have begun to incorporate laboratory biomarker studies, it is hoped that CAM investigators will be motivated to develop rigorous systematic studies with biomarkers to move this science forward.

Conclusions

There is currently a wide range of possible biomarkers that can be applicable to CAM research and clinical applications. However, a coordinated effort is warranted from academic institutions, industry, and the government to systematically develop and validate clinically relevant cancer biomarkers and to define their applicability in cancer prevention, guiding diagnosis (prognosis, prediction relative to CAM), treatment (dose and schedule guidance), and examining treatment response/resistance for cancer patients considering or undergoing CAM treatment.

It has been acknowledged that there are several important challenges with anti-cancer natural products, including inadequate specificity of sources, biological variability and heterogeneity of starting material, and the presence of mixtures of a variety of inert and therapeutic activity compounds. Application of standard techniques, such as chemical purification, synthetic chemistry, and cellular pharmacology in in vitro, preclinical, and clinical studies, are essential to understanding the activity of natural products. In this regard, systematic studies with PHY906 including quality control studies have provided a good model on how this natural product was processed through a rigorous path of preparation in a cGMP facility to maintain specificity and reproducibility for therapeutic applications.

It is conceivable that the lessons learned from the anti-cancer natural product studies, particularly PHY906, can be used to guide investigators interested in the pre-clinical and clinical development of herbal medicines. These are (a) quality control to ensure the availability of reproducible and validated high-quality herbal medicine for pre-clinical and clinical studies; (b) in vivo animal studies to establish biological activity, safety profile, and potential dosing schedules; (c) well-designed molecular studies for drug target(s) identification and validation; (d) a systems biology approach to investigate potential mechanisms of action; (e) characterization of in vivo metabolism of specific herbal medicine to determine formation of active and inactive metabolites; (f) a rigorous design of clinical trials with well-defined endpoints using cGMP guided herbal medicine preparation to determine clinical activity; (g) incorporation of translational pharmacodynamic (PD) biomarkers in clinical trials; and (h) a comprehensive bioinformatics analysis to identifying therapeutically relevant PD biomarkers and potential bioactive component(s) to develop a herbal medicine starting from laboratory to clinic.

In summary, strategies that incorporate an understanding of molecular mechanisms of action of complementary and alternative therapies, with those that employ more open-ended, physiology-based approaches, have the potential to yield new, rigorous, and quantitative strategies to assess the effects and impacts of these cancer CAM therapies. Importantly, understanding molecular mechanisms of action in conjunction with the identification of rational biomarkers for sensitivity and response are equally important to fully exploit these valuable compounds and to benefit cancer patients seeking CAM treatment.

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Compliance with Ethical Standards

Conflict of Interest Aniruddha Ganguly declares that he has no conflict of interest.

David Frank has received research funding from Gilead and Cstem; has received compensation from Kymera for service as a consultant; has a patent issued, licensed, and receives royalties for STAT Modulators; and has a patent pending for targeting the transcription factor NF-kB with harmine.

Nagi Kumar declares that she has no conflict of interest.

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Edward Chu is a member of the scientific advisory board of Yiviva.

Human and Animal Rights and Informed Consent All reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards (including the Helsinki declaration and its amendments, institutional/national research committee standards, and international/national/institutional guidelines).

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