

## Commentary

# Development of Dietary Phytochemical Chemopreventive Agents: Biomarkers and Choice of Dose for Early Clinical Trials

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### Abstract

In view of safety concerns surrounding the use of pharmaceuticals such as nonsteroidal anti-inflammatory drugs and tamoxifen as cancer chemopreventive agents, potentially innocuous phytochemicals derived from the diet are considered attractive alternatives. However, results from cancer chemoprevention trials of dietary agents have been disappointing to date, as promising activities observed in rodent models and cells *in vitro* have not translated into clinical success. This may be partly due to the development process for these agents, which is complex for a number of reasons; the definitive end point, inhibition of carcinogenesis, requires large numbers of individuals followed-up over many years. Furthermore, whereas biomarkers are frequently used as surrogate efficacy end points to expedite the process, biomarker assessment and validation has proven difficult because dietary agents exert multiple actions with an unknown hierarchy of biological importance. These factors have made determining the dose for clinical investigation extremely challenging, and at present, there are no defined strategies for rationally identifying the most appropriate doses. In this commentary, the complexities involved in the development of dietary chemoprevention agents are discussed, and a tentative route towards selection of the optimal clinical dose is proposed. The approach highlights the need to conduct long-term preclinical studies with realistic concentrations that are achievable in human tissues and the importance of efficacy biomarkers that are intrinsically linked to the key mechanisms of action. A more logical design of studies should increase the likelihood that the encouraging preclinical results observed for many phytochemicals translate into tangible clinical benefit.

In the light of safety concerns posed by pharmaceutical agents such as nonsteroidal anti-inflammatory drugs and tamoxifen, potentially innocuous phytochemicals derived from the diet are considered attractive alternatives for development in cancer chemoprevention. Resveratrol, genistein, folate, and curcumin are examples of dietary agents which have undergone extensive mechanistic and preclinical efficacy investigation (1), yet no such agents have been approved for routine cancer chemoprevention. One reason is that their clinical development is at least as complex as that of molecularly targeted drugs. New anticancer drugs are designed to act on specific targets, whereas dietary agents exert a plethora of actions with an unknown hierarchy of biological importance. Furthermore, the end point of definitive clinical studies of chemopreventive agents is challenging, as the ultimate proof of efficacy, inhibi-

tion of carcinogenesis, requires large numbers of individuals followed-up over many years. Results from several such clinical trials of dietary phytochemicals conducted to date have been disappointing because promising activities observed in rodent models and cells *in vitro* have not translated into clinical success. Indeed,  $\beta$ -carotene supplementation caused unexpected detrimental effects in humans, which is inconsistent with the epidemiologic data that suggested it might be of value in the prevention of lung cancer (2, 3).

In an effort to expedite and facilitate the development of dietary agents in chemoprevention, biomarkers are frequently used as surrogate end points of efficacy. Ideally, such markers of pharmacologic efficacy should be intrinsically related to the mechanisms of action responsible for chemopreventive effects. However, the assessment and validation of appropriate efficacy biomarkers has been equally difficult, as the chemopreventive relevance of the numerous mechanisms which these agents engage is unclear. In turn, the determination of an appropriate dose for clinical investigation has been complex as measurement of the dose-response relationship for efficacy, particularly in the short to medium term, is controversial. In this commentary, some of the complexities involved in the development of dietary chemoprevention agents are discussed, and a tentative route towards identifying the optimal dose to be administered in clinical trials is proposed.

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## Development of Dietary Chemoprevention Agents to Date

There is no consensus for how to determine appropriate doses of dietary phytochemicals in clinical chemoprevention studies despite the fact that this conundrum was first raised more than 10 years ago (4). Most often, supradietary doses, inconsistent with the epidemiologic data on which the choice of agents is founded, have been administered. These doses tend not to correlate with available preclinical efficacy and pharmacokinetic data. For example, the absorption of oral vitamin C has been shown to plateau at 400 mg/d (5), yet several clinical trials have administered doses higher than this even after the publication of these findings (6–8).

Some preclinical studies have shown that supradietary doses of phytochemicals may be harmful. Genistein and  $\beta$ -carotene for example, exhibited chemopreventive properties at low doses but were procarcinogenic at high doses both *in vitro* and in animal models of cancer *in vivo* (9, 10). Four lung cancer chemoprevention trials of  $\beta$ -carotene supplementation have been carried out to date, recruiting over 100,000 subjects in total; although no difference was noted in two studies (11, 12), supplementation significantly increased the risk of lung cancer incidence in the other two trials (2, 3). Some authors have postulated that the increased risk of lung cancer noted in the smoker cohort recruited into the Beta-Carotene and Retinol Efficacy Trial was due to an inappropriately high dose (2, 13). Clinical data suggest that the dose-response relationship for some dietary phytochemicals can be nonlinear. A nested case control study of colorectal patients with matched references showed that colorectal cancer risk was a bell-shaped curve in relation to folic acid levels, with decreased risk of disease in the lowest and highest quintiles (14). A fasting blood sample was taken from each participant in this study for folate analysis but there was no dietary intervention.

In some trials, phytochemicals have been administered at dietary doses as constituents of food. Folic acid and genistein, for example, have been ingested in the form of spinach (15) and bread rolls (16), respectively. These trials afforded relevant pharmacokinetic information as the dose administered was clinically feasible, but not efficacy data in terms of either beneficial or detrimental effects on biomarker end points. Studies of this type can be difficult to interpret due to the presence of multiple agents with potential pharmacological activity and the food matrix, which may influence absorption and pharmacokinetics. Ascribing chemopreventive efficacy to an individual food component is therefore potentially more complex than for single purified agents.

The choice of efficacy biomarker in clinical dose finding studies is also challenging as many biomarkers have been used to date without being validated in terms of correlation to chemopreventive activity and/or clinical efficacy, which means that their significance is unclear. On the basis that antioxidation is an important mechanism via which many phytochemicals may exert chemopreventive activity, malondialdehyde-deoxyguanosine ( $M_1dG$ ) for example, has been used as a surrogate efficacy biomarker in clinical studies with curcumin in patients with colorectal cancer (17, 18).  $M_1dG$  is a type of oxidative damage to DNA formed endogenously by the reaction of deoxyguanosine with the peroxidation products of either lipids (malondialdehyde) or DNA (base propenal).  $M_1dG$  is mutagenic in mammalian cells (19) and

may therefore be a contributing factor in cancer initiation and subsequent mutation accumulation during the promotion phase of carcinogenesis. Consequently, because analysis of  $M_1dG$  can provide a quantitative measure of cellular oxidative DNA damage, this lesion has been investigated as a potential efficacy biomarker for chemopreventive agents that might reduce the generation of reactive oxygen species.  $M_1dG$  has been found in liver, breast, colon, lymphocyte, and pancreatic tissues of healthy individuals at 1 to 120 adducts per  $10^8$  nucleotides (20). Increased levels have been shown to correlate with gender, diet, age, alcohol, smoking, and body mass index (21). Results from animal studies have been promising; curcumin decreased  $M_1dG$  levels by 39% in  $Apc^{Min+}$  mouse intestinal adenomas (22). Furthermore, when administered for a week to patients with colorectal cancer, curcumin significantly reduced  $M_1dG$  in colorectal neoplastic tissue compared with preintervention levels (18). However, the value of this adduct as a chemopreventive end point is currently still unclear as it may reflect variables other than efficacy.

Due to the lack of validation data demonstrating a clear correlation with chemopreventive activity and mechanistic understanding, the conditions under which efficacy biomarkers should be used are not well understood. Vitamin C, for example, has been shown to be an antioxidant (23) and as such should theoretically decrease the levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), another DNA adduct which is a marker of oxidative damage. In clinical chemoprevention trials in healthy volunteers, however, vitamin C decreased the levels of cellular 8-oxodG in some studies (6, 7) but not in others (8). These studies administered vitamin C at different doses for different durations and measured the levels of 8-oxodG in different biofluids including sperm and blood. It is unclear whether the variability in 8-oxodG levels may reflect these different conditions or the fact that it is an inconsistent biomarker.

## Proposed Development Plan for Identifying the Optimal Clinical Dose of Dietary Chemopreventive Agents

In order to determine the optimal clinical dose of dietary chemopreventive agent to be administered, *in vitro* and animal studies must provide an indication of the efficacious dose range and the surrogate end point against which efficacy can be measured. Typically, the first stage in the preclinical development of dietary chemopreventive agents comprises *in vitro* screening in cell lines. Numerous phytochemicals and vitamins have been shown to interfere with carcinogenic pathways in different cell types, but in most cases, the hierarchy of importance of these mechanisms is not understood. It is therefore difficult to mine data arising from such experiments for the identification of possible biomarkers. If, however, the relative mechanistic importance of the different effects could be established, this should allow more accurate predictions of the most relevant molecular markers to be made.

Difficulties in translating *in vitro* responses to clinically useful biomarkers are further complicated by the issue of dose and treatment durations employed in these studies. It has long been known that phytochemicals exert chemopreventive efficacy in rodent models at doses achieving submicromolar ( $<10^{-7}$  mol/L) concentrations in the biophase, yet *in vitro*

investigations typically make use of higher concentrations (in the  $10^{-5}$  mol/L range) to elicit biochemical changes consistent with chemopreventive efficacy (24). Resveratrol showed a biphasic effect in endothelial progenitor cells *in vitro* on a pathway potentially germane to carcinogenesis: at 1  $\mu$ mol/L, it increased nitric oxide synthase expression, whereas at 60 nmol/L, it decreased expression. This biphasicity was confirmed in a murine model of aorta repair *in vivo*. Resveratrol administered at 10 mg/kg increased endothelial nitric oxide synthase expression in injured arteries and increased the number of endothelial progenitor cells in the circulation (25), but a higher dose of 50 mg/kg, failed to elicit these responses, illustrating that supradietary doses may exert effects very different to those elicited by dietary doses. Consequently, it is conceivable that a particular biochemical event studied *in vitro* is actually not important for chemoprevention in the tissue in which malignancy is to be prevented. Ultimately, in the clinical setting, chemopreventive agents will be taken long-term. This scenario contrasts with the design of the vast majority of *in vitro* cell-based studies reported, which traditionally involve treatment for periods of hours or days. Short-term experiments are practically easier to conduct and the mechanisms identified may be the same as with chronic exposure, but it is also possible that important mechanisms may be overlooked, or results might be misleading, if elicited chemopreventive effects require a long time period to become manifest. Regardless of the treatment duration, the design of such *in vitro* experiments must ensure that the relevant agent is administered, which may be a metabolite rather than the parent compound, and that the dosing frequency takes into account the stability of the agent in the medium.

The apparent discrepancy between short-term and long-term exposure is exemplified by folic acid in colorectal cancer. Folic acid at concentrations of 0.6 to 10  $\mu$ g/mL inhibited DNA synthesis and induced cell cycle arrest when incubated with Caco-2 colon cancer cells *in vitro* for 48 hours (26). In rats, dietary supplementation with folic acid (8 mg/kg/d) from 4 weeks of age significantly increased the incidence and burden of azoxymethane-induced small and large intestine tumors compared with the folate-deficient group (27). Folate analysis of whole blood in the folate-supplemented or folate-deficient rats yielded levels of 0.7 and 0.1  $\mu$ g/mL, respectively. These data show that the exposure of colonic cells to folate at similar concentrations produced opposing effects, with antiproliferative action in the *in vitro* paradigm but accelerated tumor growth in rats *in vivo*.

Dietary chemopreventive agents have been administered to animals mostly via the oral route, in the diet, or via gavage, which mimics the route of human intake. Extensive *in vivo* data supports the chemopreventive efficacy of dietary agents in rodent models of carcinogenesis (1). For example, resveratrol has been shown to delay tumor development in murine models of gastrointestinal (28), mammary (29), and lung cancer (30). Once the orally effective dose, defined as a dose which delays or prevents the development of malignancy, has been established in animal models, *in vitro* studies can be designed to tease out mechanisms intrinsically linked to chemoprevention, rather than mechanisms which may turn out to be collateral. Key components of the affected chemopreventive pathway identified may then be exploited as putative biomarkers. It seems logical that in such experiments, cells should be

chronically exposed to the phytochemical under study for months rather than days, at concentrations commensurate with those measured in the target tissue *in vivo*. An example of such a study is the exposure of endothelial progenitor cells to resveratrol at a dietary dose of 29 nmol/L for 20 days, which resulted in decreased cell number and increased cellular nitric oxide levels (31). Although this study addressed the effects of resveratrol on cardiovascular disease, it shows that resveratrol can modulate a pathway linked to carcinogenesis at a concentration unable to exert biochemical changes when investigated in short-term cell cultures.

This experimental approach requires knowledge of pharmacokinetics in the rodent at the efficacious dose, but such information is often not obtained in the same animals used for the efficacy studies, making it difficult to correlate the variables of dose and target tissue concentration with chemopreventive activity *in vivo*. An illustration of the necessity for detailed pharmacokinetic data in understanding the mechanisms of action and dose optimization comes from a study on  $\beta$ -carotene, which exerted a biphasic efficacy profile in the azoxymethane-induced colon carcinogenesis rat model (10). Doses <200 ppm (~0.6 mg  $\beta$ -carotene/kg rat weight/d) were preventive, whereas doses >1,000 ppm (~3 mg  $\beta$ -carotene/kg rat weight/d) increased the incidence of aberrant crypt foci. Such biphasicity may be due to the nonlinear relationships between dose and agent or metabolite levels in the biophase. The authors proposed that these opposing effects could be due to  $\beta$ -carotene behaving as an antioxidant at low doses and as a pro-oxidant at high doses, although no explanation is given as to why this is the case.

Even when the efficacious tissue concentration is known, disentangling the mechanistic hierarchy is extremely difficult because the carcinogenic cascade involves many pathways with which chemopreventive agents can conceivably interfere. Global analyses of genomic, proteomic, and metabolomic changes induced by the phytochemical under study, after long-term exposure of cells to concentrations consistent with those seen in the target tissue after administration of active doses in rodents, may help pinpoint mechanisms high up in this hierarchy. cDNA microarray analysis of resveratrol has identified the androgen receptor pathway as a potentially relevant target for its chemopreventive properties in androgen-sensitive LNCaP prostate cancer cells (32, 33). *In vivo*, prepubertal exposure to genistein has been shown to inhibit dimethylbenz(a)anthracene-induced mammary tumors in rats (34). In an effort to elucidate the mechanisms of action, dietary genistein was administered to prepubertal rats, and the normal mammary tissue of young adults was subjected to proteomic analysis (34). Genistein was found to increase the levels of tyrosine hydroxylase and down-regulate vascular endothelial growth factor receptor 2. These changes may reflect only exposure to genistein, but the authors proposed that they could be more general efficacy biomarkers to show increased mammary gland maturation and therefore decreased tissue susceptibility to chemically induced cancer initiation, angiogenesis, and progression.

Ideally, the clinical relevance or link to the chemoprevention pathway of any efficacy biomarker identified from *in vitro* and animal studies, such as the examples cited above, need to be confirmed and validated before being used in the clinical evaluation of a chemopreventive agent. Although not concerned with chemoprevention *per se*, a recent study in a murine model of human pancreatic cancer illustrates this approach. Plasma

proteomic data from mice at different stages of tumor development were compared with results from corresponding human subjects. A panel of five proteins, found elevated at an early stage of tumor development in mice, were tested against blinded human samples and were able to confidently predict pancreatic cancer in patients up to 13 months before clinical diagnosis (35). This panel included TIMP1, which is involved in extracellular matrix degradation, REG1A and REG3, proteins highly secreted by pancreatic islet cells, IGFBP4, which is the smallest protein from the IGF binding protein family and has been related to tumor growth, and LCN2, which has been shown to regulate cell growth and metastasis in colon cancer. Although these proteins are considered to be clinical risk biomarkers, further validation studies may also reveal a possible role as efficacy biomarkers.

Proteomic, genomic, and metabonomic profile changes induced by phytochemicals in patients could potentially be used as efficacy end points which might bypass the need to understand specific mechanistic hierarchies. Proteomic analysis has predicted the different stages of ovarian (36), breast (37), and pancreatic cancer (35) in patients. Again, although these examples are currently relevant only to cancer diagnosis, they provide proof-of-principle that such profiling can help determine the different stages of carcinogenesis and therefore perhaps indicate chemopreventive efficacy. In the pancreatic and ovarian cancer studies, proteomic profiles were evaluated in plasma samples, which can be easily obtained as peripheral surrogate biomarkers. In the breast study, however, nipple aspiration and ductal lavage fluid was examined, involving procedures which can cause discomfort if repeated samples are required.

Clinically acceptable efficacy biomarkers for chemopreventive interventions should be measurable noninvasively in biofluids. Accordingly, changes in surrogate efficacy end points should correlate with alterations in the target tissue consistent with chemopreventive activity. This relationship is difficult to ascertain in humans due to the limited accessibility of tissues, and it is therefore essential that potential surrogate efficacy biomarkers are validated in animal models prior to application in clinical trials. This is particularly important as variations in many of the plasma or urine biomarkers currently under investigation have not yet been established to correlate with changes in the specific tissue of interest (38).

Once the efficacious dose in animals has been established, and validated markers of pharmacologic efficacy have been identified, pilot clinical trials can begin. Such studies should commence with a dose equivalent to the efficacious dose in animals. If this dose in humans elicits efficacy biomarker changes, the minimum dose that induces maximal biomarker response should be determined, involving dose escalation and de-escalation. If this dose fails to affect biomarkers in humans, cautious dose escalation should be considered.

## Conclusion

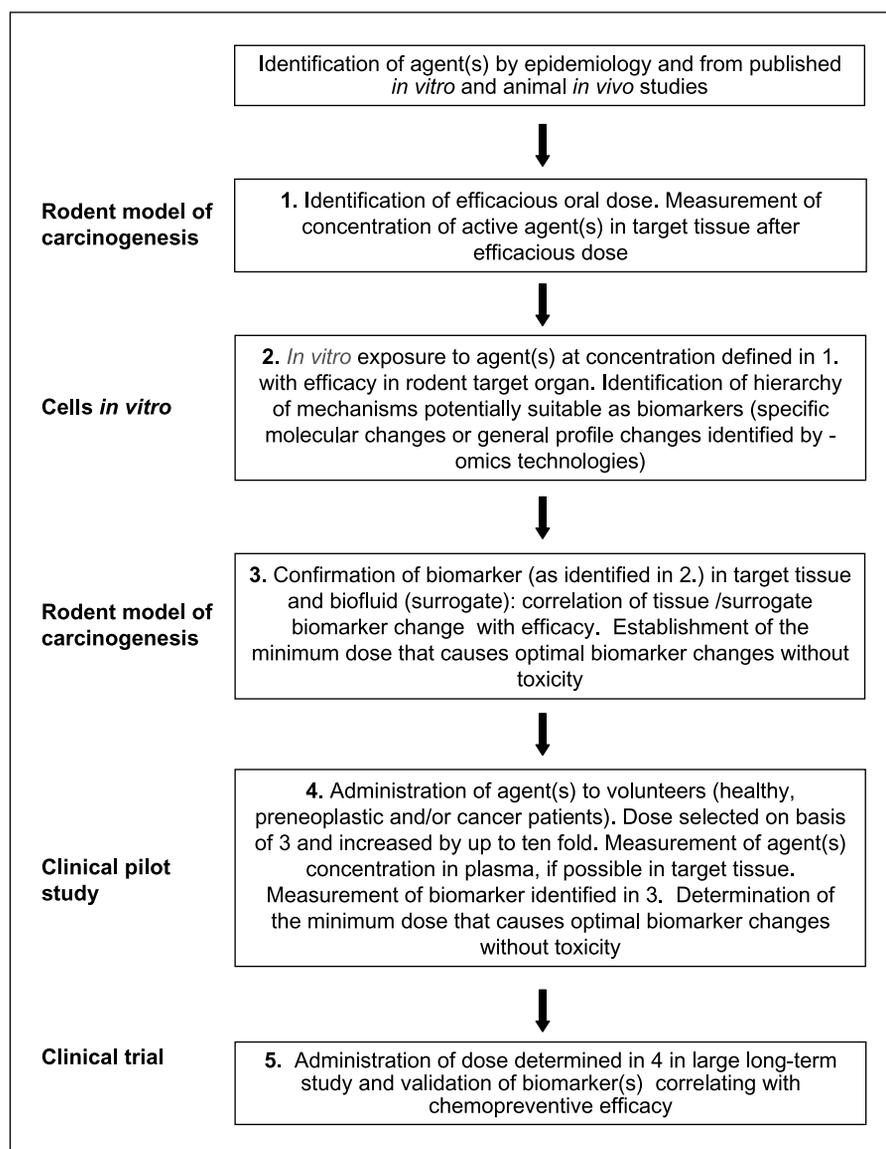
The overwhelming preclinical and clinical data published to date have used supradietary doses of dietary chemopreventive agents. The average dietary intake of genistein in Europeans for example is <1 mg/d (39), yet in a study in healthy postmenopausal women, 600 mg of genistein was administered daily for 84 days (40). The significance of data from trials at supradietary doses can only be determined with fur-

ther preclinical studies to distinguish which concentrations are clinically achievable and what end points are clinically relevant. A standardized rationale is needed for the determination of the appropriate dose to be taken forward into early clinical trials. There is little knowledge from prospective trials of the efficacy of dietary doses of agents which, based on epidemiologic data, may exert cancer chemopreventive effects.

Figure 1 describes a tentative outline of suitable steps in the development of chemopreventive phytochemicals. Initially, the minimum oral dose with proven efficacy should be identified in rodents, with efficacy defined both in terms of preventing or delaying the onset of malignancy and inducing optimal biomarker changes. This dose must be safe in animals as any side effect would be unacceptable in a human chemoprevention setting. The mechanistic hierarchy engaged by the agent should be characterized to allow the identification of relevant surrogate efficacy end points. Such characterization might be achieved in cells *in vitro* at the concentrations previously determined in rodent studies as being active target tissue levels. In addition to the application of long-term culture studies in developing dietary chemopreventive agents, it might be more important to incorporate the measurements of putative efficacy biomarkers identified *in vitro* (even in short-term culture experiments or molecular studies) early on in *in vivo* rodent studies. Parallel experiments can be done to define the roles of the putative efficacy biomarkers in carcinogenesis. This approach should help identify more relevant efficacy biomarkers for early phase clinical studies.

Which cell types are the most suitable for use in such *in vitro* experiments is debatable. Malignant cell lines have been widely used in chemoprevention studies in the absence of alternatives with preneoplastic properties. Benign immortalized cell lines tend to lack malignant phenotypic features vital to detect chemopreventive events, whereas malignant cells are likely to have different genomic and proteomic patterns compared with early neoplastic or normal cells. The use of any such cell type seems potentially suitable, irrespective of whether it is malignant or transformed, as long as the connection between the hypothesis to be tested and the biological nature of the cells is robust; however, multiple cell lines must also provide a consensus outcome. Global proteomic, genomic, and metabonomic changes might help identify mechanistic hierarchy and pinpoint relevant biomarkers which need to be confirmed and validated in animal models before use in clinical trials. Alternatively, these global changes may themselves be used as efficacy biomarkers if they can be correlated with the desired activity, thereby potentially overcoming the need to identify mechanistic hierarchy. Dose escalation or de-escalation should be considered depending on efficacy biomarker response to the initial dose, as outlined above.

The scheme proposed here, with emphasis on detailed preclinical studies, is undoubtedly costly, but this cost is arguably insignificant compared with the cost of performing clinical studies at inappropriate doses. In the context of targeted anticancer drug development, it has been proposed that "much of the success of targeted drug development rests on high-quality basic science", encompassing the "need to develop biomarkers that can be used early in the clinical development process" (41). In this article, costs per patient were estimated to approach US \$7,000. These points are probably equally pertinent to the development of dietary chemoprevention agents. The difference



**Fig. 1.** Proposed steps in the development of dietary chemopreventive agents.

is the lack of funding by trial sponsors in the case of dietary agents.

The scheme suggested here may help to overcome the consequence of poor oral bioavailability, a property of many phytochemicals (42), by employing conditions determined in rodent studies using the oral route of administration. It does not, however, resolve the conundrum of how to extrapolate agent dose from one species to another. For chemotherapy drugs, the starting dose for phase I studies in humans has traditionally been calculated as 10% of the lethal dose ( $LD_{10}$ ) in rodents with dose escalation until unacceptable toxicity is seen. This is not applicable in the chemopreventive setting as any toxicity would be unacceptable in healthy individuals. The dose of dietary chemopreventive agent to be investigated in clinical trials should therefore not be the maximum tolerated dose, but rather, the minimum dose that can induce the optimal biomarker response without any adverse effects as outlined above. Recommendations from the U.S. Food and Drug Administration state that dose conversions between species should be based on body surface area (43), as this is more

accurate than conversions based on body weight alone (44). Choosing an appropriate dose is complicated by the fact that for some dietary constituents, efficacy is linked to baseline nutrient levels, with individuals who are deficient benefiting more than those who are well nourished. Vitamin C for example, decreased the risk of prostate cancer development in individuals with a baseline level of  $<49 \mu\text{mol/L}$ , but increased the risk in participants with higher baseline concentrations of  $\geq 49 \mu\text{mol/L}$  (hazard ratio, 0.52 versus 1.48, respectively; ref. 45).

Skepticism abounds as to whether we will ever be able to translate the encouraging cancer chemopreventive efficacy shown preclinically by some dietary phytochemicals into tangible clinical benefit. Perhaps a rational design of studies aimed at defining the "right" doses, taking some of the considerations discussed above into account, may ultimately aid to confound this skepticism.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Cancer Prevention Research

## Development of Dietary Phytochemical Chemopreventive Agents: Biomarkers and Choice of Dose for Early Clinical Trials

Edwina N. Scott, Andreas J. Gescher, William P. Steward, et al.

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