

Mechanisms of Cancer Chemopreventive Agents: A Perspective

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- Chemoprevention
- Resveratrol
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Abstract

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A fundamental question addressed by drug development programs is how agents being tested function on a molecular level. Using resveratrol, curcumin and EGCG as examples, it is clear that a definitive mechanism of action for cancer chemopreventive agents is not available despite decades of exhaustive research. This is profoundly evident based on the myriad of biological responses that have been observed at the cellular level, and even more overwhelming when considering gene expression data that are now available. The situation is confounded further when chemopreventive agents are used in combination, even though superior clinical responses are antici-

ated. The best hope for delineating tangible, meaningful mechanisms resides in the use of complex physiological systems and computer models to decipher the most critical pathways that are appropriate for targeting with chemopreventive agents, their analogues, and combination treatments. Definitive answers concerning clinical efficacy are only available through human trials. Given the enormity of these tasks, together with the urgency of continuing the fight against cancer, it is adequate to move ahead with chemopreventive drug development on a semi-empirical basis, bearing in mind the importance of limiting toxic side effects.

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Introduction

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For individuals less than 65 years of age, cancer is now the leading cause of death in the United States [1]. Since this disease may stealthily progress for a decade or more prior to diagnosis, and only limited routine and robust early diagnostic markers are known, at risk individuals are advised to take preventive measures. Some cancer chemoprevention agents are available having the ability to prevent, delay, or reverse the risk of cancer development and/or progression. Those approved by the U.S. FDA include selective estrogen receptor modulators (SERMS), aromatase inhibitors, and celecoxib. In the general population, a diet highly enriched in fruits and vegetables may have preventative value; examples of natural products that show promise as cancer chemopreventive agents include epigallocatechin gallate (EGCG), capsaicin, resveratrol, curcumin, 6-gingerol, and lycopene [2], [3], [4].

Chemopreventive compounds can be identified in various ways. Some are isolated from plants with known medicinal properties, largely gleaned from

epidemiological studies, while others are identified in massive screens of libraries of randomly collected samples. Irrespective of the method of identification, compounds with chemopreventive promise are ultimately purified and subjected to structure elucidation. Although structure identification is an important first step, it usually does not provide an explanation as to why a particular compound is active. Answering this question is exceedingly complex.

Considering pharmaceutical agents that have been discovered throughout history, only a few are believed to have a clearly defined mechanism of action. For example, penicillin inhibits the formation of peptidoglycan cross-links in bacterial cell walls, 5-fluorouracil serves as a pyrimidine analogue, taxol stabilizes tubulin, tamoxifen is an SERM, and methotrexate interferes with one-carbon metabolism. Identifying a clearly defined mechanism of action for the majority of drugs, however, is the equivalent of finding a single needle in multiple haystacks, all equally complex. In most cases, the very composition of each haystack is largely uncharacterized or even yet undiscovered.

The complexity of mechanistic definition is particularly apparent with cancer chemopreventive agents. As described herein, the anticancer effects observed from a single chemopreventive agent are the outcome of a combination of several distinct sets of intracellular effects, rather than limited to one established biological pathway. To elaborate on the mechanistic complexity of chemopreventive agents, as examples, we have surveyed the reported mechanisms of action of three compounds: resveratrol, curcumin, and EGCG. Presently, it is clear that a meaningful sequence of critical mechanistic events cannot be defined in a straightforward manner. These three chemicals also exemplify the structural diversity of chemopreventive agents (● Fig. 1).

Resveratrol

Resveratrol is a stilbene that is found in several plants, the primary dietary source being grapes. It can function as a chemopreventive agent capable of inhibiting all stages of cancer development. Modes of action identified for resveratrol include induc-

tion of phase II drug-metabolizing enzymes, inhibition of cyclooxygenase (COX), and cellular differentiation [5]. Resveratrol inhibits cytochrome P450, cell invasion, transformation, and angiogenesis [6]. Resveratrol has been shown to up-regulate antioxidant enzymes, such as glutathione peroxidase, catalase and quinone reductase. It inhibits lipid peroxidation, ornithine decarboxylase (ODC), protein kinases, and cellular proliferation [7]. Resveratrol effectively induces apoptosis modulated through multiple pathways including up-regulation of p53, activation of caspases, decreases in Bcl-2 and Bcl-x_l, increases in Bax, inhibition of D-type cyclins, and interference with NF-κB and AP-1 mediated cascades [8].

A multitude of *in vitro* and *in vivo* studies implicate resveratrol in a large web of anticancer pathways (recently reviewed in [8], [9]). Resveratrol treatment resulted in growth arrest at G1 and G1/S phases of the cell cycle by inducing the expression of p21 and p27 [10]. It reduced inflammation via inhibition of prostaglandin production and COX-2 activity. Resveratrol has been shown to regulate cathepsin D, inhibit hypoxia-induced protein, and down-regulate telomerase. Resveratrol pretreatment suppressed activation of ERK2, JNK and p38 in association with inhibition of protein kinase C (PKC) and protein tyrosine kinase [11]. Resveratrol blocked activation of NF-κB through suppression of IκB activation, inhibited activation of MEK, and abrogated TNF-induced caspase activation.

Curcumin

Curcumin (diferuloylmethane), a yellow pigment from the rhizomes of turmeric, has been recognized as a chemopreventive agent due to its antitumor, antioxidant, antiproliferative, and proapoptotic effects. Curcumin suppresses transformation, proliferation, angiogenesis, and metastasis. Similar to resveratrol, curcumin mediates its anticancer effects through regulation of various transcription factors, growth factors, inflammatory cytokines, and protein kinases (reviewed in [12]).

Many mechanisms of action for curcumin have been identified, including modulation of the expression of genes involved in proliferation, apoptosis, invasion, metastasis, angiogenesis, and resistance to chemotherapy [13], [14]. Curcumin inhibits cell-cell adhesion and blocks cell cycle transition from G2 to M [15]. It suppresses cytochrome P450 and decreases P-glycoprotein expression [16], [17], [18]. Curcumin inhibits the catalytic activity of ERK1/2, activates caspases 8 and 3, down-regulates cyclin D1, suppresses the activation of NF-κB, AKT-PI3K, AP-1, STATs, TNF, MAPK, PKC, and modulates the expression of PPAR-γ, β-catenin, and Nrf-2 [13], [19], [20], [21], [22]. Curcumin inhibits histone acetyltransferase [23] and down-regulates the expression of p53, EGR-1 and c-myc [24]. Curcumin treatment activates proapoptotic members of the Bcl-2 family and reduces the activity of EGFR and *HER2/neu* [25]. Curcumin up-regulates enzymes such as catalase, glutathione transferase, glutathione peroxidase, and superoxide dismutase (SOD). Curcumin inhibits production of IL-8 by tumor cells and augments the cytotoxic effects of chemotherapeutic drugs [26].

Epigallocatechin Gallate (EGCG)

EGCG is an antioxidant polyphenol that is found in green tea. It exhibits a wide variety of anticancer properties, including inhibi-

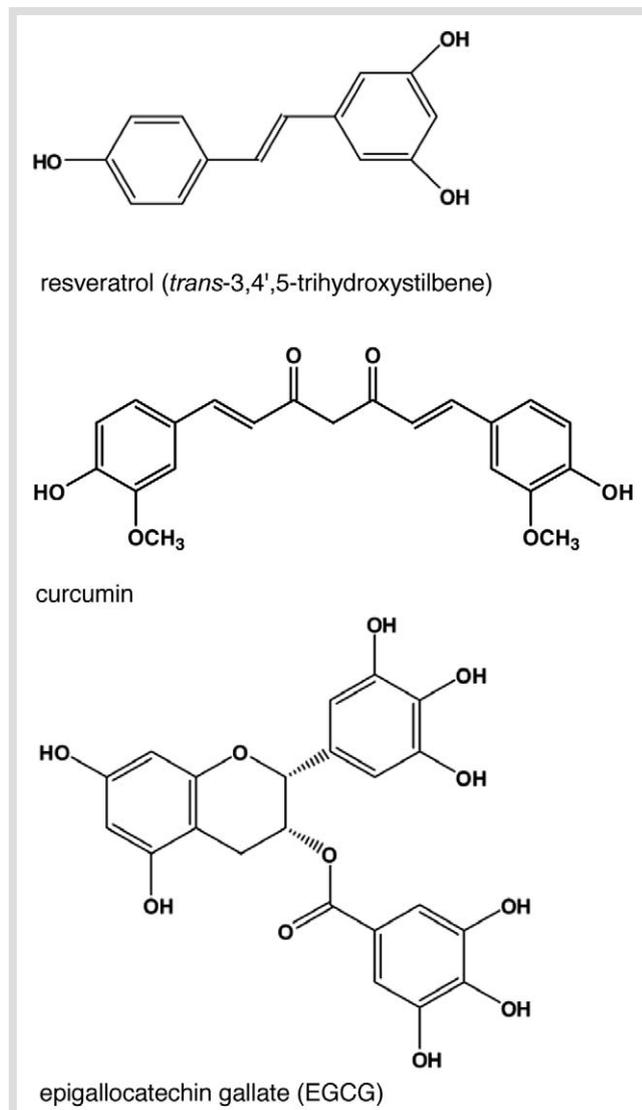


Fig. 1 Structures of resveratrol (*trans*-3,4',5-trihydroxystilbene), curcumin, and epigallocatechin gallate (EGCG).

ition of extracellular mitotic signals, inhibition of the cell cycle at G1 phase, suppression of iNOS, and induction of apoptosis (reviewed in [27]). EGCG has been reported to inhibit invasion and angiogenesis, processes that are essential for tumor growth and metastasis [28]. Similar to resveratrol and curcumin, mechanisms contributing to the anticarcinogenic and antimutagenic effects of EGCG include antioxidant activity, induction of phase II enzymes, blocking carcinogen formation, inhibition of carcinogen binding to DNA, and inhibition of DNA synthesis and cell proliferation [29], [30], [31]).

EGCG has been reported to act through a myriad of mechanisms [27]. It suppresses MMP-9 secretion and phosphorylation of focal adhesion kinase and ODC activity. EGCG activates caspases 3 and 9, SOD activity, and catalase activity. EGCG inhibits DNA synthesis [29], MAPK signaling [32], NF- κ B activity, STAT3, PI3K activation [33], [34], raf, MEK, ERK, JNK, p38 kinase, EGFR, and I κ K [27]. EGCG reduces signaling via P13K-AKT-NF- κ B mediated through inhibition of ERBB2 receptor tyrosine phosphorylation [35]. EGCG causes G0/G1-phase arrest and induction of apoptosis in human epidermoid carcinoma cells, but not in normal human epidermal keratinocytes, indicating that at least some of its antiproliferative effects are cancer-cell specific [36].

Microarray Analyses

▼ Of the chemopreventive agents examined above, several targets are modulated by each of the three, such as NF- κ B. Should we conclude that NF- κ B is a critical target? Another possibility is there is investigator bias towards examining effects on the NF- κ B pathway, and other, possibly undiscovered, targets are not being considered. Perhaps hypothesis-driven research is limiting the ability to discover the unexpected, and we are only discovering mechanisms that we are seeking. One way to limit investigator bias is to perform microarray studies, which have also been used to confirm existing hypotheses. The results from selected microarray experiments involving resveratrol, curcumin, and EGCG are summarized in ► **Table 1**. Column three lists how many genes exhibited a greater than two-fold up- or down-regulation, although it should be recognized that lesser-fold changes may also have biological significance. Columns four and five contain a sampling of commonly recognized genes. In the first example, microarray analyses were performed to identify genes that are regulated by resveratrol in androgen-sensitive prostate cancer cells (LNCaP), human ovarian cancer cells (PA-1), and renal cell carcinoma cells (RCC54). The most com-

Table 1 Microarray results from selected studies involving treatment with resveratrol, curcumin, or EGCG

Chemopreventive Agent	Cell Type, Dose, Time	2× Change	Genes Up-Regulated	Genes Down-Regulated	Ref
Resveratrol	LNCaP 100 μ M 48 h	555 of 2400	P300 (5.09) Glutathione transferase (2.91) Bak protein (2.14) Pig7 (2.27) CRABP II (2.1)	PPAR (0.3) NF κ B p65 (0.47) Phospholipase D (0.31) TGFB (0.12)	[10]
Resveratrol	LNCaP 100 μ M 24, 48 h	553 of 2,400	PIG7 (2.23) Bak (2.16) p21 (2.7) p300 (5.09) Apaf-1 (4.4) Glutathione reductase (2.9)	PSA (0.10) ARA 24 (0.01) NF κ B p65 (0.47) PPAR (0.3)	[37]
Resveratrol	LNCaP 75, 150 μ M 0 – 60 h	1,600 of 42,000	Quinone reductase Phase II enzymes	PSA AR Cyclins D, E, A, B	[38]
Resveratrol	RCC54 25, 50 μ M 24 h	633 of 2059	GADD45 (3.07) CRABP II (3.28) TRAF-1 (1.58) Protein-tyrosine phosphatase (1.4) Rb binding protein 1 (4.7)		[39]
Resveratrol	PA-1 50 μ M 24 h	118 of 7448	NQO-1 (12.4) p21 (4.6)		[40]
Curcumin	ECV304 1 μ g/mL 24 h	27 of 2400	p21WAF1/CIP1 p53	cyclin B1 cdc2	[41]
Curcumin	CL1 – 5 10 μ M 24 h	152 of 9600	Hsp27 (2.78) Hsp70 (3.75) Hsp40-like protein (3.21)	MMP14 (0.65) Neuronal cell adhesion molecule (0.54) Integrin α 6 (0.67) Integrin β 4 (0.63)	[42]
Curcumin	MCF-7 25, 50 μ g/mL 24 h	104 of 214	TRAF6, GADD45, BCL2L2, PIG11, PIG3, PCNA, CDC10, JNK1, RBP2	TRAIL, TNFR, AP13, IGFBP3, PKB, IGFBP, TRAIL-R2, TNF β	[43]
EGCG	LNCaP 12 μ M 12 h	25 of 250	Protein-tyrosine phosphatase	PKC alpha PI3K homolog	[44]

prehensive of these studies identified 1,600 genes that were up- or down-regulated more than two-fold following a single treatment with resveratrol. Changes were reported in genes regulating apoptosis (Bak, Apaf-1), differentiation, signal transduction (CRABP II, TRAF-1), proliferation (protein tyrosine phosphatase), transcription factors, cell adhesion, tumor suppression (Rb binding protein), cell cycle (p300), growth factors (TGF β , GADD45), p53 (PIG7, NQO-1, p65 NF- κ B homologue, p21), and GST (glutathione transferase). Specifically in prostate cancer cells, resveratrol induced apoptosis by activating p53 signaling mechanisms and by blocking androgen signaling pathways including prostate-specific antigen (PSA) and the androgen receptor (AR). Collectively, these results confirm that resveratrol modulates more than one set of functionally related targets.

Microarray studies performed with cells treated with curcumin or EGCG treatment yielded similarly complex results, confirming these agents modulate gene expression through multiple pathways involving hundreds of genes or more. Curcumin up-regulated cyclin dependent kinase (CDK) inhibitors, such as p21 and p27, and down-regulated cyclin B1 and cdc2 [41]. A study following curcumin treatment in a lung adenocarcinoma model indicated that several invasion-related genes were suppressed, including matrix metalloproteinase 14 (MMP14), neuronal cell adhesion molecule, and integrins. Additionally, several heat-shock proteins (hsp) were induced. Gene expression was altered up to 14-fold in breast cancer MCF-7 cells as compared to only 1.5-fold in the MCF-10A normal breast cell line following treatment, indicating that curcumin is capable of selectively suppressing the growth of cancer cells. A microarray study of kinases and phosphatases using LNCaP cells indicated that EGCG induced a subset of genes inhibiting cell growth, mostly belonging to the G-protein signaling network. PKC α , the only PKC isoform implicated in cancer, was repressed while the other PKC isoforms were not affected.

These microarray data are limited by the number of genes on the arrays themselves and the quality of the data mining analysis. Newer technologies enable single chip genome-wide expression analysis using oligonucleotide or cDNA microarrays to measure, in a massively parallel fashion, the mRNA levels of many or all genes in a genome. Such genome-wide expression analysis has successfully been used to investigate the regulatory networks controlling a variety of cellular processes in yeast [45]. However, functional interpretation of microarray data remains limited by multiple protein products of each mRNA, posttranslational modifications, protein-protein interactions, protein-DNA interactions, protein-RNA interactions, RNA-RNA interactions, and methylation state. Additionally, most genes have several alternative transcripts and many also have alternative promoters [46]. Furthermore, amplification of signaling pathways could be more critical than mRNA or even protein abundance alone.

Combination Chemoprevention

Analyzing the effects of chemopreventive agents using genome-wide or proteome-wide analysis would allow investigators to predict multi-target approaches involving more than one chemopreventive agent. The combination drug approach was first successful in treating tuberculosis with a cocktail of antibiotics, each with a different mechanism of action. Such an approach is now standard in cancer chemotherapy, where different drugs given in combination reduce the likelihood that a tumor develops resistance to a particular treatment. It is logical to extend

this approach to the field of chemoprevention, especially if we consider that dietary chemopreventive agents are naturally present in the diet in combination. **Table 2** lists the results from a selection of studies retrieved during a literature search that report the effects of treatment with combinations of chemopreventive agents.

As a whole, the results indicate that combination treatment is more effective than single-agent treatment. This is presumably due to simultaneously attacking tumor development on multiple fronts, which reduces the ability of a tumor cell to develop resistance to a single treatment. Treatment with agents that act synergistically allows for reduced dosing of individual agents, which can reduce single-agent toxic effects and make chemopreventive levels more easily attainable in the diet. For example, combination treatment with low doses of cholesterol-lowering statins, celecoxib (COX-2 inhibitor), DFMO (polyamine inhibition), and NSAIDs were successful. Diallyl sulfide, found in garlic, was effective in preventing tumor development when given in combination with either quercetin (anti-inflammatory) or Selenomethionine (raf/MEK/ERK inhibitor). Combination treatments with agents that exhibit a broad spectrum of chemopreventive mechanisms, such as curcumin/EGCG or curcumin/quercetin were also successful. Indole-3-carbinol, found in cruciferous vegetables and having anticarcinogenic, antioxidant, and anti-atherogenic effects, was chemopreventive when paired with putrescine, a polyamine. Combination treatment with all-*trans*-*N*-(4-hydroxyphenyl)retinamide (antiproliferative/proapoptotic) and tamoxifen (modulator of growth factors/hormonal activity) targeted multiple mechanisms at once. Other successful combinations include ellagic acid (antioxidant/proapoptotic) given with selenomethionine (antioxidant), urodeoxycholate paired with sulindac, DFMO with piroxicam, tomato with garlic, and beta-carotene in combination with vitamin E. The observed anticancer benefits resulting from treatments with combinations of chemopreventive agents indicate that a diet rich in as many of these compounds as possible is a reasonable approach. However, this approach may not always be possible, especially in developing countries. It is necessary to determine the most affordable and available combinations that are effective against the broadest range of targets as possible. This needs to be addressed at the mechanistic level. At this time, we are not certain if the observed synergistic responses are due to perceived mechanisms or other factors that have not been identified or realized.

Systems Biology

The individual mechanisms that have been proposed to date as being important in defining the action of chemopreventive agents are important pieces of a yet incomplete puzzle. It has become apparent that such mechanisms are too complex to be understood using the classic reductionist approach of identifying linear pathways. Such a narrow approach was historically necessitated due to experimental limitations. The evolution of large-scale, high-throughput technologies has led to a paradigm shift away from reductionism in favor of systems biology. In collaboration with mathematicians and computer scientists, biologists have created complex algorithms to identify clusters of pathways, or networks, representing extensive interactions among different components from the subcellular to organism level [58]. Systems biology offers the field of cancer chemoprevention the potential to identify comprehensive mechanisms of action

Table 2 Combination treatments involving chemopreventive agents

Agents	Model	Findings	Reference
Celecoxib DFMO Statins NSAIDs	Colon cancer	Combinations at low dosages inhibit carcinogenesis more effectively and with less toxicity than if given alone	[47]
EGCG Curcumin	Normal, premalignant and malignant human oral epithelial cells	Synergistically inhibit cell growth	[48]
Curcumin Quercetin	Adenomas in familial adenomatous polyposis	Decreased number and size of polyps	[49]
Indole-3-carbinol Putrescine	SW480 colon tumor cell line	Synergistically caused growth inhibition and necrosis	[50]
Ursodeoxycholate Sulindac	Mouse model of polyposis	Prevents intestinal adenomas at lower doses than with sulindac alone, less toxicity	[51]
All- <i>trans</i> - <i>N</i> -(4-hydroxyphenyl)retinamide Tamoxifen	<i>N</i> -Methyl- <i>N</i> -nitrosourea treated rats	Synergistically prevent tumor recurrence	[52]
DFMO Piroxicam	Azoxymethane-induced colonic neoplasias	Synergistically reduces the number, size, and incidence of colon tumors	[53]
Diallyl sulfide Se-methylselenocysteine	DMBA-induced mammary tumor	Combination regimen more effective than single-agent	[54]
Ellagic acid Selenomethionine	DMBA-induced mammary tumor	Combination regimen more effective than single-agent	[54]
Diallyl sulfide Quercetin	DMBA-induced mammary tumor	Combination regimen more effective than single-agent	[54]
Tomato Garlic	Male Swiss albino mice	Combination regimen more effective than the single-agent in inhibiting DMBA-induced genotoxicity and oxidative stress	[55]
S-Allylcysteine Lycopene	Gastric cancer	Modulatory effects on glutathione redox cycle antioxidants	[56]
Beta-carotene Vitamin E	Oral cancer	Combination treatment results in regression of oral leukoplakia	[57]

involving a multitude of interactions. Both new and preexisting data derived from a broad spectrum of experimental models can be processed in this way to confirm the importance of existing mechanisms as well as to define new mechanistic pathways.

Conclusions

At the present time, we submit that the key mechanism by which any known chemopreventive agent mediates a reduction in tumorigenesis remains ill-defined. The situation is even more confounded in combination chemopreventive work. Most studies performed to date have examined *in vitro* activities. Results from *in vivo* studies often show much more modest benefits, stemming from problems of bioavailability, toxicity, and physiological dosing limitations. In a typical case, irrespective of perceived mechanism, the biological and physiological complexity of a mammal is required to establish efficacy. Moreover, even animal models are not sufficient to predict efficacy in human beings. The failure of β -carotene in clinical trials [59] well exemplifies this point. As illustrated with resveratrol, curcumin and EGCG, a great deal can be learned about the mode of action of a chemopreventive agent. It may be, however, that a critical, straightforward pathway leading to the chemoprevention of cancer will never be known. It may be necessary to finally accept a superb therapeutic response as being empirical in nature and due to a fortuitous sequence of events leading to a good outcome. Of utmost importance is the ability to facilitate a predictable clinical response in the absence of toxicity.

Nonetheless, it is clear that many contemporary basic and clinical scientists, as well as health authorities and regulatory agents, will not find the proposition of empiricism to be sufficiently satisfying. In order to realistically approach a true definition of critical mechanism, we suggest the greatest hope lies in exploring the action of chemopreventive agents and analogues on a genome-wide and proteome-wide scale. Large data sets generated in such experiments require proper analysis and interpretation, which is not a trivial task. This presents a conundrum since modern-day science is not capable of simply disregarding the molecular mechanism leading to a favorable therapeutic outcome and then proceeding with confidence. This is fundamentally equivalent to accepting the untenable philosophy of ignorance is bliss. Certainly, a great deal of work remains to be done for the accurate definition of chemopreventive mode of action, but we should be willing to forge ahead on a semi-empirical basis in our fight against this dreadful disease.

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