Quantitation of chemopreventive synergism between (–)-epigallocatechin-3-gallate and curcumin in normal, premalignant and malignant human oral epithelial cells

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An *in vitro* model for oral cancer was used to examine the growth inhibitory effects of chemopreventive agents when used singly and in combination. The model consists of primary cultures of normal oral epithelial cells, newly established cell lines derived from dysplastic leukoplakia and squamous cell carcinoma. Two naturally occurring substances, (–)-epigallocatechin-3-gallate (EGCG) from green tea and curcumin from the spice turmeric were tested. Cells were treated singly and in combination and effects on growth determined in 5-day growth assays and by cell cycle analysis. Effective dose 50s and the combination index were calculated with the computerized Chou–Talalay method which is based on the median-effect principle. Agents were shown to differ in their inhibitory potency. EGCG was less effective with cell progression; the cancer cells were more resistant than normal or dysplastic cells. In contrast, curcumin was equally effective regardless of the cell type tested. Cell cycle analysis indicated that EGCG blocked cells in G_1, whereas curcumin blocked cells in S/G_2M. The combination of both agents showed synergistic interactions in growth inhibition and increased sigmoidicity (steepness) of the dose-effect curves, a response that was dose and cell type dependent. Combinations allowed for a dose reduction of 4.4–8.5-fold for EGCG and 2.2–2.8-fold for curcumin at ED_{50}s as indicated by the dose reduction index (DRI). Even greater DRI values were observed above ED_{50} levels. Our results demonstrate that this model which includes normal, premalignant and malignant oral cells can be used to analyse the relative potential of various chemopreventive agents. Two such naturally-occurring agents, EGCG and curcumin, were noted to inhibit growth by different mechanisms, a factor which may account for their demonstrable interactive synergistic effect.

Introduction

The development of oral cancer is a multi-step process requiring initiation (irreversible genetic changes), promotion (development of a visible premalignant lesion) and progression (the development of some of these lesions into a malignant tumor). Premalignant lesions for oral cancer are a well established clinical entity carrying a risk of 0.3–28% (for leukoplakia) and 50–90% (for erythroplakia) for progression into a malig-

nancy (1). Patients who have been cured of an initial upper aerodigestive tract neoplasm are also at high risk (20–30%) for developing a second primary tumor over the next several years (2–5). Those patients are potential candidates for chemopreventive intervention.

Our understanding of chemoprevention therapy is still in its infancy. Many clinical studies, as well as basic research, have focused on chemoprevention with retinoids (2,6). In double blind randomized trials, 13-*cis* retinoic acid has been shown to inhibit promotion and progression of leukoplakia as well as inducing clinical regression; 13 *cis*-retinoic acid has also been clinically effective in preventing the development of second primary tumors of the upper aerodigestive tract (2,5). Retinoids have several negative aspects including severe toxicity which could result in low compliance and the reappearance of the leukoplakia after cessation of treatment (6). Thus, the search for better chemopreventive agents is still indicated.

The use of naturally occurring substances that are derived from the diet for chemoprevention provides a strategy to inhibit cancer that should have limited toxicity. Two such agents, green tea and the spice curcumin are being extensively investigated. Green tea, mainly through its major constituent (–)-epigallocatechin-3-gallate (EGCG*) has demonstrated anti-carcinogenic activities in several animal models including those for skin, lung and gastro-intestinal tract cancer (7,8). *In vitro* studies have shown that green tea can arrest the growth of human mammary and lung cancer cell lines (9). Epidemiological studies on green tea and cancer are limited, but several investigators have demonstrated positive correlations between green tea consumption and a lower incidence of gastric (10) and esophageal (11) cancer. Turmeric has long been used as a yellow spice in Indian food and as a naturally occurring medicine for the treatment of inflammatory diseases (12). Curcumin is the major phenolic antioxidant and anti-inflammatory agent in the spice turmeric, and has also been shown to have anti-carcinogenic properties in animal models including skin, gastrointestinal tract and mammary gland (13–17). *In vitro* studies have shown that curcumin can inhibit growth of human myeloid leukemia cells (14,18) and epidermoid carcinoma (19). Curcumin has also been shown to inhibit induced expression of several proto-oncogenes (20), protein kinase activity (21), as well as the binding of mutagens to cellular DNA (22).

In the present study, we are using a model for oral cancer to examine the ability of EGCG and curcumin to inhibit the growth of oral cells, when used as single agents and in combination. The model consists of primary cultures of normal oral epithelial (NOE) cells, a cell line established from dysplastic leukoplakia and thus considered premalignant, a more progressed leukoplakia subline and an oral squamous cell carcinoma cell line. It is well recognized that epidemiological studies often show preventive activity with broad-based food categories, while individual nutrients may not show correlations (23). As with standard chemotherapy, chemopreventive strat-

egies which use a combination of agents will probably be more effective (24).

Materials and methods

Cell culture

Normal oral epithelial (NOE) cells were derived from anterior tonsillar pillar mucosa obtained from routine pediatric tonsillectomies as described by Xu *et al.* (25). Briefly, pieces of anterior tonsillar epithelium were plated onto 60-mm Primaria culture dishes (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) in Amniomax-c100 medium (Gibco BRL, Grand Island, NY) and epithelial cells were allowed to outgrow for 7–10 days. Outgrowths were harvested and passaged in serum-free keratinocyte growth medium (KGM®, Clonetics Corp., Walkersville, MD). All experiments were performed on first passage NOE cells plated in Primaria culture dishes. MSK Leuk1 was established from a dysplastic leukoplakia lesion adjacent to a squamous cell carcinoma of the tongue in 46-year-old non-smoking female cancer patient. MSK Leuk1 cells are immortal and non-tumorigenic (26). Cells are routinely maintained in KGM and passaged using 0.125% trypsin–2 mM EDTA. A more progressed subline was developed by selecting for cells that would grow in high calcium serum containing media (DMEM/F12 plus10% FCS). These leukoplakia cells which have lost their response to calcium/serum are termed Leuk1s and were maintained in KGM. The 1483 HNSCC cell line has been described previously (27). Some 1483 cells were acclimatized to and routinely maintained in KGM for these experiments.

EGCG and curcumin studies

EGCG, a gift from the Thomas J.Lipton Tea Company, and curcumin (Fluka Chemical Corp., Ronkonkoma, NY) were dissolved in water and ethanol, respectively, and stored as a 0.01 M stock at –20°C. All manipulations with these agents were performed under subdued lighting.

Cell cycle

Cell cycle analysis was performed on nuclei isolated and stained with ethidium bromide as described (28,29). Cells were trypsinized, rinsed in PBS and treated with Nusse I reagent (10 mM NaCl, 3.4 mM Na-Citrate, 0.03% NP-40) for 1 h and nuclei released by addition of an equal volume of Nusse II reagent [78 mM Citric acid (anhydrous), 250 mM sucrose]. Cells were analysed in the Flow Cytometry Core Facility on a FACScan (Becton Dickinson, San Jose, CA) using multicycle computerized program for data analysis.

Growth studies

For the inhibition of cell proliferation studies, cells were plated onto 96-well plates and allowed to attach for 24 h. Agents were then added at appropriate serial diluted concentration (six wells/concentration) and cells allowed to grow for 5 days. Growth was measured by DNA fluorescence in an assay modified from that described by McCaffrey (30). Following 5 days growth, the wells in plates were washed twice with 200 µl/well HBSS (Ca^{++}, Mg^{++}, 0.01 M Hepes), fixed with 100 µl/well of 4% paraformaldehyde for 20 min and washed once with HBSS. Cells were then stained for 2–3 h with 100 µl/well of Hoechst 33342 (5 µM, 0.001% Triton-x 100 in water). The plates were than washed twice with water and allowed to dry overnight in the dark. Fluorescence was read the next day on a microtiter fluorometer (Fluoroskan, Flow Labs) interfaced to an IBM-PC computer.

Median-effect principle for dose-effect analysis and the combination index studies

The multiple drug effect analysis based on the median-effect principle was used to examine drug interactions (31–33). This involves plotting dose effect curves for each agent and for multiple diluted, fixed ratio combinations of agents using the median effect equation:

$$f_a/f_u = (D/D_m)^m.$$

In this equation, D is the dose, D_m is the dose required for 50% effect (e.g. 50% inhibition of cell growth), f_a is the fraction effected by D (e.g. 0.9 if cell growth is inhibited by 90%), f_u is the unaffected fraction, $(1–f_a)$, and m is the coefficient of sigmoidicity of the dose-effect curve; $m = 1$, >1 and <1 indicate hyperbolic, sigmoidal and negative-sigmoidal dose-effect curves, respectively, for an inhibitory drug. The dose-effect curve is plotted using a logarithmic conversion of this equation to: $\log(f_a/f_u) = m \log(D)–m \log(D_m)$ for the median-effect plot: $x = \log(D)$ versus $y = \log(f_a/f_u)$, which determines the m (slope) and D_m (anti-log of x intercept) values. A combination index (*CI*) is then determined with the classic isobologram equation of Chou–Talalay (31,33):

$$CI = (D)_1/(Dx)_1 + (D)_2 /(Dx)_2$$

where $(Dx)_1$ is the dose of agent 1 (EGCG) required to produce×percentage effect alone and $(D)_1$ is the dose of agent 1 required to produce the same×percentage effect in combination with $(D)_2$. Similarly, $(Dx)_2$ is the dose of agent 2 (curcumin) required to produce×percentage effect alone and $(D)_2$ is the dose required to produce the same effect in combination with $(D)_1$. The denominators of the *CI* equation above, $(Dx)_1$ and $(Dx)_2$ can be determined by $Dx = D_m[f_a/(1–f_a)]^{1/m}$ (31–33). Different values of *CI* may be obtained for solving the equation for different values of f_a (e.g. different degrees of inhibition of cell growth). *CI* values of <1 indicate synergy, >1 indicate antagonism and $=1$ indicates additive effect. Computer programs (34,35) based on median-effect plot and the *CI* equation have been used for determining dose-effect parameters (m, D_m and linear correlation coefficient r) for each agent alone and their fixed ratio combinations, and for quantification of *CI* values.

The dose-reduction index (DRI) (33–35) provides a measure of how much the dose of each drug in a synergistic combination may be reduced at a given effect level, (i.e. at x% inhibition) compared with the doses of each drug alone. Toxicity toward the host may be avoided or reduced when the dose is reduced. Based on the above *CI* equation, $(DRI)_1 = (D_x)_1/(D)_1$ and $(DRI)_2 = (D_x)_2/(D)_2$.

Results

Growth studies

A 5-day growth assay was used to examine the potential for EGCG and curcumin (10^{-2}–10^{-8} M) to modulate cell growth within our carcinogenesis model. The model consists of normal, premalignant and malignant oral epithelial cells. The ED_{50}s for the various cell lines are shown in Table I. EGCG and curcumin were found to be growth inhibitory to all four cell lines. However, the extent of inhibition was dependent on both the cell line and agent. There was an increase in resistance to the inhibitory activity of EGCG as the cells progressed from NOE cells through dysplasia to HNSCC within our carcinogenesis model. As shown in Table I, the ED_{50} for EGCG increased from 5.9 µM for NOE to 17.8 µM for the 1483 HNSCC cells. In contrast to EGCG, the inhibitory effect for curcumin was relatively constant with an ED_{50} ~3.5–5.2 µM for all cell types (Table I).

As shown in Table I, NOE cells have lower ED_{50}s to the individual and combined agents as compared to the leukoplakia or cancer cell lines. However, the shape of the dose effect curves, which are depicted by the m value in Table I ($m = 1$, >1 and <1 indicates hyperbolic, sigmoidal and negative sigmoidal curve, respectively), indicate that NOE has a slightly sigmoid dose-effect curve, MSK-Leuk1 has increase sigmoidicity, and MSK-Leuk1S and 1483 have marked increased sigmoidicity. Since a larger m value indicates a steeper dose-effect curve, NOE cells have shallow dose-effect curves (lower m values) and will be less responsive to small changes in high agent concentrations than the more progressed cells.

Flow cytometry

Flow cytometric studies showed that EGCG and curcumin inhibited growth by different mechanisms. EGCG blocked all cells in the G_1 phase of the cell cycle whereas curcumin blocked cells in S/G_2M. Representative data for NOE cells are shown in Table II, but similar results were found for all cells. Since these agents were active at different phases of the cell cycle, i.e. different checkpoints, studies were undertaken to test the hypothesis that combination treatment with these agents would be more effective than treatment with a single agent.

Combination studies

Using 5-day growth assays, agents were examined singly and in combination. A representative dose–response assay for the MSK Leuk1s cell line is shown in Figure 1. The dose–response curves for that cell line are typical of those found for all cell lines within this model of oral progression, and the combination

Table I. Dose-effect relationship parameters for egcg and curcumin in oral cancer model

Cell type	EGCG			Curcumin			(1:1) EGCG:curcumin		
	D_m (µM)	m	r	D_m (µM)	m	r	D_m (µM)	m	r
NOE	5.99 ± 2.5	1.23 ± 0.07	0.93 ± 0.03	3.45 ± 0.72	1.53 ± 0.12	0.89 ± 0.04	1.28 + 1.28 (×4.7)[b] (×2.7)	2.21 ± 0.59	0.89 ± 0.05
MSK Leuk1	7.15 ± 1.62	1.83 ± 0.36	0.95 ± 0.02	3.49 ± 0.51	2.29 ± 0.43	0.94 ± 0.02	1.61 + 1.61 (×4.4) (×2.2)	4.05 ± 1.01	0.91 ± 0.03
MSK Leuk1s	9.01 ± 1.09	2.39 ± 0.15	0.97 ± 0.01	4.28 ± 0.67	3.70 ± 1.18	0.94 ± 0.04	1.54 + 1.54 (×5.9) (×2.8)	4.09 ± 1.31	0.92 ± 0.05
1483	17.8 ± 7.0	2.52 ± 0.70	0.82 ± 0.15	5.18 ± 0.89	3.17 ± 0.67	0.93 ± 0.05	2.10 + 2.10 (×8.5) (×2.5)	4.52 ± 1.64	0.90 ± 0.04

[a]Potency, shape (sigmoidicity) and conformity of dose-effect curve (linear correlation coefficient) are represented by D_m, m, and r, respectively, where D_m (ED_{50}) is the antilog of x-intercept in µM, m is the slope of the median-effect plot signifying the shape of the dose-effect curve ($m = 1$, >1 and <1 indicates hyperbolic, sigmoidal and negative sigmoidal curve, respectively), and r is the linear correlation coefficient of the median-effect plot. Data shown are pooled results of minimum of three experiments (mean ± SE).
[b]Dose-reduction index (DRI) (33–35) were determined by $(DRI)_1 = (Dx)_1/(D)_1$ and $(DRI)_2 = (Dx)_2/(D)_2$ (see Materials and methods). The DRI values at ED_{50} (or D_m) are given in the parentheses (e.g. 5.99 µM/1.28 µM = 4.7 DRI for NOE).

Table II. Effects of EGCG and curcumin on NOE by cell cycle analysis

Treatment	Cell cycle phase (%)		
	G_1	G_2	S
Control	55 ± 1	15 ± 2	30 ± 2
EGCG	71	11	18
Curcumin	29	40	31

Log phase cultures of NOE cells were exposed to 5.5 µM EGCG or 5 µM curcumin for 24 h. Cells were prepared for cell cycle analysis as described in Materials and methods.
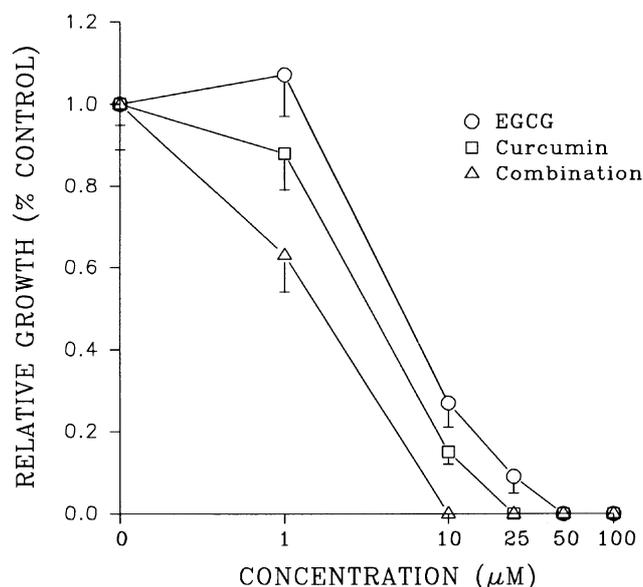

Fig. 1. Dose effect relationship for MSK Leuk1s in 5-day growth assays. Each point is the average of six wells/dose. Combination is at a 1:1 ratio for EGCG:curcumin.

of EGCG and curcumin appears to be more effective than either agent alone. Data were examined using median effect analysis to determine the type of interactions which occurred, i.e. antagonism ($CI > 1$), additivity ($CI = 1$) or synergism ($CI < 1$). As shown in Table III, the combination index (CI) was <1 for all cell types at ED_{50} and above, indicative of synergistic effects. However, at low dose-effect combination regimens ($\leqslant ED_{40}$), NOE and Leuk1 responded differently than the more progressed Leuk1s and 1483 cells. NOE and Leuk1 cells showed an additive to slightly antagonistic effect at low doses but a synergistic effect only at high doses while Leuk1s and 1483 showed a synergistic effect at both low and high doses (Table III, Figure 2).

As occurs in synergistic interactions, the DRI was >1 (Table I). To achieve an ED_{50}, the DRI in Table I indicates the fold decrease in agent concentration needed. With MSK Leuk1 an ED_{50} is produced by 7.15 µM EGCG or 3.49 µM curcumin, but a 1:1 combination of agents will produce this ED_{50} at 1.61 µM, a DRI of 4.4-fold decrease for EGCG and 2.2-fold decrease for curcumin. Also shown in Table I is an increase in the m value in the combination treatments as compared to single agents for all cell types. Thus, combination treatment produced steeper dose-effect curves than single treatments indicating that small changes in doses will produce greater effects, i.e. increased growth inhibition.

Discussion

The concept of combination chemoprevention seeks to increase the chemopreventive effectiveness of agents, while decreasing toxicity by dose reduction. Two independent *in vivo* studies in 1981 demonstrated that combinations of selenium and retinyl acetate were more effective than either agent alone in suppressing mammary induced tumorigenesis (36,37). Combination studies have attempted not only to increase effectiveness, but also lower side effects due to toxicity which may occur at therapeutic levels (24). Synergistic interactions were identified between beta carotene and alpha-tocopherol in an *in vivo* hamster cheek pouch carcinogenesis model (38) and between beta carotene and anti-cancer alkylating agents *in vitro* with human tongue squamous carcinoma cells (39). In the present study, a synergistic inhibition of growth was quantitatively demonstrated following treatment of normal, premalignant and malignant oral cells with a 1:1 combination of EGCG and curcumin at different concentrations. To our knowledge, this is the first time that the interactions of two chemopreventive

Table III. Interaction of EGCG and curcumin combinations in cells at different stage of carcinogenesis: combination indices at different effect levels

Cell type	Combination index (CI) at:				
	ED_{30}	ED_{40}	ED_{50}	ED_{75}	ED_{95}
NOE	0.90 ± 0.17	0.79 ± 0.22	0.70 ± 0.08	0.52 ± 0.03	0.33 ± 0.06
MSK Leuk1	1.02 ± 0.08	0.89 ± 0.04	0.79 ± 0.05	0.58 ± 0.09	0.38 ± 0.12
MSK Leuk1s	0.56 ± 0.03	0.55 ± 0.03	0.54 ± 0.03	0.51 ± 0.03	0.46 ± 0.04
1483	0.79 ± 0.02	0.74 ± 0.05	0.69 ± 0.08	0.59 ± 0.13	0.46 ± 0.21

CI value <1, $=1$, >1 indicates synergism, additive effect, and antagonism, respectively.
The *CI* values were calculated by the Chou–Talalay method (31,33) based on the median-effect equation and the classic isobologram equation (31,33), using computer software (34,35). *CI* values shown are mean ± SE with a minimum of three experiments.
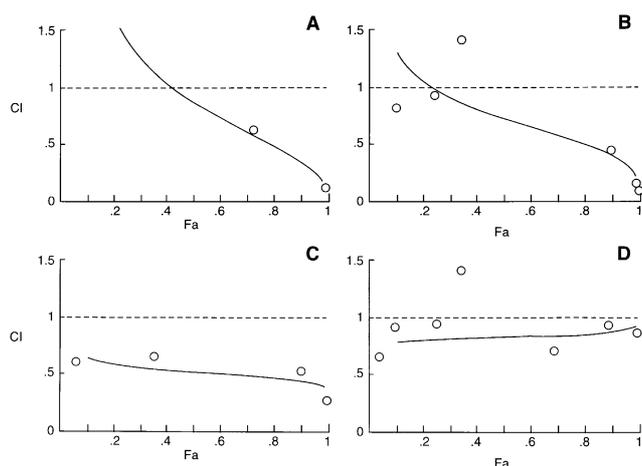

Fig. 2. Combination index (*CI*) versus fraction affected (f_a) plots obtained from median- effect analysis of Chou–Talalay (32,33). (**A**) NOE; (**B**) MSK Leuk1; (**C**) MSK Leuk1s; (**D**) 1483. Curves with solid lines are computer simulated f_a–*CI* plots based on parameters (*m* and D_m values) for EGCG, curcumin and their equimolar (1:1) combinations. Open circles are actual combination data points. [Note, in (A) several low value data points are off scale.] *CI* <1, $=1$ and >1 indicates synergism, additive effect and antagonsim, respectively.

agents have been quantitatively determined in terms of combination indices.

The cells used in this study represent various stages along the progression to oral cancer. The NOE cells were derived from pediatric tonsillectomies; these tissues would be expected to have had limited exposure to environmental carcinogens and are considered by our laboratory to represent a baseline 'normal' with respect to carcinogenesis (26). MSK Leuk1 cells were derived from a dysplastic leukoplakia specimen, and are immortal and non-tumorigenic. Since dysplastic leukoplakia is a premalignant lesion for oral cancer (1), MSK Leuk1 can be considered premalignant (initiated and progressed) given its source of origin and immortality. MSK Leuk1s cells were selected for loss of the normal epithelial differentiative response to calcium/serum (40) and are a more progressed, non-tumorigenic subline. 1483 is an oral squamous carcinoma cell line developed from a previously untreated tumor of the retromolar trigone and is tumorigenic (27)**.**

Using median effect analysis to identify dose-effect parameters and the interactions between chemopreventive agents, i.e. antagonism, additivity, synergism, unique interactions between EGCG and curcumin have been identified. As single agents, both were effective in suppressing growth of all oral cells (normal, premalignant, malignant). Interestingly, EGCG and curcumin as single agents showed shallow dose-effect

curves in NOE and MSK Leuk1 cells (i.e. lower *m* values) whereas MSK Leuk1s and 1483 cells showed steeper dose-effect curves (i.e. higher *m* values). In combination treatment, however, differences in sensitivities were identified among the cell populations that might have clinical relevance. First, combination treatment was more effective than either agent alone with dose-reduction index (DRI) $>$ 1. The DRI in the combination for EGCG were 4.4–8.5-fold and for curcumin were 2.2–2.8-fold (Table I). Even higher DRI values were observed at above the ED_{50} effect levels. Secondly, at low EDs, normal and less progressed cells showed a slight antagonism or additive effect with CI $\geqslant$ 1 with respect to agent-induced growth inhibition (Table III, Figure 2). Clinically, this could translate into a protective effect for normal mucosa, while progressed tissues respond synergistically. Lastly, the dose-effect curves of the combination in the more progressed cell lines were much steeper than those of the normal or less progressed leukoplakia as depicted by their higher *m* values (Table I). It is of interest to note that the *m* values (sigmoidicity or the steepness) in combinations are all greater than the individual agents alone. This suggests that appropriate combinations could be established which would have limited effects (for example, toxicity) against normal cells, but be quite effective for progressed cells. Additionally, the normal cells in our model are in log phase growth and, thus, in a higher proliferative state than present within mucosal epithelium. *In vivo*, normal cells would probably respond less than under our experimental conditions.

Our use of combinations was based on cell cycle results showing differential growth arrest. EGCG blocked cells in G_1, whereas curcumin induced an S/G_2M block. To our knowledge, this is the first study to examine cell cycle effects of EGCG and curcumin. Based on differential mechanisms for growth inhibition, we hypothesized that combination treatment would be more effective than single agent since cells could be simultaneously blocked at multiple checkpoints. This paradigm could serve as a model for a continuing mechanistic approach to chemoprevention. In addition to validating the strength of our model, our results could serve as a rationale for combination treatment with other agents. For example, retinoic acid induces a G1 block in HL60 leukemia cells (41). In this respect, we have shown that combinations of retinoic acid and various non steroidal anti-inflammatory agents, including curcumin, produces synergistic inhibition of growth of 1483 cells (42).

Since green tea and curcumin have been reported to produce a variety of biological effects (7,8,43), multiple pathways are probably involved in the final outcome that was experimentally measured, i.e. DNA fluorescence as a measure of cell growth. Proliferation, cell death and differentiation are some of the

physiological processes that will affect cell number in our growth assay. Although differential cell cycle checkpoint blockage was identified, it is not known whether cells are permanently blocked or slowly cycling. Cells not specifically blocked may also be cycling at a slower rate. Whether cell death or differentiation occur at certain doses or combinations during the 5-day growth assay is also at present unknown. Thus, the underlying mechanism(s), signal transduction pathways, leading to growth inhibition induced by single agents and combinations remain to be elucidated. Factors other than differential cell cycle blockage may be important. Additionally, our assay measures growth and comparison of ED_{50}s assumes similar uptake and metabolism. It is possible that differences between cell systems could also be related to these parameters that would affect intracellular concentrations. However, differences were identified between MSK Leuk1 and MSK Leuk1s, cells with identical parentage, suggesting that differences besides uptake or metabolism are being identified.

Using a 96-well plate growth assay suitable for large scale screening and computerized pharmacodynamic and statistical analysis, synergistic interactions have been demonstrated between two chemopreventive agents. EGCG and curcumin are naturally occurring substances being consumed on a daily basis by large populations in India and the Far East. In one study (44) intake of 1 g/day of EGCG produced negligible side-effects. Curcumin is also expected to have limited toxicity, is under development by the National Cancer Institute's chemo-prevention drug development program and clinical trials for prevention with dysplastic leukoplakia are planned (45). The model being used in the present study allows examining effects of chemopreventive agents on oral cells which are at different stages along the pathway to cancer. These are the very cells that are targeted in clinical prevention studies and the model is being used to both screen and study mechanism of action.

Acknowledgements

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