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Ginger phytochemicals exhibit synergy to inhibit prostate cancer cell proliferation

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Abstract

Dietary phytochemicals offer non-toxic therapeutic management as well as chemopreventive intervention for slow-growing prostate cancers. However, the limited success of several single-agent clinical trials suggest a paradigm shift that the health benefits of fruits and vegetables are not ascribable due to individual phytochemicals rather may be ascribed to but to synergistic interactions among them. We recently reported growth-inhibiting and apoptosis-inducing properties of ginger extract (GE) in *in vitro* and *in vivo* prostate cancer models. Nevertheless, the nature of interactions among the constituent ginger biophenolics, viz. 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol, remains elusive. Here we show antiproliferative efficacy of the most-active GE biophenolics as single-agents and in binary combinations, and investigate the nature of their interactions using the Chou-Talalay combination-index (CI) method. Our data demonstrate that binary combinations of ginger phytochemicals synergistically inhibit proliferation of PC-3 cells with CI values ranging from 0.03-0.88. To appreciate synergy among phytochemicals present in GE, the natural abundance of ginger biophenolics was quantitated using LC-UV/MS. Interestingly, combining GE with its constituents (in particular, 6-gingerol) resulted in significant augmentation of GE's antiproliferative activity. These data generate compelling grounds for further preclinical evaluation of GE alone and in combination with individual ginger biophenols for prostate cancer management.

Keywords

antiproliferative; ginger; phytochemicals; prostate cancer; synergy

INTRODUCTION

Although the clinical armamentarium of chemotherapeutic drugs has been expanding with remarkable momentum, the rate of cancer incidence and mortality is exponentially increasing. The barriers to the development of “tumor-eradicating” drugs can perhaps be attributed to the fact that carcinogenesis in humans is a complex, multistep process that involves dysfunction of several molecules including oncogenes and tumor suppressors²⁰. These molecules exquisitely control multifarious signaling circuitries that dictate cell

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survival and death pathways and their genetic alterations vary temporally; consequently, the acquisition of malignant phenotypes can be quite variable across different cancer types. Thus, it appears that the current mono-targeted therapy approach using rationally designed pharmacological inhibitors against a particular target has its own limitations. Furthermore, since tumors represent a heterogeneous population, a multi-targeted approach using single agent or a combination regimen might offer an edge over the more prevalent mono-targeted strategy. Therefore, an ideal chemo- preventive or therapeutic regimen should be relatively non-toxic and concurrently hit multiple targets to achieve superior anticancer benefits.

Nature abounds in fruits and vegetables that are concoctions of several molecules called phytochemicals present at low concentrations. Epidemiological studies suggest that a high intake of fruits and vegetables is linked to a reduced cancer risk^{47; 10; 25}. Phytochemicals present in fruits and vegetables (e.g., carotenoids, polyphenolics, anthocyanins, terpenes, alkaloids) are functionally pleiotropic; they possess multiple intracellular targets, affecting different signaling cascades usually altered in cancer cells with limited toxicity to normal cells^{11; 41}. They have been shown to simultaneously target multiple neoplastic events by preventing DNA damage, modulating inflammation, and inhibiting tumor cell proliferation, thereby reducing overall cancer risk^{14; 45; 27; 37}.

Several reports suggest that the anticancer benefits of fruits and vegetables are due to an additive or synergistic interplay of the complex mixture of phytochemicals present in them^{27; 28}. This emerging notion further lends support to the premise of concomitantly targeting multiple pathways. For example, studies of green tea polyphenols have demonstrated that EGCG and at least four other catechins are necessary for maximum *in vivo* efficacy, suggesting that synergistic inter-reactivity or dependency on other components of the whole food is required for optimal activity⁴. Similarly, the highest antioxidant activity was realized with the combination of polyphenols in pomegranate juice as opposed to the constituent polyphenols alone³⁴. In a comparable investigation of cranberry extract, enhanced antiproliferative activity was attributed to the synergistic and additive interactions of its main components, including anthocyanins, proanthocyanidins, and flavanol glycosides³³. Such studies thus offer a plausible explanation as to why clinical trials with pure, single phytochemicals, such as α -tocopherol, β -carotene, and vitamin C, have met with limited success^{19; 28; 40}. It is thus likely that disrupting the natural balance of phytochemicals as it exists in fruits and vegetables by extracting individual phytochemicals from the “food matrix”^{23; 22} may result in sub-optimal health benefits.

Ginger (*Zingiber officinale* Roscoe, *Zingiberaceae*) is one of the most commonly consumed dietary agents worldwide. Ginger is widely used as an aid for digestive disturbances, including dyspepsia, colic, nausea, vomiting, gastritis, and diarrhea. The main bioactive constituents of ginger extract (GE) have been reported to be 6-gingerol (6G), 8-gingerol (8G), 10-gingerol (10G), and 6-shogaol (6S)^{43; 50}. Our laboratory recently demonstrated significant anticancer benefits of whole ginger extract (GE) both *in vitro* and *in vivo* prostate cancer models²⁴.

In this study, we evaluate the antiproliferative efficacy of the most-active ginger phytochemicals (6G, 8G, 10G, and 6S) as single agents as well as in binary combinations in prostate cancer PC-3 cells. Our data showed the existence of strong synergistic interactions in the binary combinations of 6G, 8G, 10G and 6S. In particular, robust synergy was evident between 8G and 10G. Additionally, the ability of these ginger biophenolics to augment the activity of whole GE in prostate cancer cells was explored. The dose reduction index (DRI) that quantitates the magnitude by which the dose level of whole GE can be reduced upon employing a combination regimen of GE and ginger phytochemicals was calculated to emphasize the translational relevance of ginger extract in prostate cancer prevention and

therapy. Although numerous studies have shown the anticancer properties of individual ginger phytochemicals (albeit at higher doses)^{38; 39; 36; 26}, ours is the first systematic study to report the nature of the interactions among ginger biophenolics and emphasize the significance of designing phytochemical mixtures that can exert superior anticancer benefits.

MATERIALS AND METHODS

Cell line, media and reagents

Human prostate cancer, PC-3 cells obtained from American Type Culture Collection (Manassas, VA) ATCC, were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Hyclone, UT) and 1% antibiotic (penicillin/streptomycin). The MTT dye (Thiazolyl Blue Tetrazolium Bromide, 98% TLC) and Dimethyl Sulfoxide (DMSO) were from Sigma-Aldrich (St. Louis, MO). Cells were cultured at 37°C with 5% CO₂. The ginger phytochemicals, 6-gingerol (6G), 8-gingerol (8G), 10-gingerol (10G), and 6-shogaol (6S) were purchased from Chromadex, Inc. (Santa Ana, CA) and their purity was confirmed by HPLC analysis.

Preparation of ginger extract (GE) and ginger standards

GE was prepared as described previously²⁴. Briefly, fresh ginger was obtained from a local farmer's market, and grated ginger was then soaked in methanol for 4 consecutive days. The supernatant collected daily was pooled together, concentrated *in vacuo* (Buchi Rotovap, Buchi, Switzerland), and freeze-dried to a solid form. GE and the standard stock solutions of gingerol phytochemicals were prepared at a concentration of 100 mg/ml and 10 mM, respectively, in DMSO. All experiments were performed using a single batch of GE in order to avoid batch-to-batch variation and maximize constancy. However, the variation among different batches of ginger extracts was determined based upon the quantitative values of 6, 8, 10 gingerols.

In vitro proliferation assay

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was employed to evaluate the proliferative capacity of cells. Essentially, MTT is a colorimetric assay, which utilizes the colorless tetrazolium dye and converts it into a colored formazan salt, which can be quantified by measuring absorbance at 570 nm. Briefly, a 96-well format was used to seed 100 µl medium containing cells at a density of 5×10^3 cells per well. After 24h of incubation, cells were treated with gradient concentration of GE, gingerols, and shogaol (Figure 1A, B), which were dissolved in DMSO. The final concentration of DMSO in the culture medium was maintained at 0.1%. After 48h of incubation, the spent medium was removed and the wells were washed twice with PBS. 100 µl of fresh medium and 10 µl of MTT (5 mg/ml in PBS) was added to the wells and cells were incubated at 37°C in dark for 4h. The formazan product was dissolved by adding 100 µl of 100% DMSO after removing the medium from each well. The absorbance was measured at 570 nm using a Spectra Max Plus multi-well plate reader (Molecular Devices, USA).

Experimental design of the binary combination studies

IC₅₀ values of GE, 6G, 8G, 10G and 6S were obtained and a series of combinations at varying concentrations were tested against PC-3 cells. For 6G, 8G, 10G and 6S the concentrations used in combinations were 0.075(IC₅₀/2), 0.125(IC₅₀/2), 0.25(IC₅₀/2), 0.5(IC₅₀/2), 0.75(IC₅₀/2), 1(IC₅₀/2), 1.25(IC₅₀/2). For GE, the concentrations were 0.075(IC₅₀), 0.125(IC₅₀), 0.25(IC₅₀), 0.5(IC₅₀), 0.75(IC₅₀), 1(IC₅₀), 1.25(IC₅₀). Gingerols and shogaol were added in combination with GE and with one another at the aforementioned concentrations. In each combination, a constant ratio of the IC₅₀ for each drug was

maintained. The following combinations were tested: 6G+8G, 8G+10G, 10G+6G, 6S+6G, 6S+8G, 6S+10G, GE+6G, GE+8G, GE+10G, GE+6S.

High-performance liquid chromatography and mass-spectrometric detection

Liquid chromatographic separations were achieved on a HP1100 series Instrument (Agilent Technologies, Wilmington, DE) equipped with a photodiode array detector, using an Agilent XDB reversed phase C-18, 1.8 μ m, 4.6 $\times$ 50 mm, ODS-2 column. The solvent gradient system consisted of solvent A (2.5% formic acid in water) and solvent B (2.5% formic acid in acetonitrile (ACN)). The gradient elution was 20% B for 45 min, followed by 80% B for 20 min and 95% B over the next 5 min, which was held at an isocratic composition of 95% B for 20 min, reconditioning to initial mobile phase concentration for 10 min with a flow rate of 0.2 ml/min. 0.5 mg/ml of GE, 6G, 8G, 10-G and 6S dissolved and filtered in pure methanol (10 μ l) were injected into the system and the resultant spectra were observed at 280 nm. Standard calibration curves for gingerols and shogaol were obtained and the area under the curve was considered to quantify their amount in GE.

The HPLC-MS analyses were performed in tandem with HPLC-UV using the same column and mobile phase as described above on HP1100 series instrument (Agilent Technologies, Wilmington, DE) interfaced to an Agilent 6400 Series Triple Quadrupole LC-MS/MS equipped with an electrospray ionization source, which was operated in negative-ion mode. The MS analysis was performed in multiple reaction monitoring (MRM) mode. The nebulizer and collision gases were nitrogen and helium, respectively, and the former was set at 40 psi. A drying gas temperature of 300°C, drying gas flow rate of 9 liter/min and capillary voltage of ± 3 kV were the spray chamber parameters to quantify the ginger phytochemicals in GE. The quantitation of 6G, 8G, 10G and 6S in GE was performed using MRM, where each of these phytochemicals were identified based on their fragmentation patterns using the transitions; 6G 293 $\rightarrow$ 99, 8G 321 $\rightarrow$ 127, 10G 349 $\rightarrow$ 153, and 6S 275 $\rightarrow$ 139. Collision energy of 15 eV was used to obtain the product ions at a fragmentor voltage of 135V.

Determination of CI and DRI

Dose effects were analyzed using the computer software, CalcuSyn (Biosoft, UK), beginning with single-agent dose-response curves, followed by dose-response curves involving combinations of two test agents, as described previously³². The Chou-Talalay drug combination method was employed to yield the combination index (CI) and dose reduction index (DRI) values. The CI values thus obtained are based on the multiple drug-effect equation of the enzyme kinetic models of Chou-Talalay. For mutually exclusive drugs exhibiting similar modes of action, where $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and drug 2 alone, respectively, causing $\times$ % inhibition, the combination index is described as $CI = (D)_1 / ((D_x)_1 + (D)_2 / (D_x)_2)$. A CI value <1 , $=1$ or close to 1, or >1 indicates synergistic, additive, or antagonistic interaction, respectively (Suppl. Table 1). Further, the factor by which the dose of each agent may be reduced at a given effect when in combination compared with the dose when each agent is used alone can be measured by the DRI value. This value holds clinical importance since the dose reduction could result in reduced toxicity while retaining the therapeutic efficacy. The dose-effect relationship was derived through mathematical induction method using enzyme kinetic models. The correlation between the dose and effect in its simplest form can be described as, $Fa = 1 / [1 + (D_m/D)^m]$, where D is the dose of the agent, and Fa is the fraction affected by the dose. D_m is the median-effect dose signifying the potency and m is an exponent signifying the sigmoidal shape of the dose-effect curve, where the former is determined from x-intercept and the latter by the slope of the median-effect plot. A DRI >1 is ideal, as the greater the DRI value, the higher the dose reduction is

for a given therapeutic effect. The number of agents in combination as well as the combination ratio affects the DRI value.

Statistical analysis

All experiments were repeated at least three times. Results were expressed as the mean values $\pm$ standard deviation. Student's t-test was used to perform statistical analysis. The *p*-values were calculated using Excel software in reference to the control. The criterion for statistical significance was $p < 0.05$.

RESULTS

Ginger phytochemicals inhibit prostate cancer cell proliferation

We have previously shown that GE is non-toxic and inhibits prostate cancer growth both *in vitro* and *in vivo*²⁴. Since the major bioactive constituents of ginger are gingerols and shogaols, the antiproliferative activity of GE could be attributed to these components of ginger extract, viz., 6G, 8G, 10G and 6S. Thus, we first determined the half-maximal growth inhibitory concentration (IC₅₀) for GE and its phytochemicals in prostate cancer, PC-3 cells. To this end, we used an *in vitro* cell proliferation assay, MTT, to quantify the percent survival of PC-3 cells in the presence of test agents. The IC₅₀ values obtained were 75 μ M (22.07 μ g/ml) for 6G, 10 μ M (3.22 μ g/ml) for 8G, 50 μ M (17.53 μ g/ml) for 10G, 4 μ M (1.12 μ g/ml) for 6S (Figure 1A), and 250 μ g/ml for GE, (Figure 1B). The carbon chain length of ginger phytochemicals (Figure 1C) has been reported to play a role in the differential potencies of the gingerols and shogaols¹⁵. On similar lines, the results observed in our study showed a variability of IC₅₀s in the context of prostate cancer cells. In addition, our data suggested a significant difference in the IC₅₀ values of ginger constituents compared to whole ginger extract. As evident from Figure 1A and B, the constituent ginger phytochemicals were more potent than GE.

HPLC-UV quantification of ginger phytochemicals in GE

To gain further insights into the obvious differences in the IC₅₀s of ginger phytochemicals and GE, our next step was to quantitate the natural abundance of constituent phytochemicals in ginger extract. HPLC-UV analysis was employed to investigate the relative abundance of 6G, 8G, 10G and 6S in GE (Figure 2). We first obtained the calibration curves of commercially available pure ginger phytochemicals at various concentrations using the analytical method described in *Materials and methods*. Next, GE was injected into the system to obtain the HPLC-UV profile, where all the peaks corresponding to individual ginger phytochemicals were identified and manually integrated. The areas under the curve for each of these peaks in GE were used to calculate their abundance (Figure 2).

The quantitation table in Figure 2 indicates that 6G is the most abundant phytochemical present at 6.12% in GE. This result is consistent with literature reports, which suggest that 6-gingerol is the most abundant constituent of fresh ginger^{17; 49; 3}. Our data showed that the other ginger phytochemicals, 8G, 10G, and 6S were present at 1.81, 2.61 and 1.42%, respectively, in GE. As shown in Figure 1A, the activity (IC₅₀) of 6G alone computed to 22.07 μ g/ml in PC-3 cells. Accounting for the natural abundance of 6G at ~6% in GE, its contribution to the IC₅₀ of GE calculates to be 15 μ g/ml. This is equivalent to a ~32% reduction in the IC₅₀ of 6G when it is present in the "food matrix"^{23; 22} with other ginger phytochemicals compared to 6G as a single agent. Similarly, 10G at an abundance of 2.61% in GE, exhibited a ~57% reduction in IC₅₀ when in its native environment compared to 10G as a single agent. These data suggested the existence of a synergistic relationship between ginger phytochemicals, which is perhaps responsible for their enhanced efficacy at lower-

dose levels when present in GE. To validate these hypotheses, we next investigated the nature of interactions among individual ginger phytochemicals.

GE phytochemicals exhibit synergism

To explore the nature of interactions among ginger constituents, we designed binary combination regimens at fixed ratios of pure ginger phytochemicals (Materials and methods). The concentrations chosen were lower than their respective IC_{50} values. PC-3 cells were treated with combinations of 6G+8G, 8G+10G, 10G+6G, 6S+6G, 6S+8G, 6S+10G, GE+6G, GE+8G, GE+10G, and GE+6S at various concentrations, and the fractions of cells affected (Fa) upon these treatments were determined using the MTT assay. These data were then computed using the CalcuSyn software (Version 2.1) to obtain the corresponding combination index (CI) values. A drug combination showing $CI < 1$ is considered as synergistic, > 1 as antagonistic, and equal to 1 as additive (Suppl. Table 1).

Our results showed that the combination of 6G+8G (Figure 3A) exhibited significantly strong synergistic interactions for concentrations $0.25(IC_{50}/2)$, $0.5(IC_{50}/2)$, $0.75(IC_{50}/2)$, with CI values of 0.276, 0.158, 0.191, respectively (Suppl. Tables 1 and 2). Interestingly, the corresponding CI vs. Fa graph in Figure 4A demonstrated that two out of the five concentrations tested lie above the red dotted line, signifying that their interactions were antagonistic, with CI values > 1 (Suppl. Table 3). Similarly, the combination of 8G+10G (Figure 3B) at concentrations $0.125(IC_{50}/2)$, $0.25(IC_{50}/2)$, $0.5(IC_{50}/2)$, $0.75(IC_{50}/2)$, resulted in CI values less than 0.3 (Suppl. Table 2), indicating strong synergism. The CI vs. Fa line plot in Figure 4B demonstrated that all combinations tested displayed synergy (Suppl. Tables 2 and 3). Three out of the five 10G+6G combinations (Figure 3C) exhibited synergism, with CI values > 0.5 (Suppl. Table 3), whereas the other two were observed to be antagonistic in nature (Suppl. Table 3). Similar to 8G+10G, we found that all combinations of 6G+6S (Figure 3D) were strongly synergistic, as the CI values (0.05-0.11) lie below the red dotted line. (Suppl. Table 2). Furthermore, at low concentrations of $0.075(IC_{50}/2)$, $0.125(IC_{50}/2)$, synergistic interactions were observed for 10G+6G combinations (Figure 3E) with CI values of 0.44 and 0.49, respectively (Suppl. Table 3). In comparison, slight synergism was observed at a concentration of $0.5(IC_{50}/2)$, with CI value of 0.87 (Suppl. Tables 1 and 3). The concentrations $0.25(IC_{50}/2)$ and $0.75(IC_{50}/2)$ interacted in a nearly additive manner lying right below the red line (Figure 4E), with CI values of 0.96 and 0.94, respectively. Finally, strong synergism was observed for 6S+10G (Figure 3F) at a concentration of $0.075(IC_{50}/2)$ with a CI value of 0.24 (Suppl. Table 2). Instead, concentrations of $0.125(IC_{50}/2)$ and $0.25(IC_{50}/2)$ exhibited moderate synergism with CI values of 0.44 and 0.58, respectively. The concentration $0.5(IC_{50}/2)$, showed a high CI value of 0.87, which indicated slight synergy (Suppl. Table 1 and 3). However, the concentration $0.75(IC_{50}/2)$ was nearly additive as its CI value lies above the red dotted line at 1.07 (Suppl. Table 3, Figure 4F).

These observations suggest that the majority of binary combinations of ginger biophenolics affected cell proliferation in a synergistic manner. The CI versus Fa plots in Figure 4 indicate that 83% of gingerol combinations (6G+8G, 8G+10G, 6G+10G) inhibited PC-3 proliferation, with Fa values ranging between 0.4 and 0.5 (Figure 4A-C) synergistically. Furthermore, 90% of the gingerol and 6-shogaol combinations, (6S+6G, 6S+8G and 6S+10G) exhibited synergism irrespective of the fraction of cells affected. In fact 80% of the 6G+6S combinations were observed to display very strong synergism ($CI < 0.1$) (Suppl. Table 2). Having determined significant synergism between pure phytochemicals derived from ginger at low concentrations, we next asked if an increase in the efficacy of GE could be achieved by increasing the ratio of these biophenolics in the extract.

GE phytochemicals augment the antiproliferative activity of GE

Considering the low abundance of the individual GE phytochemicals (<7% in GE), we next examined if increasing their individual amounts in GE would result in an enhanced antiproliferative effect (Figure 5). To address this, we used a similar experimental design for combinations of GE and constituent phytochemicals, as was used for ginger phenolics to obtain the Fa and CI values.

Our results showed that GE and 6G displayed remarkable synergism in combination at all concentration levels, with CI values ranging from 0.12-0.64 (Figures 5A, 6A, Suppl. Tables 4 and 5). The pairing of 8G with GE (Figure 5B) resulted in moderate to slight synergism, despite also exhibiting strong antagonism toward antiproliferative activity in PC-3 cells (Figure 6B, Suppl. Table 5). Although only 2 out of 5 concentrations of GE+10G exhibited synergy with CI values of 0.49 and 0.02 (Figure 6C and Suppl. Tables 4, 5), the growth inhibitory effect was robust, with Fa values of 0.51 and 0.75, respectively (Figure 5C). Precisely, the concentration $1.25(IC_{50}/2)$ of 10G combined with $1.25(IC_{50})$ of GE resulted in a CI value of 0.02, which corresponded to very strong synergism (Suppl. Tables 1, 4). Lastly, three out of five concentrations of GE+6S resulted in synergy (Figure 6D).

Our data in Figures 5 and 6 demonstrate synergy in 65% of GE combinations with individual phytochemicals. More interestingly the addition of individual phytochemicals to GE exhibited an enhancement of its antiproliferative activity as observed in terms of fraction affected, specifically for GE+6G and GE+10G, where Fa values were greater than 0.5 (Figures 5A, 5C). This is further supported by the dose-reduction index (DRI) values of GE and 6G, whose half-maximal inhibitory concentrations were ~9.7 and ~49 fold lower in combination than when dosed individually.

DISCUSSION

Our study is the first of its kind to provide encouraging evidence that ginger phytochemicals synergize with each other as well as in combination with GE to potentiate GE's anticancer activity. The combination of ginger phytochemicals with GE was identified as more potent than either agent alone in inhibiting prostate cancer growth *in vitro*. This effect is likely due to synergistic interactions. Synergy was determined by the Chou-Talalay method, now accepted to be the most accurate for combinatorial drug studies, which considers both mutually exclusive and non-exclusive drugs as well as sigmoidal and hyperbolic dose-effect curves^{9; 7; 8}. Several studies have reported synergistic interactions among phytochemicals at low concentrations, which would mimic their natural abundance^{30; 48}. Therefore, the dose ranges we chose to implement with these binary combinations involved sub-half maximal inhibitory concentrations of each phytochemical to assess 'real' synergy. Specifically, the dose concentrations chosen for combinations of ginger phytochemicals with GE were those that affected less than 50% of the prostate cancer cells as single-agents. However, upon combination, the Fa values were significantly enhanced to more than 50%, suggesting remarkable synergy. For example, GE+6G and GE+10G exhibited Fa values greater than 0.5 (Figure 5A and 5C, Suppl. Tables 4 and 5) and the CI versus Fa plots indicated that these particular combinations exerted strong synergistic activity further augmenting the antiproliferative property of GE (Figure 6A and 6C). As shown in Figure 5C, 31.25 μ M of 10G upon combination with 250 μ g/ml of GE increased the antiproliferative activity of the latter by ~40%. Given that 10G is present at a ~3% level in GE, this significant augmentation may be ascribed to an addition of 11 μ g to the natural abundance of 10G (~9 μ g) (Figure 2B). This is further supported by the fact that 10G exhibited synergism with other ginger phytochemicals, particularly 6G, 8G and 6S (Figures 3B, 3C, and 3F). In addition, 250 μ g/ml of GE when combined with 37.5 μ M of 6G, exhibited ~48% higher Fa when compared to GE alone (Figure 5A). Thus, on similar lines, supplementing 11 μ g of 6G

to its natural composition of 15 μg (~6% in GE, Figure 2B) exhibited a remarkable enhancement in the fraction affected compared to either agent alone. These findings are consistent with previous studies that have also shown that increasing the abundance of a phytochemical within an extract enhances its anticancer activity⁴⁸. Although these combination studies have revealed strategies to significantly improve activity, the mechanisms underlying this improved inhibition are yet to be unraveled. The robustly enhanced activity of combination therapy could yield avoidance of single-agent drug resistance in cancer cells and offer a multi-targeted approach in inhibiting proliferation^{21; 44}. Extensive literature suggests that each ginger phytochemical functions via a multitude of different mechanisms, as ginger polyphenols possess several pharmacological and physiological properties³⁵. For example, 6S has been shown to exhibit antioxidant and anti-inflammatory properties by enhancing the free-radical scavenging activity selectively in cancer cells¹⁵. Further, 10G has been reported to slowly increase the intracellular calcium Ca^{2+} levels in a concentration-dependent manner, leading to death in colon cancer SW480 cells by releasing Ca^{2+} from the endoplasmic reticulum⁶. The major pungent phytochemical, 6G, possesses anti-inflammatory and antioxidant properties and has been shown to exert anti-prostatic activity through various mechanisms like depolarization of mitochondrial membrane potential, thereby increasing the expression of pro-apoptotic molecules like Bax and Bak^{17; 18; 49; 5; 46; 6; 3}.

Quantitative reports from randomized human clinical trials^{12; 29} have indicated the safety profile of GE, suggesting that a daily dose of ginger extract as high as 5 g in its dry form does not cause any toxicity with the exception of mild stomach upset in individuals who infrequently consume spicy foods. However, human subjects administered with large doses (~6 g daily) showed signs of gastric irritation and loss of protective intestinal mucosa¹². The quantitative data as shown in Figure 3 suggests that 6G, 8G, 10G and 6S are present at 6.12, 1.81, 2.61 and 1.42% respectively (Figure 2), in our preparation of ginger extract²⁴. A recent pharmacokinetic study⁵⁰ indicates that human subjects dosed with 250 mg GE containing about 5.38 mg of 6G, achieved a mean maximal blood plasma concentration of 0.9 $\mu\text{g}/\text{ml}$ 6G. Assuming a linear increase in the achieved plasma level, a dose of 250 mg GE with 6G at a ~6% level would yield ~2.5 $\mu\text{g}/\text{ml}$ of 6G in plasma. Since the *in vitro* IC_{50} of 6G in PC-3 cells is 75 μM (Figure 1A), it translates to 22 $\mu\text{g}/\text{ml}$ based upon a molecular weight of 294 for 6G. Thus, a human blood plasma level of 2.5 $\mu\text{g}/\text{ml}$ 6G is possibly achievable upon consuming 250 mg of GE. However, this concentration would not match up with the half-maximal dose-effect *in vitro*. In order to obtain a 6G plasma concentration that corroborates with its IC_{50} in prostate cancer cells (22 $\mu\text{g}/\text{ml}$), one perhaps would need to consume a large quantity (2.2 g) of GE. In light of our results, a synergistic combination of 250 $\mu\text{g}/\text{ml}$ of GE with 37.5 μM (~11 $\mu\text{g}/\text{ml}$) of 6G (Figures 5A and 6A) would decrease the GE dose by 42% (i.e., to 1.275 g) in order to yield an equivalent effect. Further, if 6-gingerol was to be consumed alone, and assuming that bioavailability is not a limiting factor, a daily dose level of 6g/kg bw of 6-gingerol would be required to achieve its *in vitro* IC_{50} dose. Such high doses of individual phytochemicals may not be relevant to the *in vivo* anti-carcinogenic process, thus limiting the potential efficacy of individual ginger phytochemicals in humans. Several lines of evidence indicate that a single constituent isolated from a complex phytochemical mixture present in whole foods or their extracts may even lose its bioactivity or cause undesirable cancer-promoting effects, as in the case of β -carotene^{1; 19; 31; 28; 40}. Contrastingly, several studies have reported that intake of whole foods rich in β -carotene, such as carrots, may reduce the risk of lung cancer^{42; 16}. For example, a case-controlled study involving the consumption of carrots exhibited an inverse relationship to lung cancer¹⁶. Another study showed that C57BL/6 mice fed with carrots had the highest expression of anticarcinogenic genes when compared to the control group⁴². Therefore, it is likely that isolating a single compound from complex foods may not be

effective even at high, relatively toxic doses. However, combinations of lower, less-toxic doses of each compound, as found in whole foods, may be effective.

Our findings underscore the importance of GE supplemented with ginger phytochemicals in synergistic combinations, which may tremendously lower the concentration of the effective doses while maintaining efficacy. The natural abundance, low cost, and non-toxicity of this very common spice, are strong attributes that favor ginger as a chemopreventive as well as a potential chemotherapeutic alternative in the treatment of prostate cancer. Population-based studies suggest that Southeast Asians, who regularly consume ginger have a decreased risk for breast, prostate, gastrointestinal, and colon cancers when compared to Americans and Europeans^{13; 2}, thus strengthening the notion that ginger phytochemicals work together through common and complementary mechanisms to produce chemopreventive benefits. Specifically, prostate cancer is a potential target for chemoprevention because of its high-prevalence in American men, treatment-associated morbidity, long latency prior to premalignant lesions, and defined molecular pathogenesis³. We strongly believe that dietary supplements of synergistic combinations of GE and ginger phytochemicals could prove to be both effective chemotherapeutic and preventive agents and warrant further preclinical evaluation.

Our study presents novel data demonstrating that several ginger phytochemicals display significant synergistic antiproliferative effects in prostate cancer cells when combined at low concentrations with each other. Nonetheless, various binary combinations of ginger phytochemicals at defined concentrations relative to each other showed antagonistic effects. It is thus a subject of future research to determine how these interactions (synergistic or antagonistic) would translate in an in vivo preclinical setting, as well as in the situation of a human being. However, it is likely that an elaborate in vivo efficacy study with coupled pharmacokinetic profiling will be necessary to achieve the optimal combinations, which will yield maximum therapeutic efficacy. Our future efforts are directed towards identifying the convoluted, complementary and overlapping mechanisms by which these phytochemicals exert optimal benefits in synergistic combinations in prostate cancer models.

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Abbreviations

6G	6-gingerol
8G	8-gingerol
10G	10-gingerol
6S	6-shogol
CI	combination index
DRI	dose reduction index
Fa	fraction affected
GE	ginger extract
IC₅₀	half-maximal growth inhibitory concentration
HPLC	high-performance liquid chromatography
LC-UV	liquid chromatography with ultraviolet detection
MS	mass spectrometry

Figure 1.

Ginger extract (GE) and ginger phytochemicals inhibit proliferation of prostate cancer cells. PC-3 cells were treated with gradient concentrations at (A) 1, 10, 25, 50, 75, 100 and 250 μM for 6G (0.29, 2.94, 7.36, 14.72, 22.07, 29.43, 73.58 $\mu\text{g/ml}$), 8G (0.32, 3.22, 8.06, 16.12, 24.18, 32.24, 80.61 $\mu\text{g/ml}$), 10G (0.35, 3.51, 8.76, 17.53, 26.29, 35.05, 87.63 $\mu\text{g/ml}$), 6S (0.28, 2.76, 6.91, 13.82, 20.73, 27.64, 69.09 $\mu\text{g/ml}$), and (B) 1, 10, 25, 50, 75, 100, 250 $\mu\text{g/ml}$ of GE for 48h. The percent cell survival upon various treatments was measured using MTT assay, as described in *Materials and methods*. (A-B) Plot of percentage of cell survival versus concentrations used for determination of half-maximal concentration of growth inhibition (IC_{50}). Data shown above represent the means of three independent experiments performed in triplicate, with standard deviations represented by vertical bars ($p < 0.05$). (C)

Chemical structures of ginger phytochemicals **(i)** 6-gingerol, **(ii)** 8-gingerol, **(iii)** 10-gingerol, **(iv)** 6-shogaol.

Figure 2.

Quantitation of percent abundance of ginger phytochemicals in GE using HPLC-UV analysis. Multiple reaction monitoring (MRM) in negative electron spray ionization mode (top) and HPLC-UV profile (bottom) of GE with the corresponding peaks of 6G, 8G, 10G and 6S were used to quantitate their respective abundances. The precursor and product ion information for each phytochemical is indicated in the -ESI MRM chromatogram. The table in the inset on right refers to the retention time (RT), and percent abundance of ginger phytochemicals in GE.

Figure 3.

Ginger phytochemicals act in concert to confer antiproliferative activity. Bar graph representation of fraction of cells affected (Fa) upon treatment with (A) 6G and 8G, (B) 8G and 10G, (C) 10G and 6G, (D) 6S and 6G, (E) 6S and 8G, (F) 6S and 10G as single agents and in binary combinations. The concentrations of the ginger phytochemicals (in μM) are plotted on the x-axis, while the fraction affected is represented on the y-axis. The choice of concentrations for combinations tested has been described in *Materials and methods*. PC-3 cells were treated with the phytochemical binary combinations for 48h. Data shown above represent the means of three independent experiments performed in triplicate, with standard deviations represented by vertical bars ($p < 0.05$).

Figure 4. Ginger phytochemicals in combination employ cooperativity to display synergy. (A-F) Line plots of combination index (CI) versus fraction of cells affected (Fa) for PC-3 cells treated with various combinations of ginger phytochemicals. The various concentrations of the combinations correspond to Fig. 3(A-F). The degree of synergistic interaction was calculated using CalcuSyn software and quantitated in terms of CI. The red dotted line indicates a CI value of 1, below which the combination of ginger phytochemicals are considered to show a synergistic effect.

Figure 5.

Ginger phytochemicals act in combination with GE to enhance the antiproliferative activity. Bar graph representation of fraction of cells affected (Fa) upon treatment with (A) GE and 6G, (B) GE and 8G, (C) GE and 10G, (D) GE and 6S as single agents and in combination. The concentrations of the ginger phytochemicals (in μM) and GE (in $\mu\text{g/ml}$) are plotted on the x-axis, while the Fa is represented on the y-axis. The choice of concentrations for combinations tested has been described in *Materials and methods*. PC-3 cells were treated with the phytochemical binary combinations for 48h. Data shown above represent the means of three independent experiments performed in triplicate, with standard deviations represented by vertical bars ($p < 0.05$).

Figure 6.

Ginger phytochemicals in combination with GE exhibit synergy. (A-D) Line plots of combination index (CI) versus fraction of cells affected (Fa) for PC-3 cells treated with combinations of ginger phytochemicals and GE. The various concentrations of the combinations correspond to Fig. 5(A-D). The degree of synergistic interaction was calculated using Calcsyn software and quantitated in terms of CI. The red-dotted line indicates CI value of 1, below which the combination of ginger phytochemicals are considered to show synergistic effect.