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Synergistic *Capsicum*–tea mixtures with anticancer activity

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Abstract

We have demonstrated a synergy between a decaffeinated green tea concentrate and a vanilloid-containing *Capsicum* preparation obtained commercially. At a ratio of 25 parts green tea concentrate to 1 part *Capsicum* preparation, the resultant product exhibited efficacy in the killing of cancer cells in culture 100-times that of green tea on a weight basis. These studies were guided by assays of the putative catechin–vanilloid target protein tNOX, a cell surface growth-related enzymatic activity specific to cancer. The activity of the protein target was inhibited by the tea catechins and the *Capsicum* vanilloids. As with growth, the tea and *Capsicum* preparations evaluated were synergistic in their inhibition of the target enzymatic activity.

Introduction

Much attention to polyphenols and cancer wellness has focused on green tea (Dreosti 1996; Mitscher et al 1997; Dufresne & Farnworth 2001). In Asia, drinking tea has been regarded traditionally as a generally healthful practice (Dreosti 1996). A Japanese study showed that the onset of all cancers of patients who had consumed 10 cups of green tea per day was 8.7-years later among females and 3-years later among males, compared with patients who had consumed under three cups per day (Fujiki et al 1998). Thus, a possible relationship between high consumption of green tea and the low incidence of cancer in some Asian countries has been postulated (Ahmad et al 1997).

Animal studies involving known chemical carcinogens as well as transplanted tumour cells indicated reduced cancer risk from consumption of green or black tea catechins (Liao et al 1995; Stoner & Mukhtar 1995; Mitscher et al 1997). Cancer protective responses span the entire process of carcinogenesis including the formation and activation of carcinogens, cancer initiation, promotion and progression as well as diminished tumour growth and metastatic spread (Dreosti 1996). The former are most often attributed to antioxidant effects whereas the latter may be related to more specific mechanisms of cancer cell growth inhibition (Morré et al 2000; Cutter et al 2001).

Our research was based on the discovery of a novel cancer-specific and growth-related cell-surface protein with protein disulfide–thiol interchange and hydroquinone and NADH oxidase activities designated tNOX (Morré 1998; Morré et al 2000; Chueh et al 2002). The protein is unique in that it is associated with all forms of cancer and is absent from normal cells and tissues (Morré 1998). Activity of the protein is correlated with cancer growth and, when blocked, cancer cells fail to enlarge following division and eventually die (Morré et al 1995, 2000).

Among the most potent and effective inhibitors of tNOX are naturally occurring polyphenols exemplified by the principal tea catechin (−)-epigallocatechin-3-gallate (EGCg) (Morré et al 2000) and the vanilloid capsaicin (Morré et al 1995). These substances are safe and without adverse effects on NOX activity and growth of normal cells and tissues. Catechin–vanilloid combinations reported here are 10- to 100-times more effective than either component alone. The potential use for tea catechin–Capsicum vanilloid mixtures in cancer prevention and control is unique among

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nutraceutical products from natural sources in that a target molecule linked to the uncontrolled growth and metastasis characteristic of cancer has been identified. The tNOX target is a growth-related and cancer-specific NOX isoform (Morré et al 2000) that is emerging as a major contributor in the uncontrolled growth of cancer cells (Morré 1998). When tNOX is inhibited, cancer cells fail to enlarge following division and further proliferation is prevented. If the affected cells do not grow for at least 48 to 72 h, apoptosis is the result. NOX proteins and growth of normal cells are unaffected by tNOX inhibitors (Morré 1998).

We reported (Morré et al 2003) synergies among ester bond-containing catechins and non-ester bond-containing catechins having enhanced efficacy for slowing growth of cancer cells. Synergy is also encountered with other tNOX-blocking agents such as the Capsicum vanilloids. In this report, we have demonstrated synergies between green tea and a commercially obtained Capsicum preparation. When a green tea concentrate was utilized, the combination was 100-times more efficacious than green tea alone on a weight basis in inhibiting tNOX activity and in inhibiting the growth of cancer cells in culture.

Materials and Methods

Materials

All chemicals were from Sigma (St Louis, MO) unless otherwise specified. Green tea infusions were prepared by steeping of unrefined decaffeinated green tea (Lipton's or Sencha, Japan with equivalent results), 2 g in 10 mL water for 10 min at 90–100 °C. At the end of the infusion, the tea leaves were pressed to remove liquid.

The Capsibiol was a lyophilized preparation produced from African bird peppers (Frieda's, Los Angeles, CA) and obtained from Actibiol SA (Geneva, Switzerland). It was produced through a proprietary process by Actibiol SA in Switzerland and formulated from approved natural food sources using good manufacturing practice(s) providers. The commercially available Capsibiol–green tea mixture (Capsibiol-T) was from the manufacturer (Scientific Motive Systems, Terre Haute, IN). The genus and species of the African bird peppers utilized in the preparation of the Capsibiol was authenticated as Capsicum frutescens by Professor James Simon of Rutgers University through the auspices of the Purdue University Botanical Center. All products were compared on a weight basis dissolved in aqueous solution.

For the Figures, all components and mixtures were tested at a starting concentration (undiluted) of 125 mg dry solids mL^{-1} which for the green tea extract provided an EGCg amount equal to 100 mM. A starting dilution of 1:100 in Figures 3–7 was equal to 1.25 mg dry solids mL^{-1}.

Growth of cells

HeLa (ATCC CCL2) cells were grown in 175 cm^2 flasks in Minimal essential medium (Gibco), pH 7.4, at 37 °C with 10% bovine calf serum (heat-inactivated), plus 50 mg L^{-1} gentamicin sulfate (Sigma). Cells were harvested by scraping and taken up in 140 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 25 mM Tris, pH 7.4 to a final cell concentration of 0.1 g wet weight mL^{-1}.

The 4T1 mammary cancer cell line arose from a BALB/c C3H mouse (Miller et al 1987). The 4T1 cells were grown in DME-10, Dulbecco's modified Eagle's medium supplemented with 5% foetal calf serum, 5% newborn calf serum, 1 mM mixed non-essential amino acids, 2 mM L-glutamine, penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}).

Non-cancer MCF-10A human mammary epithelial cells were cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium containing glutamine (292 mg L^{-1}), gentamicin sulfate (50 mg L^{-1}), insulin (10 μg mL^{-1}), hydrocortisone (0.5 μg mL^{-1}), epidermal growth factor (20 mg mL^{-1}), and 5% horse serum. Medium was renewed every 2–3 days.

Growth was determined using a 96-well plate assay as described by Lin et al (1996). HeLa (5×10^4) or 4T1 (10^4) cells were distributed into each plate well (Costar tissue culture plate). The cells were grown at 37 °C for 24 h after which the substances to be evaluated were added followed by incubation for an additional 48 or 72 h as indicated. Medium was removed and the cells were washed with phosphate-buffered saline and then fixed by addition of 100 μL 2.5% (v/v) glutaraldehyde for 0.5 h followed by a distilled water wash. The cells were stained with 100 μL 1% aqueous crystal violet for 0.5 h, washed exhaustively with distilled water followed by 200 μL 33% (v/v) acetic acid for 5 min. The absorbance was determined at 580 nm using an automated plate reader. Growth was determined according to the formula $[(b - c) \times 100]/(a - c)$, where a = absorbance of cells in medium without treatment, b = cells in medium with treatment and c = medium alone (background).

Preparation of HeLa cells and cell-free extracts

HeLa S cells (grown in suspension) were collected by centrifugation and shipped frozen by a commercial supplier (Cellex Biosciences, Minneapolis, MN) in 0.1 M sodium acetate, pH 5, in a ratio of 1 mL packed cell volume to 1 mL acetate. The cells were thawed at room temperature, resuspended and incubated at 37 °C for 1 h to release the protein (del Castillo-Olivares et al 1998). The cells were removed by centrifugation at 37 000 g for 60 min and the cell-free supernatants were refrozen and stored in 1-mL samples at −70 °C.

For heat treatment, 1-mL samples of the above supernatant material were thawed at room temperature and heated to 50 °C for 10 min. The denatured proteins were removed by centrifugation (1500 g, 5 min). Full activity was retained from this step (del Castillo-Olivares et al 1998).

For protease treatment, the pH of the heat-stable supernatant was adjusted to 7.8 by addition of 0.1 M sodium hydroxide. Tritirachium album proteinase K (Calbiochem) was added (4 ng mL^{-1}) and incubated at 37 °C for 1 h with full retention of enzymatic activity and

drug response (del Castillo-Olivares et al 1998). The reaction was stopped either by freezing for determination of enzymatic activity or by addition of 0.1 M phenylmethylsulfonyl fluoride in ethanol to yield a final concentration of 10 mM phenylmethylsulfonyl fluoride.

Spectrophotometric assay of NADH oxidase

NADH oxidase activity was determined as the disappearance of NADH measured at 340 nm in a reaction mixture containing 25 mM Tris-(2[N-morpholino]ethanesulfonic acid) buffer (pH 7.2), 1 mM KCN to inhibit low levels of mitochondrial oxidase activity, and 150 μM NADH at 37 °C with stirring. Activity was measured using a Hitachi U3210 or SLM Aminco DW2000 spectrophotometer with continuous recording over two intervals of 5 min each. A millimolar extinction coefficient of 6.22 was used to determine specific activity. EGCg was added at the final concentrations indicated at the beginning of the assay and was present during the assay period.

Proteins were estimated by the bicinchoninic acid method (Smith et al 1985). Bovine serum albumin was the standard.

Statistical analyses

Experiments were repeated a total of five times. Results are averages ± s.d. The effect of ratio on growth was evaluated using the Kruskal–Wallis test with individual differences determined using Dunn's multiple comparison test. Growth responses to mixture dilutions were analysed using a two-way analysis of variance.

Results

The drug-responsive activity of an NADH oxidase preparation solubilized from the surface of HeLa cells was 50% inhibited by decaffeinated green tea extract at a dilution of 1:10^5 corresponding to an EGCg concentration of 1 μM (Table 1). The enzymatically-treated bird pepper (Capsicum frutescens) extract also inhibited but a dilution of 10^4 was required (same concentration of solids as in the green tea) to reach 50% inhibition. In contrast, 50% inhibition was achieved by a mixture of 50 parts green tea solids by weight plus 1 part Capsibiol solids by weight at a dilution of 1:10^6 equivalent to 0.1 μM EGCg i.e. there was an approximately 10-fold enhancement of the efficacy of green tea in inhibiting the drug-responsive NOX activity (tNOX) of the HeLa preparations.

The method of estimating the EC50 for inhibition of the drug-responsive NOX activity is shown in Figure 1 for the green tea–Capsibiol mixture of Table 1 and HeLa cells. Activity was estimated over the range of dilutions from 1:10^3 to 1:10^7. NADH oxidase activity expressed as nmol min^{-1} (mg protein)$^{-1}$ was comprised of two components. One was tumour cell-associated and susceptible to drug inhibition (tNOX), whilst the other was constitutive

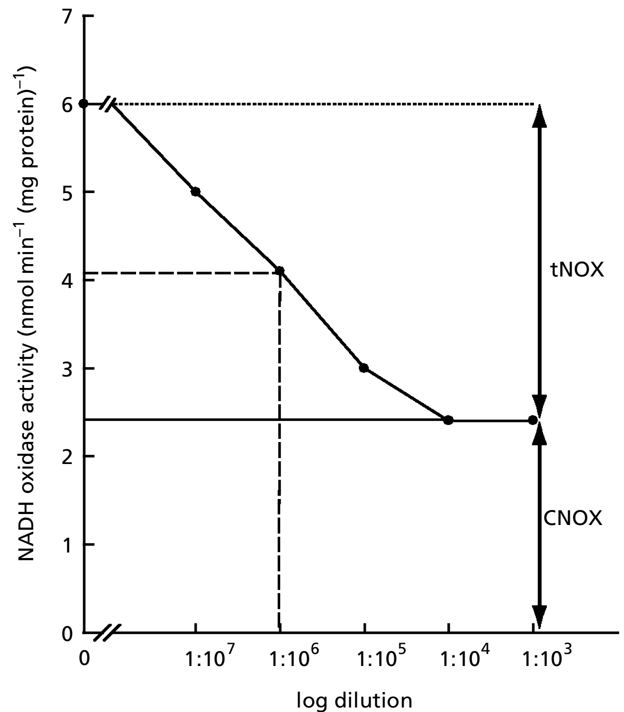

Figure 1 Dose–response of NADH oxidase (NOX) activity of partially purified and solubilized tNOX from the surface of HeLa cells to a (1:1) mixture of a green tea infusion plus liquid Capsibiol (ratio of total solids of 50:1). The NOX activity was derived from two components. One was cancer-specific and inhibited by green tea catechins (tNOX). The other was constitutive and resistant to inhibition by catechins (CNOX). The EC50 values reported in Table 1 were for inhibition of the tNOX component. The dilution of 1:10^3 was equal to 125 μg total solids mL^{-1} or 100 μM EGCg.

Table 1 Efficacy of a mixture of an infusion of decaffeinated green tea (Lipton's) and Capsibiol in inhibition of the activity of the tNOX component of NADH oxidase activity partially purified and solubilized from the surface of HeLa cells as described by del Castillo et al (1998). The green tea infusion was standardized to provide 100 mM EGCg before dilution.

	Dilution to give 50% inhibition of tNOX target activity	
	HeLa	4T1
Decaffeinated green tea infusion (undiluted = 100 mM EGCg)	1:10^5	1:10^7
Capsibiol	1:10^4	1:10^4
Decaffeinated green tea extract (50 parts) + Capsibiol (1 part)	1:10^6	1:10^8

Table 2 Growth of human cervical carcinoma (HeLa) and mouse mammary cancer (4T1) cells in culture showing synergy in growth inhibition (cell killing) by a mixture of an infusion of decaffeinated green tea (Lipton's) and Capsibiol. The green tea infusion was standardized to provide 100 mM EGCg before dilution.

	Dilution to reduce cell number by 50% after 72 h	
	HeLa	4T1
Decaffeinated green tea infusion (undiluted = 100 mM EGCg)	1:200	1:1000
Capsibiol	Not reached	Not reached
Decaffeinated green tea (50 parts) + Capsibiol (1 part)	1:2000	1:10 000

and refractory to drug inhibition (CNOX). In these studies approximately 60% of the activity of the solubilized HeLa preparations was tNOX with the remainder being CNOX. The values reported were the EC50 for inhibition of the tNOX component only.

Results with the drug-responsive NOX activity of 4T1 cells were similar although the NOX activity of these cells was more sensitive to both the decaffeinated green tea extract and to the green tea–Capsibiol mixture. For the decaffeinated green tea, the EC50 was reached at a dilution of $1:10^7$ whereas with the green tea–Capsibiol mixture, the EC50 was at a 10-times lower dilution of $1:10^8$.

A synergy between the decaffeinated green tea extract and the Capsibiol was observed with the growth of HeLa and 4T1 mouse mammary cells in culture (Table 2). Growth of HeLa cells was 50% inhibited at a dilution of 1:200 by the decaffeinated green tea extract whereas a mixture of 50 parts of the decaffeinated green tea extract (by weight of solids) plus 1 part Capsibiol inhibited by 50% at a dilution of approximately 1:2000. As with the drug-responsive NADH oxidase activity, the growth of the 4T1 mouse mammary cells exhibited a greater sensitivity to decaffeinated green tea and the combination of decaffeinated green tea with Capsibiol (Table 2). For the decaffeinated green tea alone, the EC50 was at a dilution of approximately 1:1000 whereas with the Capsibiol added, the EC50 was at a dilution of approximately 1:10 000.

The optimum ratio of 50 parts green tea to 1 part Capsibiol (w/w of dry solids) was determined based on inhibition growth of HeLa and 4T1 cells as illustrated in Table 3. Liquid Capsibiol was added to the tea leaf and dried before making the infusion to simulate the marketed product. Three lots of Capsibiol, tested individually and combined with green tea, were equally effective.

To increase the efficacy of the green tea–Capsibiol mixture, the decaffeinated green tea leaf was replaced by a decaffeinated green tea concentrate, obtained commercially from the same source as the Capsibiol. The green tea extract was 92% tea polyphenols of which 80% were catechins (4.8% (−)-epigallocatechin (EGC), 1.8% DL-catechin, 5.8% (−)-epicatechin (EC), 50.0% (−)-epigallocatechin-3-gallate (EGCg), 2.3% gallocatechin gallate (GCG) and 15.3% (−)-epicatechin gallate (ECG)). The caffeine content was < 1%. Here the optimum ratio of decaffeinated green tea extract to lyophilized Capsibiol (by weight) was found to be 25:1 based on the growth response of mouse mammary 4T1 (Figure 2A) and HeLa (Figure 2B) cells as well as on the inhibition of tNOX activity (not shown).

The green tea extract inhibited the growth of HeLa cells (Figure 3A) by 50% at a dilution of approximately 1:2000 and that of the 4T1 mouse mammary cells at a dilution of 1:20 000. Capsibiol alone was much less inhibitory (EC50 at a dilution of approximately 1:500). With the mixture of 25 parts green tea extract plus 1 part Capsibiol by weight, the EC50 for inhibition of growth was reached at a dilution of approximately 1:20 000 (Figure 3A, B), whereas with 4T1 mouse mammary cells (Figure 3C, D)

Table 3 Optimum ratio of a green tea infusion (Lipton's) to Capsibiol determined from growth of HeLa and 4T1 cells in culture over 72 h. Results are averages of three determinations ± s.d. at a green tea infusion (no Capsibiol) or a green tea infusion + Capsibiol dilution of 1:1000.

Ratio Capsibiol:green tea (w/w)	Growth (% of control)	
	HeLa	4T1
No Capsibiol	90 ± 5	30 ± 8
1:100	88 ± 7	36 ± 10
1:50	66 ± 6*	13 ± 11†
1:25	81 ± 12	20 ± 9
1:5	83 ± 7	20 ± 8

*Significantly different from no Capsibiol and the 1:100 ratio ($P < 0.007$). †Significantly different from no Capsibiol and the 1:100 ratio ($P < 0.02$).

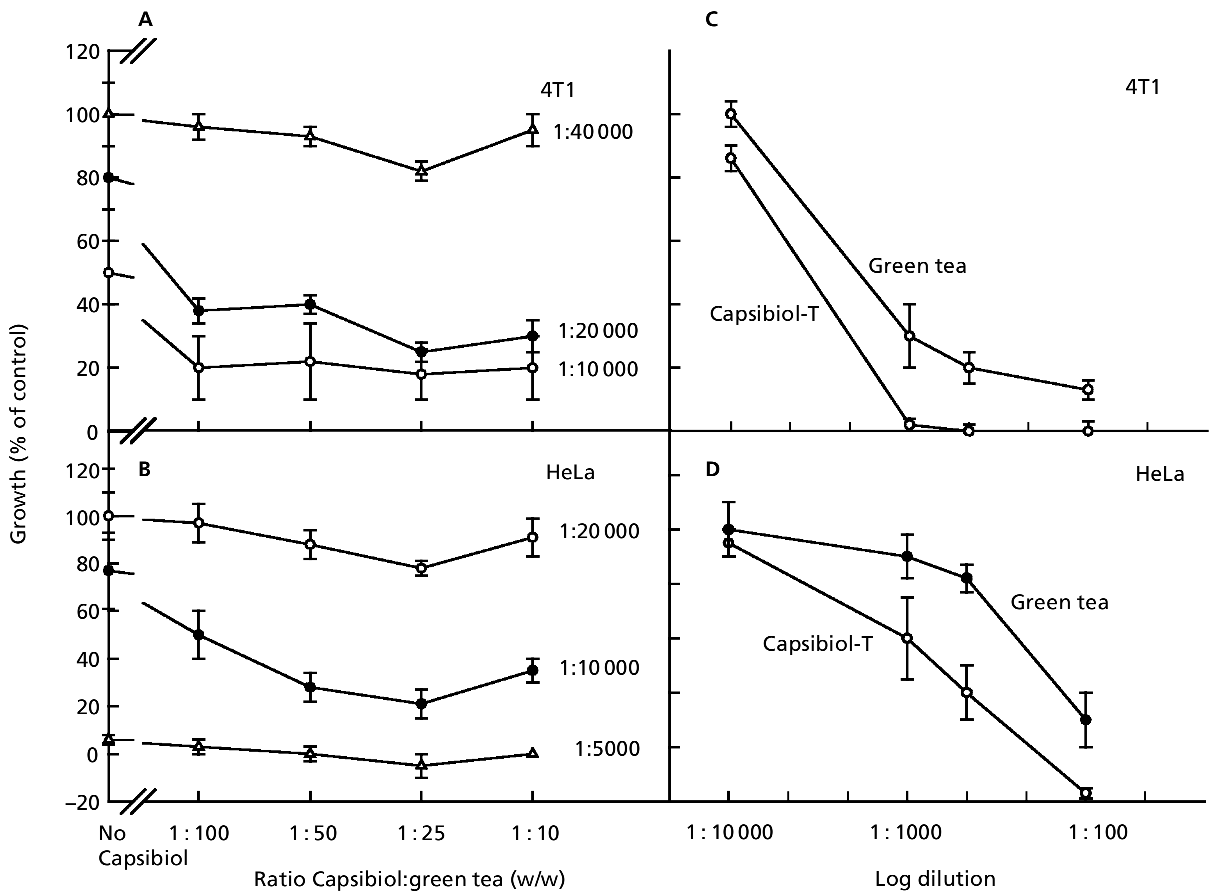

Figure 2 Optimum ratio of Capsibiol to a green tea concentrate (A, B) or Capsibiol to green tea (C, D) determined from growth of 4T1 (A, C) and of HeLa (B, D) cells in culture over 72 h. For green tea concentrate, the ratios were determined for three different dilutions in the media (1:5000, 1:10 000 and 1:20 000 for the HeLa cells and 1:10 000, 1:20 000 and 1:40 000 for the 4T1 cells). For the Capsibiol-T decaffeinated green tea comparisons, the Capsibiol-T was manufactured with a ratio of 1 part Capsibiol to 50 parts decaffeinated green tea solids on a dry weight basis. The dilution of 1:100 was equal to 1.25 mg total solids mL^{-1} Capsibiol-T (1 mM EGCg) and was compared with the indicated dilutions of a green tea infusion (Methods). Results were averages of five determinations ± s.d. The dilution of 1:10 000 was equal to 12.5 μg total solids mL^{-1} or 10 μM EGCg.

the EC50 for inhibition of growth was at a dilution of approximately 1:50 000. When the mixture of 25 parts concentrated green tea extract and 1 part lyophilized Capsibiol was compared with decaffeinated green tea, the mixture was approximately 100-times more effective in the inhibition of growth of HeLa (Figure 3E, F) and 4T1 mouse mammary cells in culture (Figure 3G, H).

A non-cancer human mammary epithelial cell line, MCF-10A, which lacks tNOX, was used as a control. The constitutive NOX activity of these cells was unaffected by green tea extracts or Capsibiol-T. No growth inhibition or apoptosis was observed at dilutions equivalent to 50 μM EGCg or less in agreement with previous findings (Morré et al 2000).

The activity of extracts of a commercially available decaffeinated green tea–Capsibiol combination (50 parts tea:1 part Capsibiol), trade name Capsibiol-T, gave growth responses (Figure 2C, D) similar to those obtained by aqueous combination of the two ingredients (Table 2).

Discussion

Our work identified tNOX, a cancer specific growth protein, as a target for EGCg to help explain the anticancer benefits of tea. NOX proteins are located at the surface of cells (Morré 1995) where they are responsible for the increase in cell size following cell division (Morré 1998). When cells divide, the cells must reach some minimum size in order to divide again. Cells in which NOX activity is blocked are unable to enlarge. Being unable to enlarge, they cease to divide and, after several days, undergo programmed cell death (apoptosis) (Morré et al 1995, 2000; Morré 1998). The growth inhibitions reported here are due largely to cell cycle arrest in G_1. Cell death by apoptosis followed after approximately 72 h of treatment.

Constitutive NOX activities (CNOX) are regulated and growth factor responsive (Bruno et al 1992). In contrast, cancer cells express a NOX activity (tNOX) that is unregulated and constitutively activated (Bruno et al

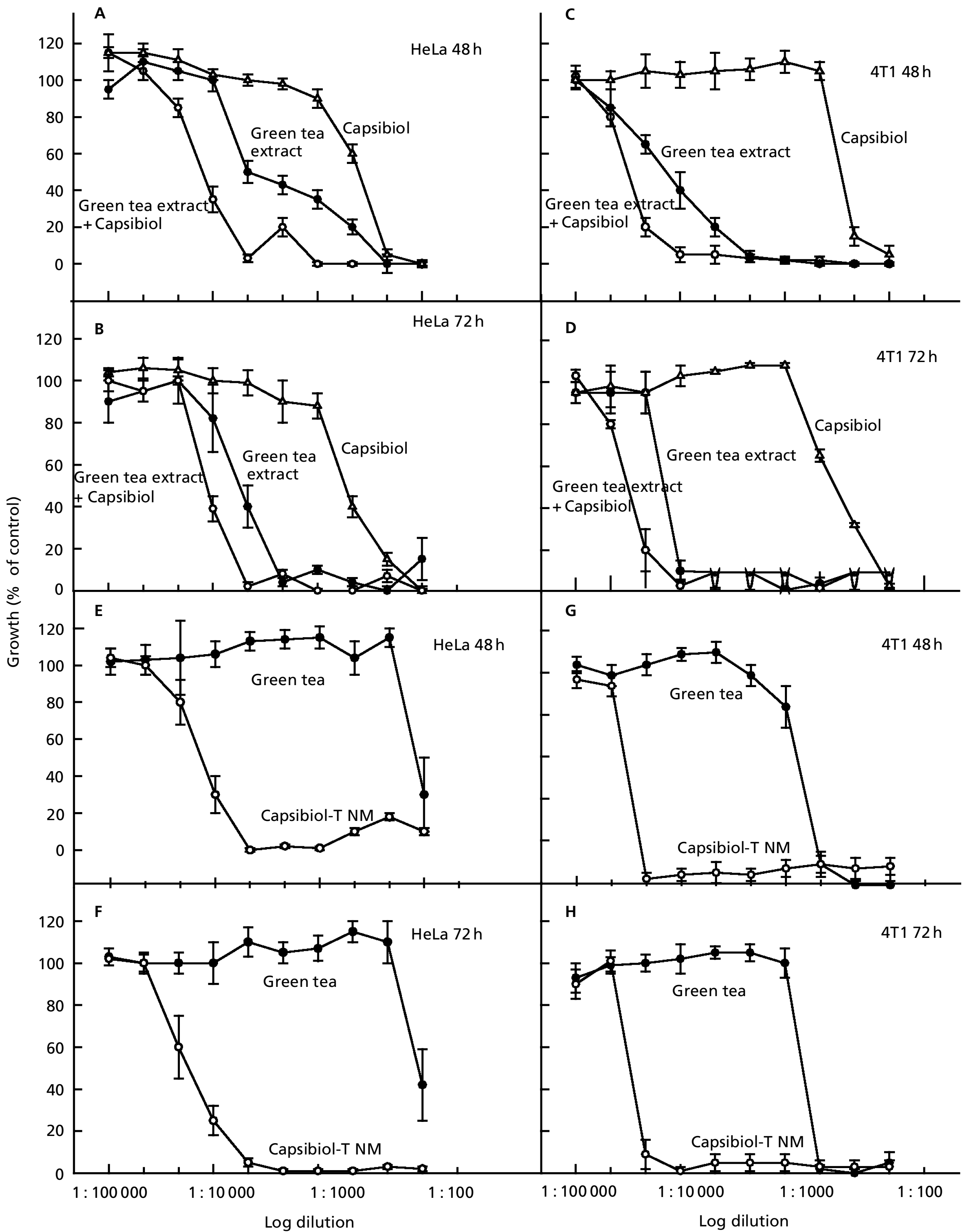

Figure 3 Dose response of HeLa or 4T1 mouse mammary cells in culture. A, B. Mixture of decaffeinated green tea extract plus Capsibiol in a ratio of 25:1 (o), compared with green tea extract alone (•) and Capsibiol alone (Δ). A. After 48 h. B. After 72 h. C, D. As for A, B except 4T1 mouse mammary cells. E, F. Green tea (Sencha, Japan) infusion compared with a green tea concentrate and a 25:1 green tea concentrate–Capsibiol-T mixture (Capsibiol-T NM) prepared on an equivalent weight basis (A–D) or a green tea infusion–Capsibiol mixture (Capsibiol-T NM) (E–H). E. After 48 h. F. After 72 h. G, H. As in E, F except 4T1 mouse mammary cells. The dilution of 1:100 was equal to 1.25 mg total solids mL^{-1} of Capsibiol-T NM (1 mM EGCg) and was compared with the indicated dilutions of the green tea concentrate (A–D) or a green tea infusion (E–H) prepared on an equivalent weight basis (Methods). Results were averages of three to five determinations ± s.d. Values separated by at least two standard deviations were highly significant.

1992; Morré et al 1995). Growth and enlargement of cancer cells also are unregulated and activated constitutively. Expression of an unregulated NOX form by cancer cells may be important to unregulated cell proliferation as cell enlargement would no longer be rate limiting to cell cycle traverse (Morré 1998).

tNOX appears to be universally expressed in all forms of human cancer (pancancer) (Morré & Reust 1997; Morré et al 1997). It is absent from normal (non-transformed) cells. While tNOX expression is likely insufficient to cause cancer, it may be necessary to achieve the unregulated cell enlargement that must accompany unregulated cell division and the loss of growth control that typifies cancer.

A major difference that distinguishes the cancer-(tumour-) specific tNOX protein and potential tea and vanilloid target (Morré et al 1995, 1996, 2000) from CNOX, the non-cancer NOX protein, is that tNOX, but not CNOX, is inhibited by agents that induce cells to differentiate and by antitumour drugs that act at cell surface targets (Morré 1998). The antitumour drugs targeted to tNOX are without effect on the activity of CNOX at therapeutic doses and, at therapeutic doses, slow the growth of cancer cells but not that of non-cancer cells (Morré et al 1995, 1996).

Among the safest and most effective tNOX blockers is EGCg, the most active anticancer constituent of green tea (Mitscher et al 1997). Capsaicin and EGCg block tNOX activity and growth of tumour cells without adverse effects on normal cells (Liao et al 1995; Stoner & Mukhtar 1995; Ahmad et al 1997; Chen et al 1998; Katdare et al 1998; Conney et al 1999; Mukhtar & Ahmad 1999; Morré et al 2000) or individuals (Pisters et al 2001). In this report, we have demonstrated that the efficacy of green tea could be enhanced 10-fold by combination with a commercially-obtained Capsicum preparation. The peppers were treated enzymatically to cleave the noneamide functions that result in neurological discomfort but seem relatively unimportant to anticancer activity.

Since the Capsicum extracts were more active than would be predicted on the basis of their capsaicin content, it was reasonable to assume that the active principle(s) in the Capsibiol that contributed to the synergy were not exclusively the pungent vanilloids. Therefore, the Capsicum extracts were standardized on the basis of their synergistic interaction in inhibition of cell growth rather than on the basis of capsaicin content, for example. That more than one constituent may contribute to the activity of Capsibiol is a reasonable expectation such that standardization to any single vanilloid or capsacinoid present in the mixture would be premature and potentially inappropriate.

In previous studies, we have examined a considerable number of synergies among green tea, green tea catechins and other plants or plant products with reported chemopreventative or anti-carcinogenic properties including proanthocyanadins, anthocyanins, blue fruit extracts, turmeric, soy isoflavones and grape extracts. None were as effective as those encountered with Capsibiol.

Assuming 2 g of green tea per cup in preparing the infusion, our findings suggested that a 350 mg capsule of the combination of green tea extract plus Capsibiol would be equivalent to drinking 16 cups of green tea based on the comparative responses of growth of 4T1 and HeLa cells. It would be beneficial to determine if this apparent 100-fold enhancement of anti-cancer activity demonstrated with cells in culture would be reflected in a comparable effect on tumour growth in-situ.

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