© *Pharmacology & Toxicology* 2003, **92**, 234–241.
Printed in Denmark . All rights reserved

Pharmacology & Toxicology
ISSN 0901-9928

Tea Catechin Synergies in Inhibition of Cancer Cell Proliferation and of a Cancer Specific Cell Surface Oxidase (ECTO-NOX)

D. James Morré[1], Dorothy M. Morré[2], Howard Sun[3], Raymond Cooper[3,4], Joseph Chang[3] and Elsa M. Janle[4]

Departments of [1]Medicinal Chemistry and Molecular Pharmacology and [2]Foods and Nutrition, Purdue University, West Lafayette, IN 47907, [3]Pharmanex, Provo, UT and [4]Bioanalytical Systems, Inc., West Lafayette, IN 47906, U.S.A.

Received August 26, 2002; Accepted December 12, 2002

Abstract: The anticancer properties of tea catechins are most frequently attributed to the principal catechin (-)-epigallocatechin-3-gallate (EGCg). Efficacy was evaluated using growth of cultured HeLa cells and inhibition of the enzymatic activity of a putative cell surface tea target enzyme, a cancer-associated cell surface-located NADH oxidase (ECTO-NOX) designated tNOX. The amounts of EGCg required to inhibit by both criteria was reduced 10 times by combination with inactive catechins such as (-)-epicatechin (EC), (-)-epigallocatechin (EGC) or (-)-epicatechin-3-gallate (ECG). Various synthetic mixtures based on purified catechins and decaffeinated tea extracts treated enzymatically to reduce the ester bond-containing catechins varying in EGCg content from 0.065 to 40% were of comparable efficacy to decaffeinated green tea extracts as long as EGCg was present and the ratio of total catechins to EGCg + EGC was about 1.5. Such mixtures appear to offer potential cancer protection and therapeutic advantages over those of EGCg alone through lowered toxicity of the mixture to normal cells and for more efficient blood delivery of orally-administered catechins to a tumour site.

Previous work in our laboratories has identified cell surface-associated proteins with ubiquinol or NADH oxidase activity, ECTO-NADH oxidases or ECTO-NOX proteins. A tumour- (cancer-) specific ECTO-NOX, designated tNOX, has been cloned and characterized (Chueh *et al.* 2002a & b) and postulated to represent the molecular target to explain the cancer specific inhibition of cancer cell growth by the principal green tea catechin (-)-epigallocatechin-3-gallate (EGCg) (Morré *et al.* 2000; Chueh *et al.* 2002b). EGCg does inhibit specifically both the tNOX activity of cancer cell lines (Morré *et al.* 2000) and the growth of cancer cell lines (Liao *et al.* 1995; Ahmad *et al.* 1997; Mitscher *et al.* 1997; Chen *et al.* 1998; Fujiki *et al.* 1998) without inhibition of the constitutive ECTO-NOX, CNOX, of non-transformed cells and tissues (Morré *et al.* 2000) or the growth of non-transformed cells in culture (Liao *et al.* 1995; Ahmad *et al.* 1997; Mitscher *et al.* 1997; Chen *et al.* 1998; Fujiki *et al.* 1998; Morré *et al.* 2000). By combining vector-forced overexpression of tNOX cDNA and antisense, we have demonstrated the tNOX target to be both necessary and sufficient to explain the anticancer properties of green tea catechins (Chueh *et al.* , unpublished results). In this report we used measurements of tNOX activity and growth of HeLa cells as the basis for an investigation of natural synergies among green tea catechins which may be important to their employment in cancer prevention and therapy.

Author for correspondence: D. James Morré, Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, 201 South University Street, West Lafayette, IN 47907–2064, USA (fax +1 765 494–4007, e-mail morre@pharmacy.purdue.edu).

Materials and Methods

Materials. All chemicals were from Sigma (St. Louis, MO, USA) unless otherwise specified. Tegreen was provided by Pharmanex (Brisbane, CA now Provo, UT, USA). Tea (Lipton decaffeinated green tea bags) infusions were prepared by sequential steeping of ca. 2 g bags of tea in 10 ml of water for 10 min. each. At the end of the infusion, bags were pressed to remove liquid.

Growth of cells. HeLa (ATCC CCL2) cells were grown in 175 cm^2 flasks in Minimal Essential Medium (Gibco), pH 7.4, at 37° with 10% bovine calf serum (heat-inactivated), plus 50 mg/l gentamycin sulfate (Sigma). Between 20 and 30 cells/mm^2 were seeded. 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 per ml.

Growth was determined microscopically by counting the number of attached cells over defined areas consisting of a grid of 1 mm squares. Numbers of HeLa cells (both attached and detached) were determined in parallel using a hemocytometer. Cell lines were from the American Type Culture Collection (Rockville, MD, USA).

Purification of plasma membranes from cultured cells. Cultured cells were collected by centrifugation for 6–15 min. at 175–1000 × g. The cell pellets were resuspended in 0.2 mM EDTA in 1 mM $NaHCO_3$ in an approximate ratio of 1 ml per 10^8 cells and incubated on ice for 10–30 min. to swell the cells. Homogenization was achieved in 7- to 8-ml aliquots with a Polytron homogenizer (Brinkmann) for 30–40 sec. at 10,500 rpm, using a PT-PA 3012/23 or ST-10 probe. To estimate breakage, the cells were monitored by light microscopy before and after homogenization. At least 90% cell breakage without breakage of nuclei was achieved routinely. The homogenates

were centrifuged for 10 min. at 175 × g to remove unbroken cells and nuclei and the supernatant was centrifuged a second time at 1.4×10^6 g × min. (e.g. 1 h at 23,500 × g) to prepare a plasma membrane-enriched microsome fraction. The supernatant was discarded and the pellets were resuspended in 0.2 M potassium phosphate buffer in a ratio of ≈ 1 ml per pellet from 5×10^8 cells. The resuspended membranes were then loaded onto the two-phase system constituted on a weight basis consisting of 6.6% (w/w) Dextran T-500 (Pharmacia) and 6.6% (w/w) Polyethylene Glycol 3350 (Fisher) in a 5 mM potassium phosphate buffer (pH 7.2) for aqueous two-phase separation as described (Morré & Morré, 1989 & 2000). The upper phase, enriched in plasma membranes, was diluted 5-times with 1 mM sodium bicarbonate and the membranes were collected by centrifugation. The purity of the plasma membrane was determined to be > 90% by electron microscope morphometry. The yield was 20 mg plasma membrane protein from 10^{10} cells.

Preparation of HeLa cells and cell-free extracts. HeLa S cells were collected by centrifugation and shipped frozen in 0.1 M sodium acetate, pH 5 in a ratio of 1 ml packed cell volume to 1 ml of acetate (Cellex Biosciences, Minneapolis, MN, USA). The cells were thawed at room temperature, resuspended and incubated at 37° for 1 hr 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 aliquots at -70°.

For heat treatment, 1 ml aliquots of the above supernatant material were thawed at room temperature and heated to 50° for 10 min. The denatured proteins were removed by centrifugation (1,500 × 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) and incubated at 37° for 1 hr 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 (PMSF) in ethanol to yield a final concentration of 10 mM PMSF.

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-Mes buffer (pH 7.2), 1 mM KCN to inhibit low levels of mitochondrial oxidase activity, and 150 μM NADH at 37° 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) with bovine serum albumin as standard.

Fluorescence microscopy. Cells were grown for 72 hr on glass coverslips placed in small culture dishes with media containing EGCg in ethanol or an equivalent amount of ethanol alone. The coverslips were rinsed and the cells fixed in methanol followed by addition of fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) as described (Wolvetang *et al.* 1994). Cells were observed and photographed at a primary magnification of 400 X.

Determination of EGCg. EGCg amounts in the hot water extracts were determined using the standardized chromatographic procedure described by Katiyar *et al.* (1992). Authentic EGCg (Sigma) was used as the standard.

Preparation of catechins with reduced levels of EGCg. Standardized green tea extract, Tegreen97® from Pharmanex (Provo, UT, USA) consisting of 97% polyphenols of which 72% of the polyphenols were catechins and 36% was EGCg (table 1) was utilized. Tannase was obtained from Kikkoman Corp. (Japan). HPLC grade acetonitrile, methanol, and ethanol were obtained from Brudick and Jackson Labs, Inc. (USA). Analytical grade chloroform, formic acid, NaH_2PO_4, Na_2HPO_4 and K_2HPO_4 were obtained from Sigma (St. Louis, MO, USA). Sephadex LH-20 resin was obtained from Amersham Pharmacia Biotech (UK).

Initial levels of the catechins in the green tea extract were determined by High Pressure Liquid chromatography (HPLC) using aqueous acetonitrile as an effluent. Tannase (37.5 mg) and green tea extract (205.3 mg) were transferred to a 250 ml Erlenmeyer flask. One hundred ml buffer solution (0.2 M Na_2HPO_4 + 0.05 M K_2HPO_4), with a pH between 6.0 and 6.2, was added to the flask. The flask was heated to 38° to 40° for 30 min. in a shaker bath.

Alternately, immobilized tannase was used for the incubation step. The modified resin was prepared by immersing a macroporous polystyrene anion exchange resin in a 0.002 M buffer of Na_2HPO_4 and Na_2HPO_4 with a pH of 6.8. 4 g of resin (wet weight) was added into 40 ml of 2.5% glutaraldehyde solution. The glutaraldehyde-resin solution was stirred for 2 hr at room temperature. The modified resin was washed with water. Tannase (4 g) was dissolved in 20 ml of buffer at pH 6.8 to which the modified resin was added. The reaction was stirred at room temperature over night. Excess enzyme was washed out with buffer.

The activated resin with immobilized tannase was placed in a packed-bed reactor. A 0.5% solution of green tea extract in buffer of pH 6.8 was eluted with buffer on the packed-bed reactor at a flow rate of 0.1 ml/min.

The resultant solution from the incubation step was evaporated under a vacuum to obtain a residue of a volume of approximately 2 ml. The residue was extracted with 10 ml of ethyl acetate. The ethyl acetate was separated and put aside. The ethyl acetate extraction was repeated. The aliquots of ethyl acetate were combined and evaporated under a vacuum. This process resulted in a residue designated 92A.

92 A (70 mg) was dissolved in 1 ml of 90% ethanol and applied to a Sephadex LH-20 column. The column was eluted with 90% ethanol. Fractions of 2.5 ml were collected with a flow rate of 0.5 ml/min. The fractions were examined using TLC (chloroform:methanol:formic acid 9.0:1.0:0.2). Fractions 27 to 36 of 80 total fractions contained gallic acid. The remaining fractions, i.e., Fractions 1–26 and 37–80, were combined and evaporated to dryness. This process resulted in a residue designated 92B.

Statistical analyses. Means and standard deviations were analyzed for statistical significance using a one-tailed T-test.

Results

Effects of decaffeinated green tea extract and individual catechins on NOX activity and growth of HeLa cells.

The response of NADH oxidase of HeLa cells and HeLa cell growth to a decaffeinated green tea extract (Tegreen) and various catechin constituents of green tea (fig. 1) are shown in fig. 2–4. For Tegreen, which contains 36% EGCg, the EC_{50} for growth of inhibition of HeLa cells over 72 hr is a single dose of between 1.25 and 2.5 μg/ml (fig. 2B). Tegreen also is effective in inhibiting the activity of the cancer-specific tNOX component of the NADH oxidase activity (ca. 50% of the total NADH oxidase) with an EC_{50} of about 0.25 μg/ml (fig. 2A). The remainder of the NADH oxidase activity was due to the constitutive CNOX that was refractory to inhibition.

When individual green tea catechins were evaluated in a

Table 1.

Percentage ratio of catechins before or after treatment of Tegreen 97® with tannase[a].

Content (%)	Tegreen 97®	92A	92B	DTG118	DTG120	DTG121
EC	6.67	18.6	18.2	48.55	24.2	27.3
EGC	3.16	28.1	37.6	26.19	45.1	44.7
ECG	15.40	0.1	0.19	0.06	0.035	0.04
EGCg	36.20	0.07	0.1	0.49	0.065	0.95
dl-C	1.83	3.1	2.1	7.64	3.28	4.09
GC	1.89	5.8	7.8	–	–	–
GCG	6.64	0.32	0.31	0.13	0.03	0.29
(EC+EGC+ECG)/EGCg	0.7:1	667:1	293:1	153:1	1067:1	76:1

[a] See fig. 2 for explanation of catechin abbreviations (GC, gallocatechin) and table 2 and text for explanations of the Tegreen 97® derivatives 92A, 92B, DTG118, DTG120 and DTG121.

similar manner (fig. 3 and 4), only EGCg (fig. 3A and 4A) exhibited significant activity with an EC_{50} for inhibition of HeLa growth of ca. 2 μM (1 μg/ml). All other catechins tested were without effect or were much less effective.

Synergies among catechins.

When individual tea catechins were combined, the possibility for synergistic interactions emerged. For example, neither 1 nM EGCg alone no 10 to 100 μM (-)-epicatechin (EC) alone were effective in inhibiting tNOX activity of HeLa cells (fig. 5). However, when combined, the mixture was strongly inhibitory. This interaction first observed with measurement of tNOX activity was recapitulated with growth of HeLa cells in culture (fig. 6). EC (100 μM) was without effect on HeLa cell growth but when combined with EGCg, the EGCg concentration required to inhibit growth was reduced by one to two log orders to lower concentrations.

Synthetic catechin mixtures.

Synthetic catechin mixtures in which the ratio of non-gallic acid esterified catechins to gallic acid esterified catechins was increased to 100 or more were developed and tested. Such mixtures were practically achieved by tannase-treatment of Tegreen (table 1). Unexpectedly, some of these preparations (e.g., DTG-120, DTG-121 and 92B) were inhibitory to the growth of HeLa cells as low as 0.5 μg/ml (e.g., DTG-120) despite an extremely low EGCg content of

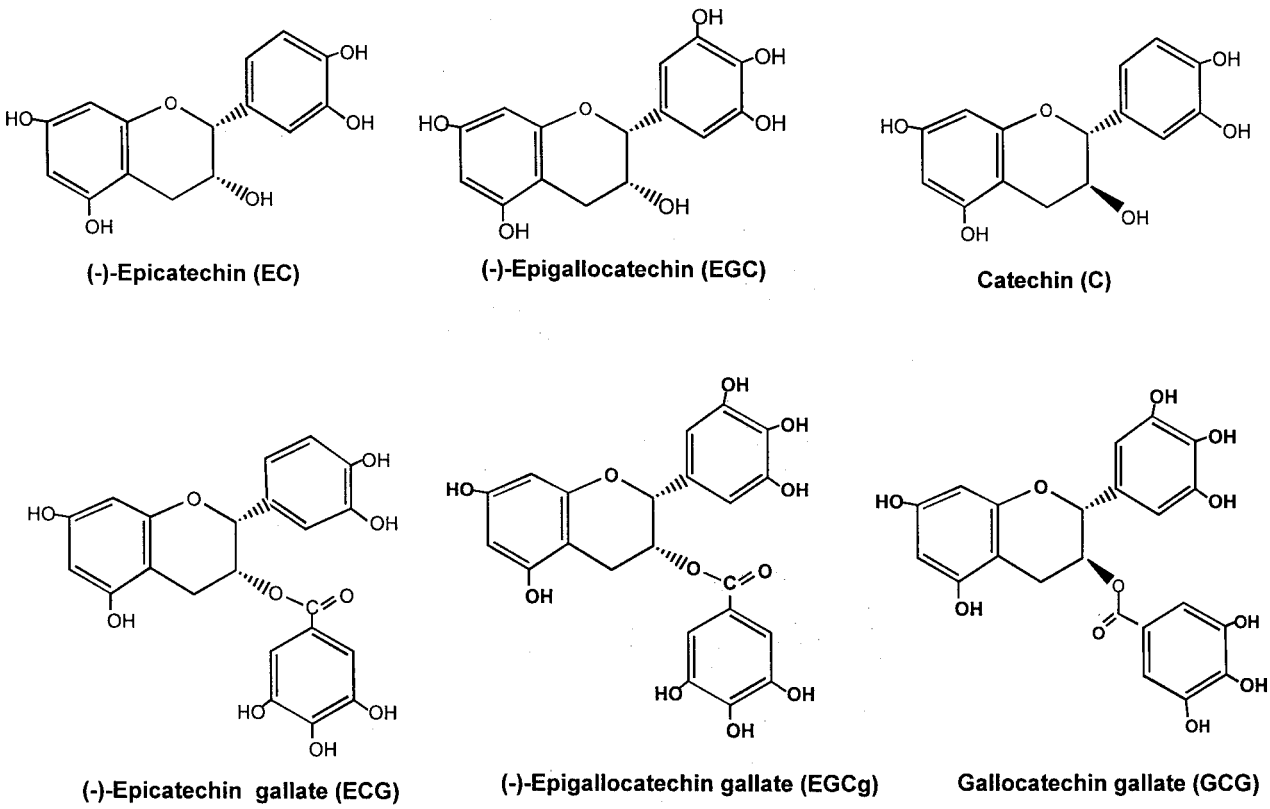

Fig. 1. Structures and abbreviations of catechins.

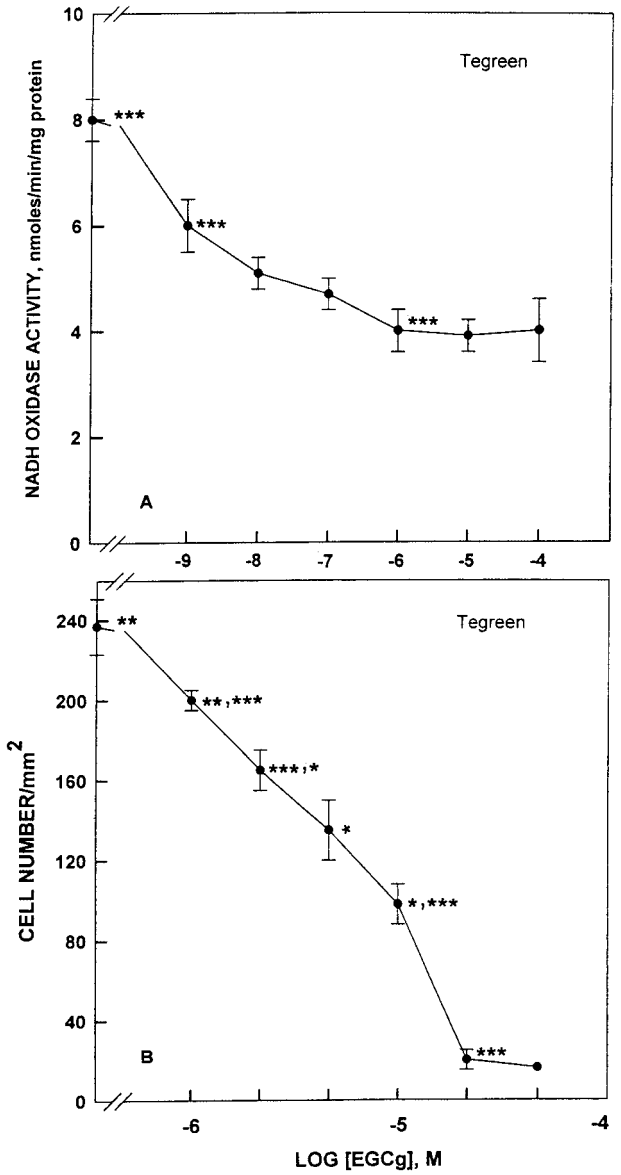

Fig. 2. Inhibition of oxidation of NADH by a solubilized tNOX preparation from HeLa cells (A) and inhibition of HeLa cell growth (B) by a decaffeinated green tea concentrate (Tegreen) containing 40% (-)-epigallocatechin-3-gallate (EGCg). Significant differences are indicated by *** $P<0.005$ in A and $P<0.001$ in B, ** $P<0.01$ and * $P<0.05$.

less than 1% of the total catechin mixture. These relationships are summarized in table 2. However, 92A and DTG-118, also with ratios of non-gallic acid ester catechins to gallic acid ester catechins of ca. 100, were much less effective in growth inhibition. These discrepancies correlated with differences in (-)-epigallocatechin (EGC) content (fig. 7).

To examine these relationships further, a synthetic mixture of catechins corresponding to that of DTG-120 was prepared and tested at 10 μM total catechins on growth of HeLa cells over 72 h following a single addition of catechins (fig. 8). The complete mixture and the complete mixture lacking ± catechin (dl-c) and/or (-)-gallocatechin-3-gallate (GCG) were comparable. However, if the small amount of EGCg present in the mixture was removed, efficacy was greatly reduced and dropped even further upon removal of EGC. The large amount of EC and ECG present were, of themselves, not growth inhibitory, but interacted synergistically with the gallic acid ester catechins to inhibit HeLa cell growth. Most important in this regard was EGC (table 2).

Discussion

In this report, we describe several interesting synergies based on inhibition of tNOX activity and growth of HeLa cells which may contribute to the anticancer efficacy of green tea catechins. Of these, only one catechin, EGCg, was required for activity (Morré *et al.* 2000). Other tea catechins, with the possible exception of ECG were without effect either on the oxidation of NADH by tNOX or on HeLa cell growth. The possible molecular basis for the synergy between the different neutral and acidic catechin fractions will be the subject of a separate report involving kinetic analyses of catechin inhibitions.

Gallic acid and caffeine when tested separately on NOX activity over the concentration range 0.01 to 100 μM were without effect. GCG at 10 μM or greater, on the other hand, stimulated NOX activity.

The relationship between inhibition of tNOX activity and growth of cancer cells is explained on the basis that substances such as the anticancer sulfonylurea, LY181984, the vanilloid capsaicin and EGCg that inhibit specifically the activity of tNOX and not that of CNOX do so by preventing cells from enlarging (Morré *et al.* 1995, 1997 & 2000; Wang *et al.* 2001) following cell division. Traverse through the cell cycle is highly dependent upon a G_1 checkpoint that monitors cell size (Morgan 1997). When inhibited by EGCg, the small cells are unable to enlarge, they fail to divide, and they begin to undergo apoptosis after about 48 to 72 hr of inhibition (Morré *et al.* 1995, 1997 & 2000; Wang *et al.* 2001).

EGCg is relatively non-toxic (Dreosti 1996; Bushman 1998; Fujiki *et al*; 1999; De Maat *et al.* 2000; Pisters *et al.* 2001). However, in the range of 50 to 100 μM a number of normal cellular functions appear to be influenced by EGCg such as inhibition of prolyl endopeptidase (Kim *et al.* 2001), proteosome activity *in vitro* and *in vivo* (Nam *et al.* 2001), EGF signaling pathway components (increases in p21 (Cipl) and p27 (Kipl) proteins and a reduction in the hyperphosphorylated form of pRN (Liberto & Cobrinik 2000; Masuda *et al.* 2001)), inhibition of TNF-alpha gene expression (Okabe *et al.* 1999), Her-2Neu signaling and transformed phenotype in breast cancer cells (Pianetti *et al.* 2002), cytokine production (Tomita *et al.* 2002), vascular endothelial growth factor receptor phosphorylation (Lamy *et al.* 2002), leukocyte elastase (Sartor *et al.* 2002), fatty acid synthetase (Wang *et al.* 2001), the PDGF-BB-induced

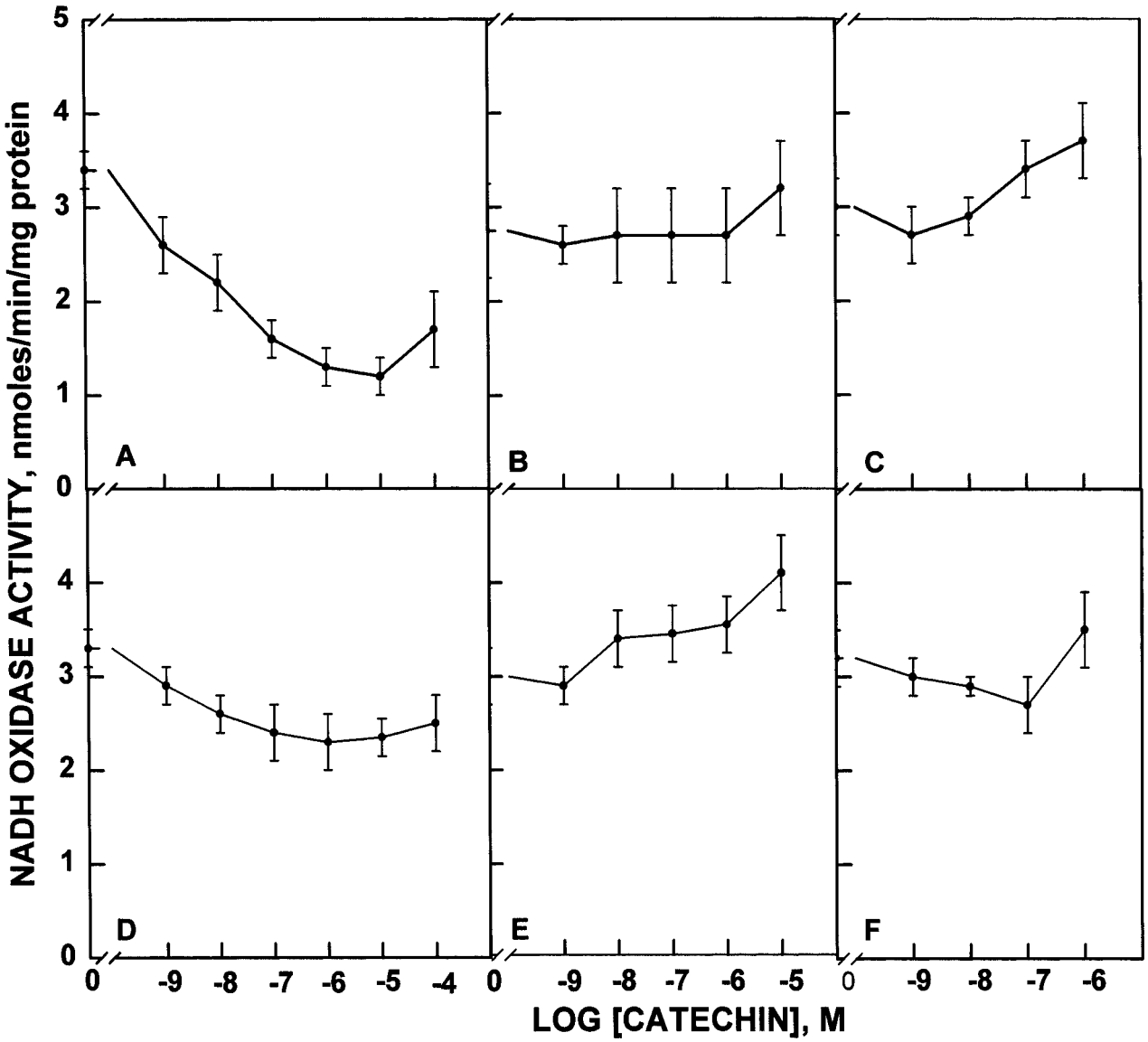

Fig. 3. Response of oxidation of NADH by solubilized preparation of tNOX from HeLa cells to green tea catechins tested singly. A. (-)-epigallocatechin-3-gallate (EGCg). B (-)-epicatechin (EC). C. (-)-epicatechin gallate (ECG). D. ± catechins. E. (-)-epigallocatechin (EGC). F. gallocatechin-3-gallate (GCG). Significant differences ($P<0.005$) are indicated by ***.

Table 2.

Summary of catechin amounts inhibitory to growth of HeLa (Human Cervical Carcinoma) cells in culture.

Catechin source		Ratio: EGCg+EC+EGC+ECG	μg/ml	
	EGCG, %	EGCg+EGC	EC_{50}	EC_{100}
Tegreen 97®[a]	40	1.6	1.25–2.5	6
DTG 92B[b]	0.1	1.5	2.5	5
DTG 120[b]	0.065	1.5	2.5	5
DTG 120R[c]	0.1	1.5	0.5	1
EGCg	95	1.0	1.0	4

[a] A decaffeinated extract of green tea containing 97% polyphenols of which 72% of the polyphenols are catechins and 36% are EGCg (table 1).
[b] Green tea preparations in which Tegreen 97® was treated enzymatically to alter the EGC to EGCg ratio.
[c] A composition duplicating that of DTG 120 reconstitued from purified individual catechins.

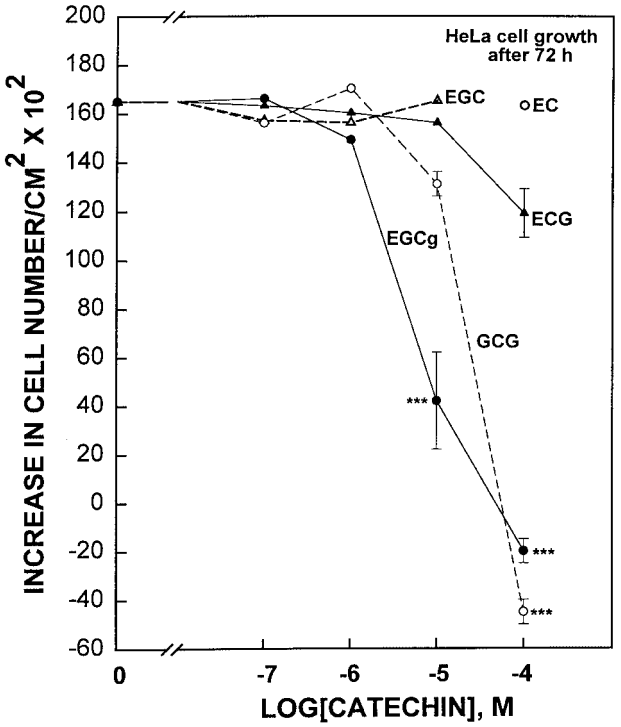

Fig. 4. As in fig. 3 except growth of HeLa cells in culture after 72 hr. Significant differences ($P<0.001$) are indicated by ***.

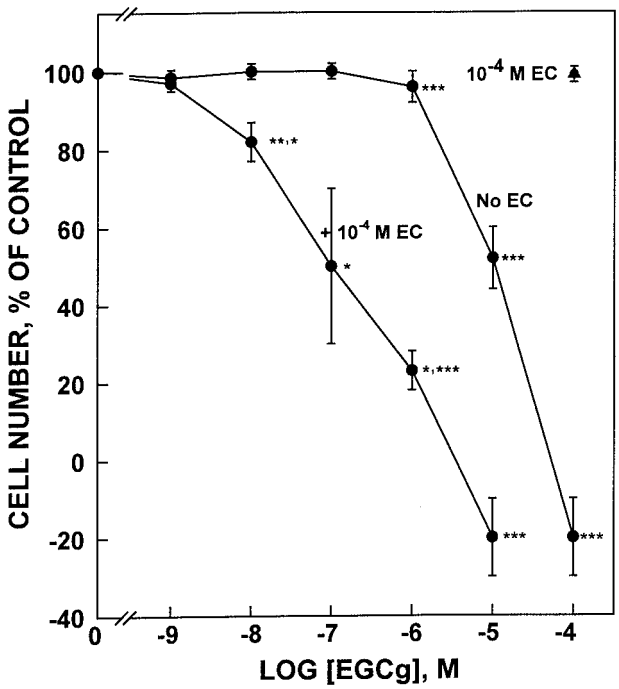

Fig. 6. Response of growth of HeLa cells after 72 hr in culture to varying concentrations of (-)-epigallocatechin-3-gallate (EGCg) alone, and EGCg in combination with 100 μM EC. Significant differences are indicated by *** $P<0.001$, ** $P<0.01$, and * $P<0.05$.

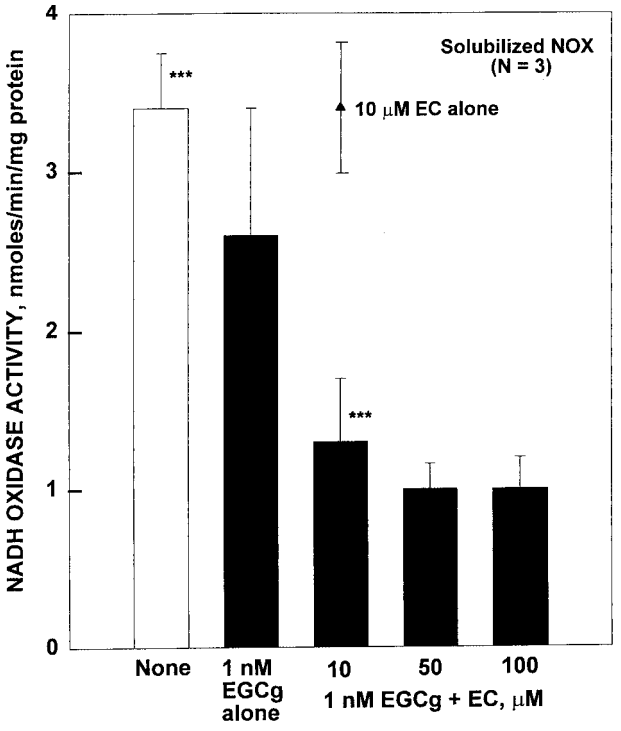

Fig. 5. Response of NADH oxidase partially purified from plasma membrane vesicles of HeLa cells to (-)-epigallocatechin-3-gallate (EGCg) alone, epicatechin (EC) alone and in various combinations. Significant differences ($P<0.002$) are indicated by ***.

intracellular signal transduction pathway in vascular smooth muscle cells (Ahn *et al.* 1999), tyrosine protein phosphorylation (Kennedy *et al.* 1998), rat brain kvl.5 potassium channel activity (Choi *et al.* 2001), topoisomerase 1 activity (Berger *et al.* 2001), UVB-induced phosphatidylinositol-3-kinase (Nomura *et al.* 2001), and other components of the MAPK signaling pathways (Chung *et al.* 2001; Katiyar *et al.* 2001) and dopa decarboxylase (Bertoldi *et al.* 2001). Most of these are high-dose responses especially with non-cancer cells and others may be a consequence of

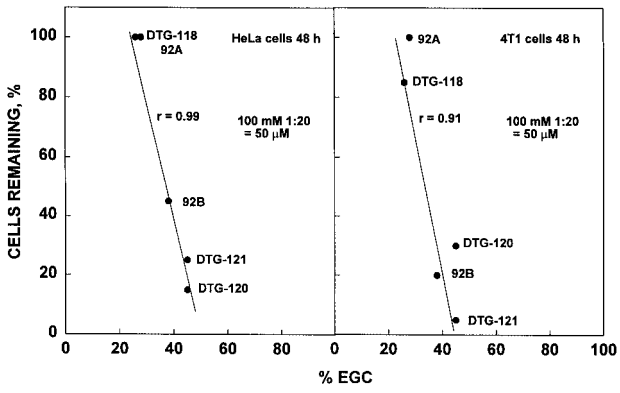

Fig. 7. Correlation between (-)-epigallocatechin (EGC) content of various tannase-treated green tea extracts shown in table 1 and growth of HeLa cells in culture after 72 hr. In the presence of small amounts of EGCg, the efficacy is highly dependent more on EGC content rather than (-)-epicatechin (EC) alone.

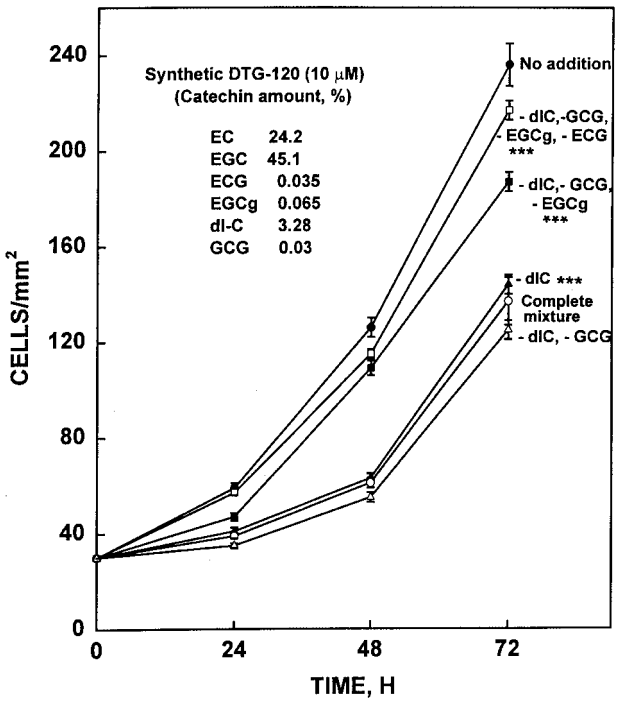

Fig. 8. Growth kinetics of HeLa cells treated with a reconstituted tea catechin mixture corresponding to DTG 120 in which each of the components was removed systematically to determine the effect on growth inhibition. Most critical was removal of (-)-epigallocatechin-3-gallate (EGCg) followed by removal of (-)-epigallocatechin (EGC) ($P<0.001$). Finally, removal of (-)-epicatechin (EC) (no addition) resulted in a further reduction in growth inhibition ($P<0.08$).

the well-established effects of EGCg on cell cycle disruption and the ability to induce apoptosis in growth-inhibited cells. Despite the relative safety of EGCg (at least 100 times more EGCg is required to inhibit normal cells than to inhibit cancer cells), green tea extracts and concentrates appear to be much safer and equally effective (Dreosti 1996; Bushman 1998; Fujiki 1999; Chang 2000).

Synergistic activity between catechins has been reported previously. EC, which by itself was inactive, enhanced apoptosis when administered together with EGCg to a lung cancer cell line (Fujiki *et al.* 1999; Suganuma *et al.* 1999). The importance of EGC in contributing to EGCg efficacy was unexpected. Induction of apoptosis by both EGCg and EGC have been observed but attributed to pro-oxidation activities (Sergediené *et al.* 1999; Yang *et al.*1999). In studies with Ehrlich ascites tumour cells, both EGCg and EGC or green tea reduced cell viability as well as both non-protein and protein sulfhydryl levels (Kennedy *et al.* 1999).

Our findings show a much greater flexibility in combining catechins to achieve tNOX inhibition and inhibition of HeLa cell growth than would be expected based on studies with EGCg alone. Even though EGCg is an essential component of all efficacious catechin mixtures, the ratio of non-gallic acid ester catechins to EGCg can be varied by a factor of 100 or more without a significant change in efficacy. Also the ratios of non-gallic acid ester catechins can be varied as well although mixtures where total catechins to EGCg+ECG was about 1.5 were most efficacious. This includes green tea and green tea concentrates.

Additionally, our findings show that, despite a high level of efficacy on a total catechin weight basis, even the most efficacious synthetic catechin mixtures were not superior to green tea concentrate by more than a factor of five or six. Thus, from standpoints of cost-effectiveness and apparent safety, standardized green tea extracts probably represent a satisfactory alternative to synthetic mixtures including pure EGCg as a source of catechins with potential anti-cancer benefits.

Acknowledgements

This work was supported in part by awards to Dorothy M. Morré from the Phi Beta Psi Sorority and to Dorothy M. Morré and D. James Morré from Pharmanex and by NIH P50 AT00477. We thank L.-Y. Wu and Brett Carnahan for technical assistance and Peggy Runck for manuscript preparation.

References

Ahmad, N., D. K. Feyes, A. L. Nieminen, R. Agarwal & H. Mukhtar: Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. *J. Nat. Can. Inst.* 1997, **89**, 1881–1886.

Ahn, H. Y., K. R. Hadizadeh, C. Seul, Y. P. Yun, H. Vetter & A. Sachinidis: Epigallocathechin-3-gallate selectively inhibits the PDGF-BB-induced intracellular signaling transduction pathway in vascular smooth muscle cells and inhibits transformation of sis-transfected NIH 3T3 fibroblast and human glioblastoma cells (A172). *Mol. Biol. Cell* 1999, **10**, 1093–1104.

Berger, S. J., S. Gupta, C. A. Belfi, D. M. Gosky & H. Mukhtar: Green tea constituent (-)-epigallocatechine-3-gallate inhibits topoisomerase I activity in human colon carcinoma cells. *Biochem. Biophys. Res. Commun.* 2001, **288**, 101–105.

Bertoldi, M., M. Gonsalvi & C. Borri Voltattomi: Green tea polyphenols: novel irreversible inhibitors of dopa decarboxylase. *Biochem. Biophys. Res. Commun.* 2001, **284**, 90–93.

Bushman, J. L.: Green tea and cancer in humans: A review of the literature. *Nutr. Cancer* 1998, **31**, 151–159.

Chang, J.: Medicinal herbs: drugs or dietary supplements? *J. Pharm.* 2000, **59**, 211–219.

Chen, Z. P., J. B. Schell, C. T. Ho & K. Y. Chen: Green tea epigallocatechin gallate shows a pronounced growth inhibitory effect on cancerous cells but not on their normal counterparts. *Can. Lttr.* 1998, **129**, 173–179.

Choi, B. H., J. S. Choi, D. S. Min, S. H. Yoon, D. J. Rhie, Y. H. Jo, M. S. Kim & S. J. Hahn: Effects of (-)-epigallocatechine-3-gallate, the main component of green tea, on the cloned rat brain Kv1.5 potassium channels. *Biochem. Pharm.* 2001, **62**, 527–535.

Chueh, P.-J., C. Kim, N. M. Cho, D. M. Morré & D. J. Morré: Molecular cloning and characterization of a tumor-associated and growth-related time keeping hydroquinone (NADH) oxidase of the HeLa cell surface. *Biochemistry* 2002a, **41**, 3732–3741.

Chueh, P.-J., D. M. Morré & D. J. Morré: A site-directed mutagenesis analysis of tNOX functional domains. *Biochim. Biophys. Acta* 2002b, **1594**, 74–83.

Chung, J. Y., J. O. Park, H. Phyu, Z. Dong & C. S. Yang: Mechanisms of inhibition of the Ras-MAP kinase signaling pathway in 30.7b Ras 12 cells by tea polyphenols (-)-epigallocatechin-3-gallate and theaflavin-3,3'-digallate. *FASEB J.* 2001, **15**, 2022–2024.

De Maat, M. P., H. Pijl, C. Kluff & H. M. Princen: Consumption

of black and green tea had no effect on inflammation, haemostasis and endothelial markers in smoking healthy individuals. *J. Clin. Nutr.* 2000, **54**, 757–763.

del Castillo-Olivares, A., F. Yantiri, P.-J. Chueh, S. Wang, M. Sweeting, D. Sedlak, D. M. Morré, J. Burgess & D. J. Morré: A drug-responsive and protease-resistant peripheral NADH oxidase complex from the surface of HeLa S cells. *Arch. Biochem. Biophys.* 1998, **358**, 125–140.

Dreosti, I. E.: Bioactive ingredients: antioxidants and polyphenols in tea. *Nutr. Rev.* 1996, **54**, S51-S58.

Fujiki, H.: Two stages of cancer preventions with green tea. *J. Cancer Res. Clin. Oncol.* 1999, **125**, 589–597.

Fujiki, H., M. Suganuma, S. Okabe, N. Sueoka, K. Imai, S. Nakachi & S. Kimura: Mechanistic findings of green tea as cancer preventive for humans. *Proc. Soc. Expr. Biol. Med.* 1999, **220**, 225–228.

Fujiki, H., M. Suganuma, S. Okabe, N. Sueoka, A. Komori, E. Sueoka, T. Kozu, Y. Tada, K. Suga, K. Imai & K. Nakachi: Cancer inhibition by green tea. *Mutation Res.* 1998, **402**, 307–310.

Katiyar, S. K., F. Afaq, K. Azizuddin & H. Mukhtar: Inhibition of uvb-induced oxidative stress-mediated phosphorylation of mitogen activated protein kinase signaling pathways in cultured human (-)-epigallocatechin-3-gallate. *Toxicol. Appl. Pharmacol.* 2001, **176**, 110–117.

Katiyar, S. K., R. Agarwal, Z. Y. Wang, A. K. Bhatia & H. Mukhtar: (-)-Epigallocatechin-3-gallate in *Camellia sinensis* leaves from Himalayan region of Sikkim: inhibitory effects against biochemical events and tumor initiation in Sencar mouse skin. *Nutr. Cancer* 1992, **18**, 73–83.

Kennedy, D. O., M. Matsumoto, A. Kojima & I. Matsui-Yuasa: Cellular thiols status and cell death in the effect of green tea polyphenols in Ehrlich ascites tumor cells. *Chem. Biol. Interact.* 1999, **122**, 59–71.

Kennedy, D. O., S. Nishimur, T. Hasuma, Y. Yano, S. Otani & I. Matsui-Yuasa: Involvement of protein tyrosine phosphorylation in the effect of green tea polyphenols on Ehrlich ascites tumor cells *in vitro*. *Chemico.-Biol. Interact.* 1998, **110**, 159–172.

Kim, J. H., S. I. Kim & K. S. Song: Prolyl endopeptidase inhibitors from green tea. *Arch. Pharm. Res.* 2001, **24**, 292–296.

Lamy, S., D. Gingras & R. Beliveau: Green tea catechins inhibit vascular endothelial growth factor receptor phosphorylation. *Cancer Res.* 2002, **62**, 381–385.

Liao, S., Y. Umekita, J. Guo, J. M. Kokontis & R. A. Hiipakka: Growth inhibition and regression of human prostate and breast tumors in athymic mice by tea epigallocatechin gallate. *Can. Lttr.* 1995, **96**, 239–243.

Liberto, M. & D. Cobrinik: Growth factor-dependent induction of p21(CIP1) by the green tea polyphenol, epigallocatechin gallate. *Cancer Lett.* 2000, **154**, 156–161.

Masuda, M., M. Suzui & I. B. Weinstein: Effects of epigallocatechin-3-gallate on growth, epidermal growth factor receptor signaling pathways, gene expression, and chemosensitivity in human head and neck squamous cell carcinoma cell lines. *Clin. Cancer Res.* 2001, **7**, 4220–4229.

Mitscher. L. A., M. Jung, D. Shankel, J.-H. Dou, L. Steele & S. P. Pillai: Chemoprotection: A review of the potential therapeutic antioxidant properties of green tea (*Camellia sinensis*) and certain of its constituents. *Med. Res. Rev.* 1997, **17**, 327–265.

Morgan, D. O.: Cyclin-dependent kinases: Engines, clocks, and microprocessors. *Ann. Rev. Cell. Dev. Biol.* 1997, **13**, 262–291.

Morré, D. J. & D. M. Morré: Mammalian plasma membranes by aqueous two-phase partition. *BioTechniques* 1989, **7**, 946–958.

Morré, D. M. & D. J. Morré: Aqueous two-phase partition applied to isolation of plasma membranes and Golgi apparatus from cultured mammalian cells. *J. Chromatog. B* 2000, **743**, 377–387.

Morré, D. J., A. Bridge, L.-Y. Wu & D. M. Morré: Preferential inhibition by (-)-epigallocatechin-3-gallate of the cell surface NADH oxidase and growth of transformed cells in culture. *Biochem. Pharm.* 2000, **60**, 937–946.

Morré, D. J., P.-J. Chueh & D. M. Morré: Capsaicin inhibits preferentially the NADH oxidase and growth of transformed cells in culture. *Proc. Natl. Acad. Sci.* 1995, **92**, 1831–1835.

Morré, D. J., L.-Y. Wu & D. M. Morré: Inhibition of NADH oxidase activity and growth of HeLa cells by the antitumor sulfonylurea, *N*-(4-methylphenylsulfonyl)-*N'*-(4-chlorophenyl) urea and response to epidermal growth factor. *Biochim. Biophys. Acta* 1997, **1355**, 114–120.

Nam, S., D. M. Smith & Q. P. Dou: Ester bond-containing tea polyphenols potently inhibit proteasome activity *in vitro* and *in vivo*. *J. Biol. Chem.* 2001, **276**, 13322–13330.

Nomura, M., A. Kaji, Z. He, W. Y. Ma, K. Miyamoto, C. S. Yang & Z. Dong: Inhibitory mechanisms of tea polyphenols on the ultraviolet B-activated phosphatidylinositol 3-kinase-dependent pathway. *J. Biol. Chem.* 2001. **276**, 46624–46631.

Okabe. S., Y. Ochiai, M. Aida, K. Park, S. J. Kim, T. Nomura, M. Suganuma & H. Fujiki: Mechanistic aspects of green tea as a cancer preventive: effect of components on human stomach cancer cell lines. *Jap. J. Cancer Res.* 1999, **90**, 733–739.

Pianetti, S., S. Guo, K. T. Kavanagh & G. E. Sonenshein: Green tea polyphenol epigallocatechin-3 gallate inhibits Her-2/Newu Signaling, proliferation, and transformed phenotype of breast cancer cells. *Cancer Res.* 2002, **62**, 652–655.

Pisters, K. M., R. A. Newman, B. Coldman, D. M. Shin, F. R. Khuri, W. K. Hong, B. S. Glisson & J. S. Lee: Phase I trial of oral green tea extract in adult patients with solid tumors. *J. Clin. Oncol.* 2001, **19**, 1830–1838.

Sartor, L., E. Pezzato & S. Garbisa: (-) Epigallocatechin-3-gallate inhibits leukocyte elastase: potential of the phyto-factor in hindering inflammation, emphysema, and invasion. *J. Leukoc. Biol.* 2002. **71**, 73–79.

Sergediené, E., K. Jönsson, H. Szymusiak, B. Tyrakowska, I. M. Rietjens & N. Cénas: Prooxidant toxicity of polyphenolic antioxidants to HL-60 cells: description of quantitative structure-activity relationships. *FEBS Lett.* 1999, **462**, 392–396.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, R. H. Gartner, E. K. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson & D. C. Klenk: Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 1985, **150**, 76–85.

Suganuma, M., S. Okabe, N. Sueoka, E. Sueoka, S. Matsuyama, K. Imai, K. Nakachi & H. Fujiki: Green tea and cancer prevention. *Mut. Res.* 1999, **428**, 339–344.

Tomita, M., K. L. Irwinl, Z. J. Xie & T. J. Santoro: Tea pigments inhibit the production of type 1 (t(H1)) and type 2(T(H2)) helper T cell cytokines in CD4(+) T cells. *Phytother. Res.* 2002, **16**, 36–42.

Wang, S., R. Pogue, D. M. Morré & D. J. Morré: NADH oxidase activity (NOX) and enlargement of HeLa cells oscillate with two different temperature-compensated period lengths of 22 and 24 minutes corresponding to different NOX forms. *Biochim. Biophys. Acta* 2001, **1539**, 192–204.

Wang, X. & W. Tian: Green tea epigallocatechin gallate: a natural inhibitor of fatty-acid synthase. *Biochem. Biophys. Res. Commun.* 2001, **288**, 1200–1206.

Wolvetang, E. J., K. L. Johnson, K. Krauer, S. J. Ralph & A. W. Linnane: Mitochondrial respiratory chain inhibitors induce apoptosis. *FEBS Lett.* 1994, **339**, 40–44.

Yang, C. S., S. Kim, G.-Y. Yang, M.-J. Lee, J. Liao, J. Y. Chung & C.-T. Ho: Inhibition of carcinogenesis by tea: bioavailability of tea polyphenols and mechanisms of actions. *Proc. Soc. Exper. Biol. Med.* 1999, **220**, 213–217.