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Black tea polyphenol theaflavins inhibit aromatase activity and attenuate tamoxifen resistance in HER2/*neu*-transfected human breast cancer cells through tyrosine kinase suppression

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Abstract

The aromatase enzyme, which converts androstenedione to oestrone, regulates the availability of oestrogen to support the growth of hormone-dependent breast tumours. In this study, we investigated the inhibitory effects of black tea polyphenols on aromatase activities. We found that black tea polyphenols, TF-1, TF-2 and TF-3, significantly inhibited rat ovarian and human placental aromatase activities. In addition, using an *in vivo* model, these black tea polyphenols also inhibited the proliferation induced by 100 nM dehydroepiandrosterone (DHEA) in MCF-7 cells. Transfection of HER2/*neu* in MCF-7 breast cancer cells appeared to be associated with an increased resistance of the cells to hormonal therapy. Interestingly, unlike the selective oestrogen receptor modulator (SERM) tamoxifen, black tea polyphenols had antiproliferation effects in breast cancer cells with hormonal resistance. The inhibitory effect of black tea polyphenols on hormone-resistant breast cancer cells suppressed the basal receptor tyrosine phosphorylation in HER2/*neu*-overexpressing MCF-7 cells. These findings suggest the use of black tea polyphenols may be beneficial in the chemoprevention of hormone-dependent breast tumours and represent a possible remedy to overcome hormonal resistance of hormone-independent breast tumours.

Keywords: Black tea polyphenols; Aromatase; Breast cancer; HER2/*neu*; Hormonal resistance

1. Introduction

Breast cancer is one of the leading causes of death in women in Western countries [1]. Approximately 60% of breast cancer patients are hormone-dependent and contain oestrogen receptors and require oestrogen for tumour growth [2]. The development of anti-oestrogens has perhaps been the primary emphasis in the field of hormonal therapy. Hormonal therapy can also be accomplished by inhibiting the production of oestrogens, e.g., inhibition of the cytochrome P450-mediated enzyme aromatase [3].

Aromatase is the enzyme complex responsible for the final step in oestrogen synthesis, via the conversion of the androgens, androstenedione and testosterone, into the oestrogens, oestrone (E_1) and oestradiol (E_2). Overexpression of aromatase in breast cancer cells may substantially influence breast cancer progression and maintenance. It has been proposed that aromatase overexpression is an indirect cause of breast cancer [4]. For example, in the aromatase-transgenic mouse model, aromatase overexpression in breast tissue causes

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premalignant lesions, such as atypical ductal hyperplasia [5,6]. As a consequence, great effort has been expended in devising pharmacological inhibitors of aromatase. Clinical trials have, indeed, now confirmed that the third-generation aromatase inhibitors, such as letrozole and anastrozole, are sufficiently powerful inhibitors of oestrogen synthesis to produce undetectable levels of circulating oestrogen [7]. They have also been shown to be more effective than tamoxifen for the treatment of metastatic or locally advanced breast cancer [8,9].

Overexpression of HER2/*neu* is found in approximately 30% of human breast cancers, and correlates with more aggressive tumours and more resistance to cancer chemotherapy [10]. Data from the literature demonstrates, that the HER2/*neu* status is a predictor for response/resistance to specific chemotherapeutic agents. Transfection of the HER2/*neu* gene to achieve amplification in ER-positive human breast cancer cells also results in the acquisition of oestrogen-independent growth that is resistant to therapy with the anti-oestrogen, tamoxifen [11]. Several studies have reported that HER2/*neu* positivity is associated with resistance to hormonal therapy [12–14].

Tea (*Camellia sinensis*) has been used as a daily beverage and crude medicine in China for several thousand years. Tea beverages are primarily manufactured as green, black or oolong tea according to the degree of fermentation involved. Polyphenols are the most significant group of components in tea, especially the catechin group of the flavanols. Green tea is thought to exert an inhibitory effect against tumourigenesis and tumour growth because of the biological activities of its polyphenols. (−)-Epigallocatechin-3-gallate (EGCG) is the major polyphenol component of green tea and a potential anti-carcinogenic factor [15]. EGCG inhibited growth of a number of tumour cell lines by inducing apoptosis [16,17]. EGCG has been described as an inhibitor of telomerase activity, supposed to be one of the major mechanisms underlying the anticancer effects of tea [18]. EGCG also inhibits endothelial cell growth *in vitro* and angiogenesis *in vivo* [19], suggesting a potential antimetastatic activity toward cancer cells. A study also demonstrated that green tea polyphenol EGCG inhibited HER2/*neu* signalling, proliferation, and the transformed phenotype of breast cancer cells [20].

The major tea beverage is black tea, especially in Western nations. For the manufacture of black tea, the fermentation' process causes green tea polyphenols to oxidise and form oligomeric flavanols, including theaflavins, thearubigin and other oligomers. Theaflavins are a mixture of theaflavin (TF-1), theaflavin-3-gallate (TF-2a), theaflavin-3′-gallate (TF-2b) and theaflavin-3,3′-digallate (TF-3). Thearubigins are the most abundant phenolic fraction of black tea and their structures are not well characterised. Black tea is assumed to be much less beneficial compared with green tea. However, reports have demonstrated that black tea could be as effective as green tea in cancer chemoprevention [21–23]. Among black tea components, theaflavins are generally considered to be the more effective components for the inhibition of carcinogenesis, but which one of these theaflavins is the most effective is unclear.

In this study, we compared TF-1, TF-2 and TF-3 with EGCG, to investigate whether black tea components can exert the same inhibitory effect on the aromatase activity.

2. Materials and methods

2.1. Reagents

EGCG, NADPH, dithiothreitol, Dehydroepiandrosterone (DHEA), 4-hydroxyandro-stenedione (4-OH-A) and Tamoxifen were purchased from Sigma (St. Louis, MO). TF-1 (theaflavin), TF-2 (a mixture of theaflavin-3-gallate and theaflavin-3′-gallate) and TF-3 (theaflavin-3,3′-digallate) were isolated from black tea as previously described in [24]. The structural formulas of these tea polyphenols are shown in Fig. 1.

2.2. Cell culture

The human breast cancer cell line, MCF-7, was obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle Medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies) and gentamicin (0.25 μg/ml). Cells were grown in a humidified incubator with 5% CO_2 at 37 °C. HER-2/*neu* stably transfected MCF7 clones were selected and cultured in media supplemented with 0.5 mg/ml G418 sulphate.

2.3. Transfection

One microgram of pcDNA3HER2 (HER2/*neu* expression vector) was used to transfect MCF-7 cells using LIPOFECTAMINE^{TM} 2000 according to the protocol provided by the manufacturer (LIFE Technologies). Transfected cells were selected for G418 resistance and single colonies were isolated, amplified and analysed for HER2/*neu* expression by immunoblotting with an anti-HER2/*neu* antibody.

2.4. Aromatase assay in MCF-7 cells

MCF-7 cells were maintained routinely in phenol red-free DMEM with 10% FCS at 37 °C and in 5% CO_2. Before starting the assays, cells were grown for at least a week in DMEM supplemented with 10% FCS–dextran-coated charcoal (DCC), that removed steroids from the FCS with DCC according to the procedure

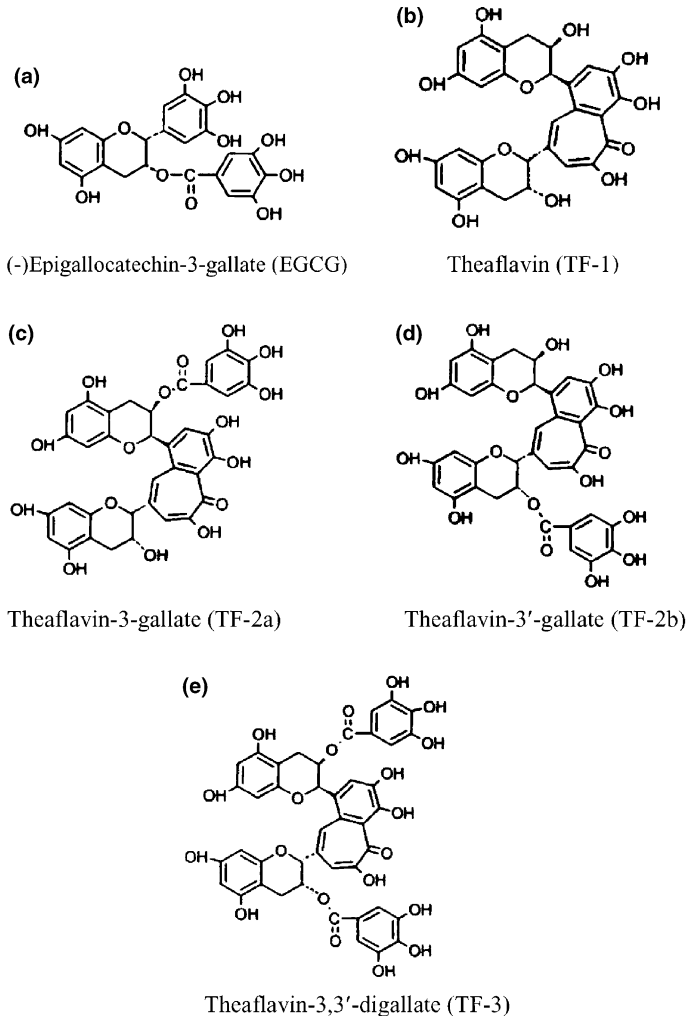

Fig. 1. Structures of (a) (−)Epigallocatechin-3-gallate (EGCG), (b) Theaflavin (TF-1), (c) theaflavin-3-gallate (TF-2a), (d) theaflavin-3′-gallate (TF-2b), (e) theaflavin-3,3′-digallate (TF-3). In the present study, TF-2 is a mixture of TF-2a and TF-2b.

described by Migliaccio and colleagues [25]; the efficiency of the stripping was controlled by electrochemiluminescence immunoassays (ECLIA). The effect on cell growth was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

2.5. Preparation of rat ovarian and human placental microsomes

Rat ovarian microsomes were prepared according to the method described by Masafumi Kudoh and colleagues [26]. Briefly, female Wistar rats of 3 weeks of age, were administered 100 IU of pregnant mare's serum gonadotropin (PMSG; Sigma Chemical Co., St. Louis, MO). After four days, the rats were sacrificed and their ovaries removed. Immediately, the ovarian tissues were rinsed with ice-cold 0.15 M KCl and homogenised in 2.5 M sucrose by a polytron homogeniser. The homogenate was centrifuged at 20 000*g* for 30 min and the resulting supernatant was centrifuged again at 148 000*g* for 60 min. The pellet of microsomes was resuspended in 0.05 M potassium phosphate buffer (pH 7.4), and centrifuged at 148 000*g* for 60 min. The resulting pellet was resuspended in the above phosphate buffer to prepare the microsomal fraction. Human placenta was freed of membranes and large blood vessels and rinsed with potassium phosphate buffer (0.067 M potassium (pH 7.4), 0.25 M sucrose and 0.5 mM 1,4-dithiothreitol) and homogenised using a Polytron homogeniser. The homogenate was centrifuged at 12 000*g* for 20 min and the supernatant was centrifuged again at 105 000*g* for 60 min. The microsomal pellet was resuspended in potassium phosphate buffer (0.067 M potassium phosphate, 20% glycerol, 0.5 mM ethylene diamine tetraacetic acid (EDTA) and 0.5 mM 1,4-dithiothreitol). The pellet was resuspended in the same buffer.

2.6. Aromatase activity assay

The aromatase assay reaction mixture (500 μl) contained the substrate [1β-^{3}H] androstenedione (0.1 μmol) (PerkinElmer Life Sciences, Inc.), rat ovarian or human placental microsomes (20 μg), NADPH (1mM final concentration) and various concentrations of test compounds dissolved in dimethyl sulfoxide (DMSO) in potassium phosphate buffer. After incubation for 10 min at 37 °C, the reaction was stopped using 500 μl ice-cold distilled water and 1.5 ml ice-cold chloroform. After a 10 min centrifugation at 3200*g*, 800 μl of aqueous phase was removed to new microfuge tubes containing 800 μl 5% charcoal/0.5% dextran. After centrifugation at 3200*g* for 10 min, 0.8 ml purified aqueous phase containing ^3H_2O was counted in liquid scintillation counter.

2.7. Enzyme kinetic assay

Inhibition kinetics using human placental microsomes were performed in which the inhibitors were evaluated at two different concentrations (5 and 10 μM). The enzyme assays were performed under initial velocity conditions of low-product formation by limiting the enzyme concentration. The results of the studies were plotted in a typical Lineweaver–Burk plot. The apparent inhibition constants, an index of the affinity of the enzyme for the inhibitor, were determined by analysis of the Dixon plot.

2.8. MTT assay

Cells were seeded in 96-well plates at a density of 1×10^4 cells per well in 200 μl of DMEM/10% FCS–DCC and incubated for 1 day to attach. Then the medium was changed to DMEM without serum and cells were adapted for a further day. Afterwards, cells were treated for 4 days with steroids and inhibitors added to DMEM/10% FCS–DCC at concentrations indicated in the respective figures. The effect on cell growth was examined by the MTT assay. Briefly, 20 μl of MTT solution (5 mg/ml; Sigma Chemical Co., St. Louis, MO) was added to each well and incubated for 4 h at 37 °C. The supernatant was aspirated, and the MTT–formazan crystals formed by metabolically viable cells were dissolved in 200 μl of DMSO. Finally, the absorbance was monitored by a microplate reader at a wavelength of 595 nm.

2.9. Western blot analysis

The cells were seeded (1.5×10^6) onto a 100-mm tissue culture dish in 10% FBS/DMEM and cultured for 24 h. Cells were washed three times with PBS and then lysed in gold lysis buffer (10% glycerol, 1% Triton X-100, 137 mM NaCl, 10 mM NaF, 1 mM Ethylene glycol tetraacetic acid (EGTA), 5 mM EDTA, 1 mM sodium pyrophosphate, 20 mM Tris–HCl, pH 7.9, 100 mM β-glycerophosphate, 1 mM sodium orthovanadate, 0.1% sodium dodecyl sulphate (SDS), 10 μg/ml aprotinin, 1 mM Phenyl methyl sulphonyl fluoride (PMSF), and 10 μg/ml leupeptin). Protein content was determined against a standardised control, using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories). A total of 50 μg of protein was separated by SDS–polyacrylamide gel electrophoresis (PAGE), and transferred to nitrocellulose filter paper (Schleicher & Schuell, Inc., Keene, NH). Non-specific binding on the nitrocellulose filter paper was minimised with a blocking buffer containing non-fat dry milk (5%) and Tween 20 (0.1%, v/v) in PBS (PBS/Tween 20). Then, the filter paper was incubated with primary antibodies specific for HER2/*neu* (Oncogene), phosphotyrosine (PY 20, Transduction Laboratories) and β-actin and followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (1:2500 dilution; Boehringer Mannheim Corp., Indianapolis, IN). Reactive bands were visualised with an enhanced chemiluminescence (ECL) system (Amersham Corp., Arlington Heights, IL). The intensity of the bands was scanned and quantified with a National Institutes of Health (NIH) image software.

3. Results

3.1. Concentration-dependent inhibition of rat ovarian and human placental aromatase activities by black tea polyphenols

Previous study shows that green tea catechins, including epicatechin gallate (ECG) and EGCG, are potential aromatase inhibitors [27]. For this reason, we further examined the *in vitro* effect of theaflavins whose structures were related to EGCG on aromatase activity. Microsomes isolated from rat ovarian and human placenta were used to determine the direct effect of black tea on aromatase activity. Incubation of microsomal tissue preparation with 0.1 μmol [1β-^{3}H] androstenedione as a substrate were performed in the presence of EGCG, TF-1, TF-2 and TF-3 with concentrations ranging from 5 to 40 μM. As shown in Fig. 2, EGCG, TF-1, TF-2 and TF-3 induced a concentration-dependent inhibition of rat ovarian (Fig. 2(a)) and human placental (Fig. 2(b)) aromatase activities. According to the computer-assisted non-linear curve-fitting method, the IC_{50} of EGCG, TF-1, TF-2 and TF-3 inhibited aromatase activity of rat ovarian microsomes were 18.27 ± 1.26, 5.72 ± 0.61, 7.33 ± 0.22, and 20.37 ± 2.57 μM, respectively (Table 1). EGCG, TF-1,

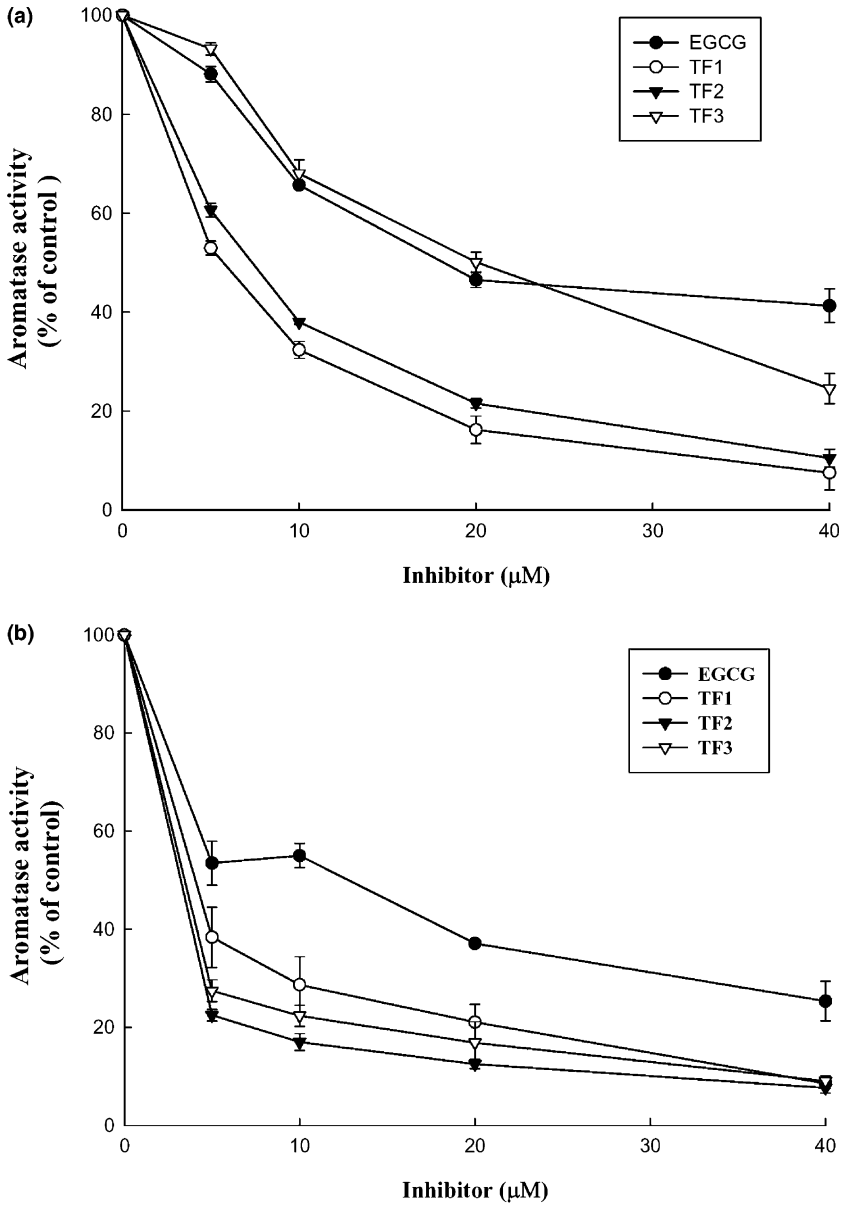

Fig. 2. The *in vitro* inhibitory effects of EGCG and theaflavins on aromatase activity from rat ovarian (a) and human placental (b) microsomes. Microsomal tissue preparations were incubated with 0.05 μM [1β-^{3}H] androstenedione and 1 mM nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of EGCG (●), TF-1 (○), TF-2 (▼), or TF-3 (▽) at concentrations ranging from 5 to 40 μM. All measurements were performed in triplicate, and values represent mean ± standard error of the mean (SEM).

Table 1
Inhibition of aromatase activities in microsomes

	Rat ovary (IC_{50}[a]) (μM)	Human placenta (IC_{50}[a]) (μM)
EGCG	18.27 ± 1.26	13.79 ± 0.89
TF-1	5.72 ± 0.61	4.17 ± 0.84
TF-2	7.33 ± 0.22	3.23 ± 0.08
TF-3	20.37 ± 2.57[b]	3.45 ± 0.18

[a] IC_{50}, concentration (μM) of compound producing 50% inhibition of aromatase activity. The IC_{50} was determined by interpolation between the appropriate data point from three individual experiments and represented as "means ± SEM".

[b] Significant difference ($p < 0.05$) between TF-1, TF-2, TF-3 and human placental microsomes.

TF-2 and TF-3 also inhibited the activity of aromatase obtained from human placenta and the IC_{50} were 13.79 ± 0.89, 4.17 ± 0.84, 3.23 ± 0.08 and 3.45 ± 0.18 μM, respectively (Table 1).

3.2. Kinetic mechanism of inhibition of human aromatase activity by black tea polyphenols

To determine the mechanism of black tea-mediated inhibition, we analysed the change in kinetic parameters of the aromatase activity in response to various concentrations of substrate and the results were

analysed by a double-reciprocal (Lineweaver–Burk) plot. As shown in Fig. 3(a), there was a dose-dependent increase in the K_m of the substrate in the presence of TF-2. The V_{max} of the enzyme reaction decreased in the presence of TF-2. Kinetic analysis indicated that TF-2 was a mix-competitive inhibitor with a K_I of 5.88 μM and a K_i of 2.38 μM (Fig. 3(b)). We also found that TF-1 and TF-3 were fmix-competitive inhibitors (data not shown).

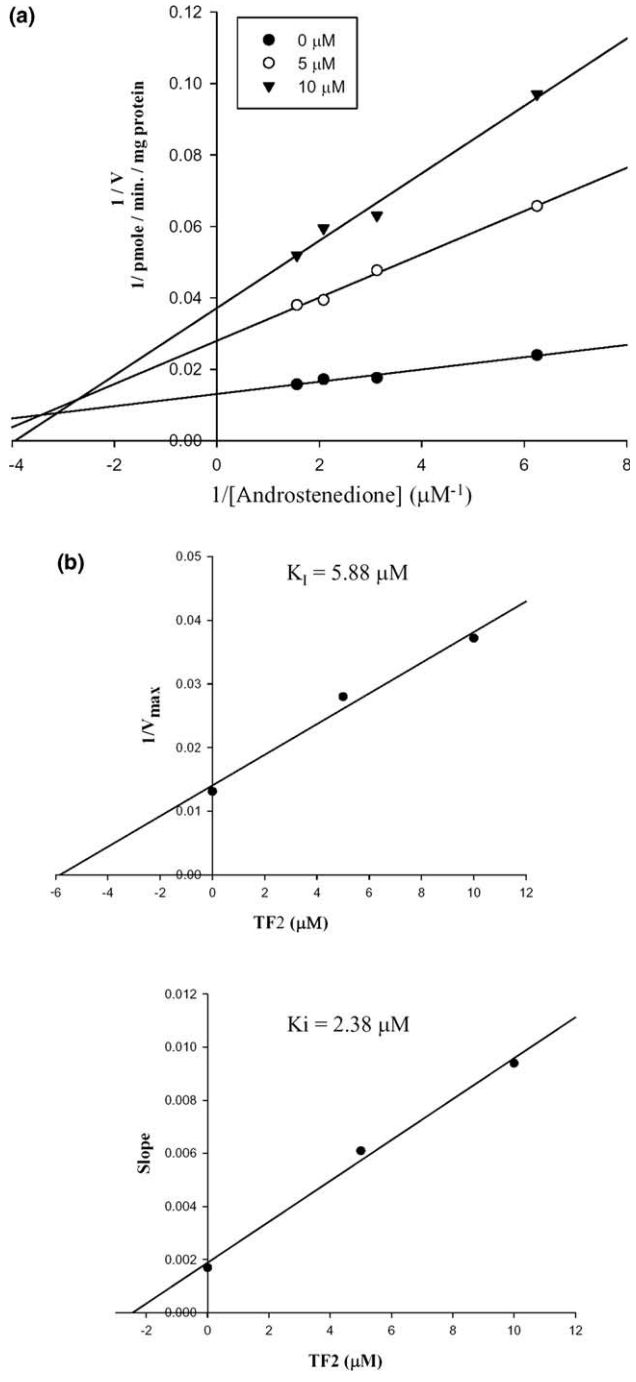

Fig. 3. Influence of TF-2 on the kinetics of human placental aromatase activity. A human placental microsome tissue preparation was incubated with [1β-^{3}H] androstenedione and 1 mM NADPH in the presence of TF-2 at concentrations of 5 and 10 μM. Aromatase activity was measured by the release of ^3H_2O into the aqueous phase. Data are presented in a Lineweaver–Burk plot (a) with the resulting K_I and K_i (b).

3.3. Effect of the black tea on the proliferation rate of MCF-7 cell lines stimulated with DHEA

In order to determine whether black tea polyphenols may act as an aromatase inhibitor and inhibit the cell proliferation of human breast cancer cells. MCF-7 cells, an oestrogen-dependent human breast cancer cell line expressing aromatase activity provides a convenient *in vivo* model to study the inhibitory effect of black tea polyphenols on the aromatase activity. IC_{50} values of tea polyphenols on human placental aromatase activities obtained in this experiment ranged from 13.79 to 3.23 μM (Table 1). For this reason, we selected a concentration of 10 μM to proceed with the study. Proliferation induced by 100 nM DHEA was inhibited by the EGCG, TF-1, TF-2 and TF-3 at a concentration of 10 μM (Fig. 4). Furthermore, the proliferation was inhibited by the aromatase inhibitor 4-hydroxy-androstenedione (4-OH-A). This indicates that black tea acts as an aromatase inhibitor and inhibits the conversion of DHEA to estradiol by aromatase. In addition, proliferation induced by 100 nM DHEA was inhibited by the anti-oestrogen ICI 182,780 at a concentration of 1 μM (Fig. 4). This indicates that stimulation of proliferation of MCF-7 cells by DHEA is a consequence of an oestrogenic effect of this steroid.

3.4. Effect of the black tea polyphenols on HER2/neu-transfected MCF-7 cell lines

Breast cancers are either oestrogen-dependent or oestrogen-independent. Many reports have suggested that coexpression of HER2/*neu* was associated with a reduced response rate of ER-positive patients to first-line hormone therapy [11–14]. The aromatase enzyme regulates the availability of oestrogen to support the growth of hormone-dependent breast tumours. Our present study identifies that black tea polyphenols act as aromatase inhibitors in MCF-7 cells, an oestrogen-dependent human breast cancer cell line (Fig. 4). To identify whether black tea has the potential to overcome hormone resistance in oestrogen-independent breast cancer, we tested the inhibitory effect of black tea on HER2/*neu* transfected MCF-7 cell lines (Fig. 5(a)). As shown in Fig. 5(b), EGCG and black tea polyphenols, TF-1, TF-2 and TF-3, have antiproliferative effects in HER2/*neu* transfected MCF-7 cell lines. Moreover, in agreement with a previous report [11], tamoxifen (a selective ER antagonist) has no antiproliferative effects in HER2/*neu* transfected MCF-7 cell lines (Fig. 5(b)). Interestingly,

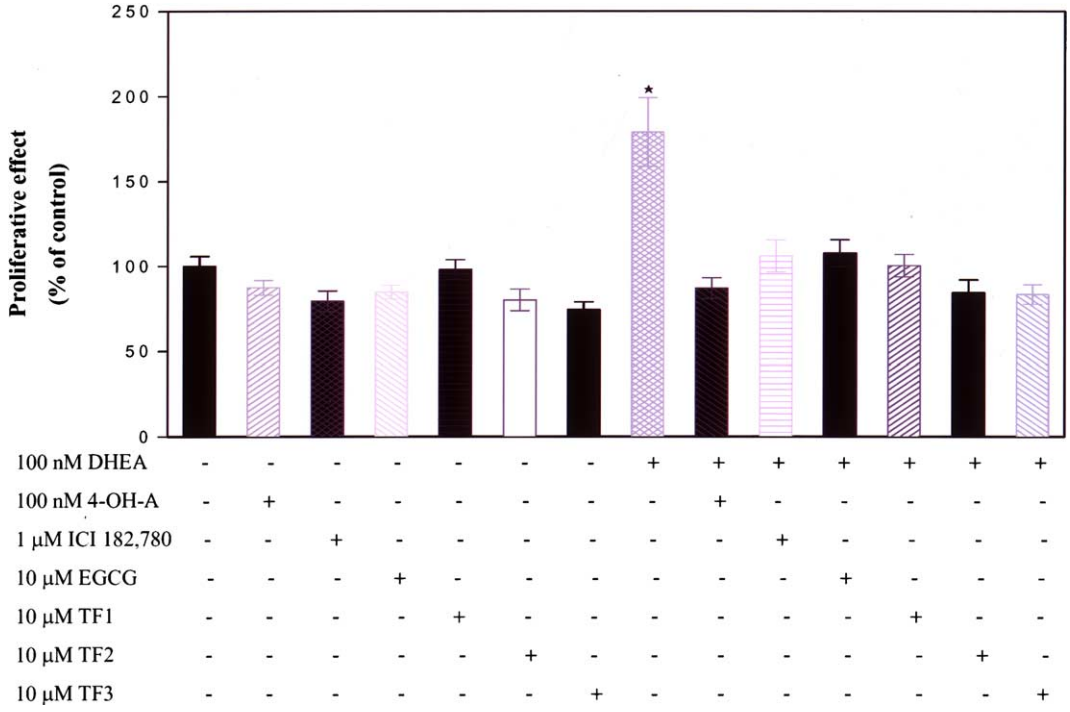

Fig. 4. Effect of the tea polyphenols, anti-oestrogen ICI 182,780 and the aromatase inhibitor 4-hydroxy-androstenedione (4-OH-A) on the proliferation of MCF-7 cells stimulated with dehydroepiandrosterone (DHEA). Oestrogen-responsive MCF-7 breast cancer cells were incubated for 4 days with 100 nM DHEA and with or without tea polyphenols in serum-free Dulbecco's modified Eagle's Medium (DMEM); control cells were incubated with ICI 182,780 only or other respective vehicles. The effect on cell growth was examined by the 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide (MTT) assay, and the percentage of cell proliferation was calculated by defining the absorption of cells without treatment as 100%. All measurements were performed in triplicate, and values represent means ± SEM. The proliferation of MCF-7 cells were significantly stimulated by DHEA ($^{*}P < 0.05$; Student's t test).

tamoxifen treatment combined with EGCG and black tea polyphenols, TF-1, TF-2 and TF-3, enhanced the antiproliferative effects of tamoxifen ($P < 0.05$; Student's t test) (Fig. 5(b)).

3.5. Black tea polyphenols attenuate tamoxifen resistance in HER2/neu-transfected human breast cancer cells through tyrosine kinase suppression

HER2/*neu* is a member of the class II receptor (ErbB) tyrosine kinase family. Recent evidence has shown that in MCF-7 cells stably transfected with HER2/*neu* and in BT-474 cells that overexpress HER2/*neu*, blockade using either a HER2/*neu* tyrosine kinase inhibitor (AG1478) or a mitogen-activated protein kinase (MAPK) inhibitor (UO126) abrogated the anti-oestrogen resistance [28]. Therefore, we next examined the effect of black tea polyphenols on the tyrosine kinase activity of HER2/*neu*. HER2/*neu* transfected MCF-7 cell lines were treated with various drugs or control vehicle at 37 °C for 24 h and the tyrosine phosphorylation levels were measured by Western blotting. We found that EGCG and black tea polyphenols, TF-1, TF-2 and TF-3 inhibited tyrosine phosphorylation in HER2/*neu* transfected MCF-7 cell lines (Fig. 5(c)). However, tamoxifen could not inhibit tyrosine phosphorylation in HER2/*neu* transfected MCF-7 cell lines (Fig. 5(c)).

4. Discussion

Despite the established chemopreventive effect of black tea polyphenols on mammary tumourigenesis [21], the effect of black tea polyphenols on the activity of aromatase have not, to our knowledge, been studied before. For *in vitro* assessment of aromatase inhibitory capability, microsomal preparations from rat ovaries and human placenta were used. Inhibition of the enzyme and the potency of the inhibitor were determined by the amount of tritiated water released in the assay. As shown in Fig. 2, black tea polyphenols, TF-1, TF-2 and TF-3, inhibited rat ovarian (Fig. 2(a)) and human placental (Fig. 2(b)) microsome aromatase activity in a dose-dependent manner. In comparison with green tea catechin, EGCG, a known aromatase inhibitor [27], the order of the inhibitory effect on the rat ovarian aromatase activity was TF-1 > TF-2 > EGCG > TF-3. However, the order of the inhibitory effect on the human placental aromatase activity was TF-2 > TF-3 > TF-1 > EGCG. Interestingly, we found that the IC_{50} of

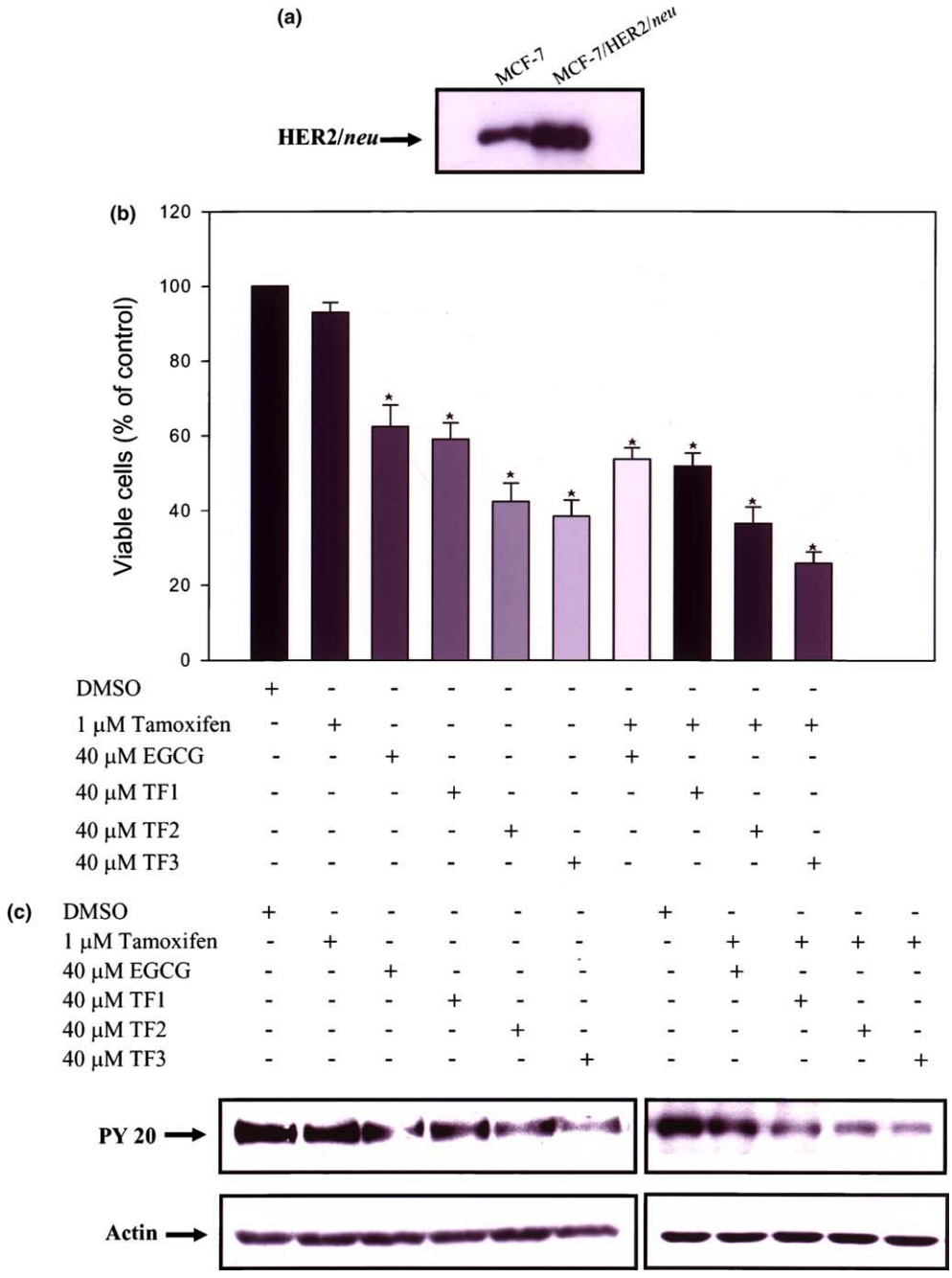

Fig. 5. Effect of the black tea polyphenols and the anti-oestrogen tamoxifen on proliferation (b) and the level of phosphorylated HER2/*neu* protein (c) in MCF-7/HER2/*neu* cells. MCF-7 cells were stably transfected with an HER2/*neu* expression vector and selected for resistance to G418. (a) HER2/*neu* protein expression level in the parental and stable clone was analysed by immunoblotting with an anti-HER2/*neu* antibody. (b) MCF-7/HER2/*neu* cells were incubated with various drugs dissolved in dimethyl sulphoxide (DMSO) or the equivalent volume of DMSO alone at 37 °C for 72 h, the effect on cell growth was examined by the MTT assay. The number of viable cells after treatment was expressed as a percentage against the vehicle-only control. This experiment was repeated three times. Bars represent the SEM. The Student's *t*-test was used to determine the significance of inhibition ($^*P < 0.05$). (c) MCF-7/HER2/*neu* cells were incubated with various drugs dissolved in DMSO or the equivalent volume of DMSO alone at 37 °C for 24 h. Immunoblotting was performed to examine the levels of phosphotyrosine (PY 20) and β-actin.

TF-1 was 3.2-fold less than EGCG on the rat ovarian aromatase activity and TF-2 was 4.3-fold less than EGCG on the human placental aromatase activity. These data suggest that black tea polyphenols are stronger aromatase inhibitors than green tea polyphenols. Among these compounds, TF-1 is the only one which does not contain a gallate group. The presence of the gallate group in the catechin molecule may be important for the chemopreventive activity of tea polyphenols. However, Satoh and colleagues showed that both the

gallate group-containing catechin and the gallate group-lacking epigallocatechin (EGC) had inhibitory effects on aromatase [27]. This concurs with our results that TF-1 significantly inhibited rat ovarian and human placental aromatase activities, and suggests that the gallate group may not be necessary for aromatase inhibition.

The mechanism by which black tea polyphenols inhibit aromatase activity appears to be multifactorial. Using a Lineweaver–Burk analysis of human placental microsomal aromatase activity to determine the kinetics of inhibition, we found that TF-2 causes an increase in the K_m of the substrate, indicating that TF-2 competes with the substrate at the substrate binding site (Fig. 3(a)). However, the V_{max} of the reaction is decreased in the presence of TF-2, indicating that non-competitive inhibition is also present. Thus, the inhibition of aromatase activity by black tea polyphenols is of a mixed type.

Because aromatase is overexpressed in breast cancer tissue, the effect of aromatase inhibitors in breast cancer cells is of particular interest. Therefore, we examined the effects of black tea polyphenols on aromatase activity in MCF-7 human breast cancer cells, an oestrogen receptor-positive cell line that has been extensively used in aromatase studies. As MCF-7 cells do not possess sufficient aromatase activity to be easily detected in microsomal preparations, an indirect assay was used to measure the activity in intact cells. DHEA mainly serves as a precursor for the biosynthesis of oestrogenic and androgenic compounds, such as 17β-estradiol, 5-androstene-3β, 17β-diol, testosterone and 4-androstene-3, 17-dione, in peripheral tissues [29]. In postmenopausal women, adrenal DHEA(S) is the main source for the production of oestrogens besides androstenedione. It has been demonstrated that DHEA is a mitogen for oestrogen-dependent MCF-7 breast cancer cells. The conversion of DHEA to oestradiol by aromatase stimulates cell proliferation and oestrogen-dependent gene expression [30]. As shown in Fig. 4, 10 μM of EGCG, TF-1, TF-2 and TF-3 inhibited the proliferation induced by 100 nM DHEA. At the same concentration, EGCG, TF-1, TF-2 and TF-3 are not toxic to MCF-7 cells (Fig. 4). Thus, our results suggest that black tea polyphenols have the potential to inhibit aromatase activities in intact cell models.

Breast cancers are either oestrogen-dependent or -independent. Despite the presence of oestrogen receptors (ERs), a certain proportion of patients do not respond to endocrine treatment. It has been reported that co-expression of HER2/*neu* is associated with a reduced response rate of ER-positive patients to first-line hormone therapy of metastatic breast cancer, from 48% to 20% [31]. Transfection of the *HER2/neu* gene to achieve amplification in ER-positive human breast cancer cells also results in acquisition of oestrogen-independent growth that is resistant to antioestrogen tamoxifen therapy [11]. This development of acquired resistance to anti-hormonal agents in breast cancer is a major therapeutic problem. Interestingly, we found that EGCG and black tea polyphenols TF-1, TF-2 and TF-3 can inhibit the proliferation of HER2/*neu*-transfected MCF-7 cells. Our study therefore provides new insights into the mechanisms of action of black tea polyphenols in overcoming the resistance to hormonal therapy.We demonstrated that black tea polyphenols inhibit the proliferation of HER2/*neu* transfected MCF-7 cells by repressing the tyrosine kinase activity. To our knowledge, this is the first time this repression of the tyrosine kinase activity has been observed. However, the mechanism is not yet clear. Neverthless, our data suggest that a HER2/*neu* tyrosine kinase inhibitor might be clinically useful in the treatment of hormone-resistant advanced breast cancer.

In conclusion, we demonstrated that black tea polyphenols strongly suppress aromatase activity *in vitro* and *in vivo*. They can also act as a HER2/*neu* tyrosine kinase inhibitor, and attenuate tamoxifen-resistant breast tumours in oestrogen-independent breast cancer cells. Further evaluation of the molecular action of black tea polyphenols is warranted to support the role of black tea polyphenols as a chemopreventive agent in the treatment of post-menopausal women with breast cancer.

Conflict of interest

None.

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