



## Effect of Methotrexate and Tea Polyphenols on the Viability and Oxidative Stress in MDA-MB-231 Breast Cancer Cells

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### Authors' contributions

This work was carried out in collaboration between both authors. Author RKO designed the study and wrote the protocols. Author TK managed the study, conducted the literature searches, and performed the experiments; authors TK and RKO analysed the results. Author TK wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

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Short Communication

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### ABSTRACT

**Aim:** To determine the effect of tea polyphenols and methotrexate on viability and reactive oxygen species (ROS) in a naturally resistant breast cancer cell line MDA-MB-231.

**Methodology:** MDA-MB-231 cells were selected as a model for methotrexate resistant breast cancer. Drug tests were performed over 72 hours at concentrations 0-100  $\mu$ M. Pre-treatments were with quercetin (QE) or epigallocatechin gallate (EGCG) for 5 hours followed by methotrexate. Cytotoxicity was measured using the MTT assay or resazurin fluorescence assay. ROS was determined using the 2', 7'-dichlorofluorescein diacetate assay. Intracellular GSH was measured using the monochlorobimane assay.

**Results:** Methotrexate was cytotoxic to MDA-MB-231 cells with IC<sub>50</sub> of 35 $\pm$ 4  $\mu$ M. The IC<sub>50</sub> value was 68 $\pm$ 9.4  $\mu$ M with QE and 83 $\pm$ 16  $\mu$ M for EGCG. The pre-treatment with QE and EGCG lowered the IC<sub>50</sub> for methotrexate by 28% ( $P = 0.009$ ) and 16% ( $P = 0.2027$ ). Intracellular ROS concentrations increased after treatment with methotrexate, QE or EGCG singly and ROS

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decreased with combination treatment compared with the response for methotrexate only. There were no significant changes in intracellular GSH.

**Conclusion:** Pretreatment with tea polyphenols partially sensitized breast cancer cells towards methotrexate and decreases intracellular ROS. More research is needed to optimize the sensitizing effect of tea phenols on the breast cancer cell response to methotrexate.

**Keywords:** Breast cancer; methotrexate; quercetin; epigallocatechin gallate; oxidative stress; anticancer effect.

## ABBREVIATIONS

*MTT* - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; *MTX* – methotrexate; *QE* – quercetin; *EGCG* - epigallocatechin gallate; *ROS* - reactive oxygen species; *GSH* – glutathione; *DMEM* - dulbecco's modified eagle's medium; *FBS* - foetal bovine serum; *DMSO* – dimethyl sulfoxide; *MTT* - thiazolyl blue tetrazolium; *PBS* - phosphate buffer saline; *DCFH-DA* - 2',7'-dichlorofluorescein diacetate; *mCB* – monochlorobimane; *IC<sub>50</sub>* - 50% inhibitory concentrations; *RFU* - relative fluorescence units; *HTA* - human tissue authority.

## 1. INTRODUCTION

Methotrexate was introduced in the 1950s to treat neoplastic disorders. Low dose methotrexate is used for the treatment of autoimmune disorders such as rheumatoid arthritis [1]. As a folic acid analogue, methotrexate has an antagonistic effect on folic acid metabolism that results in the inhibition of DNA / RNA synthesis and cell division [1]. The mechanism of methotrexate action is not specific for malignant cells and other cells such as the haematopoietic cells of the bone marrow, intestinal mucosa cells, and hepatocytes have been found to be particularly susceptible to methotrexate toxicity. Methotrexate treatment leads to increased myeloperoxidase, elevated reactive oxygen species (ROS) and increased oxidative stress. There is considerable interest in the use of natural agents to ameliorate methotrexate induced oxidative damage [2,3].

Breast cancer is the second most common cancer death in women within the United Kingdom; the incidence of breast cancer increased 90% between the years 1997 and 2010 [4]. The human breast cancer cell line MDA-MB-231 is oestrogen receptor negative and naturally resistant to methotrexate [5]. Methotrexate resistance in MDA-MB-231 cells is attributed to low levels of reduced folate carrier expression [5].

Recent investigations showed that combination treatments using methotrexate and natural antioxidants decreased the levels of drug-induced oxidative damage [2,3]. However, the effect of antioxidants on methotrexate

effectiveness has not been widely examined. Tea polyphenols exhibit antioxidant activity and, some like epigallocatechin gallate (EGCG) showed anticancer activity [6]. Recent investigations have demonstrated that tea polyphenols enhance the susceptibility of resistant breast cancer cells to anti-estrogen therapy [7], leading to their suggested use as adjuvants in cancer therapy [8]. However, few or no studies have appeared on the effects of tea polyphenols on methotrexate-induced oxidative stress or anticancer activity. A recent investigation showed that curcumin treatment can augment methotrexate cytotoxicity [9]. The hypothesis examined in this study is that *pre-treatment* with non-toxic doses QE or EGCG will chemosensitize breast cancer cells towards methotrexate and lower the intracellular levels of ROS. The experimental design involved assessing the dose-response for a methotrexate using a resistant breast cancer (MDA-MB-231) cell line before and after pre-treatment QE or EGCG. The effect of tea phenols pre-treatment on intracellular ROS was also monitored.

## 2. MATERIALS AND METHODS

### 2.1 Cell Culture

MDA-MB-231 cells (American Type Cell Culture) were cultured in Dulbecco's modified eagle's medium (DMEM; GIBCO) containing 10% foetal bovine serum (FBS) (Invitrogen) in 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C (LEEC research incubator, LEEC, UK). Cells were trypsinised, counted using the NucleoCounter (NC-3000, ChemoMetec, Denmark), inoculated to give 10,000 cells/well and 50 µl media in 96-microwell

plates (Nunc; Roskilde, Denmark). The cells were then incubated overnight to allow cell attachment.

## 2.2 Drug Tests

Methotrexate (100 mM) QE (100 mM) or EGCG (40 mM) stock solutions were prepared in dimethyl sulfoxide (Sigma Aldrich) and stored at -18°C. Each stock solution was diluted 1/5 in DMEM media and filter-sterilised (Merck Millipore Ltd, Germany). Serial 1/10 dilutions of the sterile drug solutions were performed and 50µl of the various compounds were added to inoculated wells followed by incubation for 72 hours. At the highest drug concentrations tested (100 µM) the final DMSO concentration did not exceed 0.1% (v/v) which is not toxic to breast cancer cells. As required by the experimental design, first the dose-response for MDA-MB-231 was determined by treating cells with methotrexate, QE or EGCG separately at concentrations of 0-100 µM. From this study, the non-toxic doses of QE and EGCG were established. Non-toxic concentrations of tea phenols (1-10 µM) were used to examine the effect of cell pre-treatments.

## 2.3 MTT Assay for Cell Viability

The Thiazolyl Blue Tetrazolium (MTT; Sigma-Aldrich) assay was performed essentially as described previously [10]. MTT (5 mg/ ml) was dissolved in phosphate buffer saline (PBS) and filter-sterilised then stored in the dark at 4°C. Cells treated with drug were washed three times with PBS (100 µl) and PBS left in the wells (100 µl). MTT (20 µL) was added followed by 2 hours incubation. Isopropynol-0.04N HCl solution (100 µl) was added and absorbance readings were taken at 570 nm (VERSA Max Micro plate reader; Molecular Devices, USA).

## 2.4 Resazurin Assay for Cell Viability

Resazurin (Sigma Aldrich) solution was prepared by dissolving 1.6 mg of resazurin in PBS (100 mL). The stock solution was filter-sterilized and stored at room temperature. Pre-treatments using QE and EGCG and methotrexate drug test were performed as previously described. Cells were washed twice with ice cold PBS and resazurin was added (10 µl) followed by incubation for 2 hours. Fluorescence readings were recorded with an excitation and emission wavelength ( $\lambda_{ex}/\lambda_{em}$ ) equal to 544 nm/ 620-10

nm (Fluostar Omega Fluorescence Instrument; BMG-Lab-Tech, Germany).

## 2.5 Determination of Intracellular Reaction Oxygen Species

The concentration of intracellular reactive oxygen species (ROS) was determined using 2',7'-dichlorofluorescein diacetate (DCFH-DA). Breast cancer cells were seeded in microplate wells for 24 hours and pre-treated with QE or EGCG for 5 hours. The microplate wells were washed twice with media with 50 µl left in the wells. Varying concentrations of methotrexate (50µl) were added to the cells followed by incubation for 72 hours. DCFH-DA (100µl) was added to the drug treated cells and cultures incubated for 2 hours. Fluorescence readings were read with  $\lambda_{ex}/\lambda_{em}$  =485 nm/ 520 nm. The reagent blank comprised cell-free microtitre plates containing 50 µl media and methotrexate and the DCFH-DA assay was performed as described above. ROS were also determined using MDA-MB-231 cells pre-loaded with DCFH-DA for short-term combination treatments. Cultured cells were treated with DCFH-DA (100 µl) and incubated for 1 hour. The microtitre plates were washed twice with PBS. The DCFH-preloaded cells were treated with 1 µM of methotrexate and/ or QE or EGCG for 1 hour and fluorescence readings were recording as above.

## 2.6 Monochlorobimane Assay for Intracellular GSH

Monochlorobimane (mCB) stock solution was prepared by dissolving 5 mg of mCB into 2 mL of methanol and stored at -18°C. The microtitre plates were inoculated as previously described and then treated with drugs and incubated for 1 hour. The microtitre plates were then washed twice with cold PBS (100 µl) and a further 100 µl of PBS was left in wells. Then 5 µl mCB was added to the wells and the microplate incubated for 2 hours at 37°C. Fluorescence measurements were then recorded with  $\lambda_{ex}/\lambda_{em}$  equal to 355 nm /520 nm.

## 2.7 Statistical Analysis

All experiments were repeated on 3 different occasions, with 6 replicates per drug concentration. Pooled data (n =18) were subjected to statistical analysis. One-way ANOVA tests were performed by Microsoft SPSS version 21 to analyse statistically significant

differences between means in conjunction with Tukey post hoc analysis for the separation of means.  $P < 0.05$  is considered to be statistically different. Paired t-tests performed on calculator at [www.graphpad.com](http://www.graphpad.com). To determine IC<sub>50</sub> values, data were log-transformed and plotted versus drug concentration, followed by appropriate graphic analysis as described previously [11]. Results were identical when IC<sub>50</sub> values were determined using Probit analysis.

### 3. RESULTS AND DISCUSSION

Previous research showed that QE from black tea has anti-carcinogenic and chemo-preventive properties. QE was demonstrated to reduce the viability of MDA-MB-231 cells in a dose and time dependant manner [6,7,12,13]; QE is indicated in the literature as a promising anti-cancer agent [13]. Epigallocatechin gallate (EGCG), which comprises ~30% of the antioxidants in green tea, was found to have chemo-preventative properties [6,7]. In tests using MDA-MB-231 (ER-) cells, EGCG showed synergistic effects in combination with selective estrogen receptor modulators [7]. The aim of the current study was to examine the effect of EGCG and QE on methotrexate cytotoxicity and oxidative stress in a naturally resistant breast cancer cell line. Cell response to treatment with methotrexate, EGCG and QE alone is shown in Fig. 1.

#### 3.1 Effect of Methotrexate and Tea Phenols on Breast Cancer Cell Viability

Using data from Fig. 1, the concentrations of methotrexate, QE or EGCG leading to a 50% decline in viability (IC<sub>50</sub>) were determined for MDA-MB-231 cells treated with each agent separately, when assayed using the MTT cell proliferation assay. From Table 1 the IC<sub>50</sub> value for methotrexate was significantly lower when compared to values for QE ( $P = 0.002$ ) or EGCG ( $P = 0.006$ ). There are no previous IC<sub>50</sub> values for methotrexate, QE, or EGCG determined within a *single* study. However, different investigators reported the IC<sub>50</sub> for methotrexate using MDA-MB-231 cells was 80  $\mu$ M at 24 hours of drug treatment where a soluble analogue of MTT was the cell proliferation assay [14]. To our knowledge no previous reports using resazurin to assess methotrexate effect on MDA-MB-231 cells has been published.

**Table 1. Inhibition of MDA-MB-231 breast cancer cells by methotrexate and tea phenols**

MTT assay	IC <sub>50</sub> (x 10 <sup>-6</sup> M)	p
Methotrexate	35.0±4.0*	-
QE	68.0±9.4	0.002
EGCG	83.0±16	0.006
Resazurin assay	IC <sub>50</sub> (x 10 <sup>-6</sup> M)	
Methotrexate	55.5±3.5*	-
Methotrexate-QE	40.1±4.5	0.009
Methotrexate-EGCG	46.8±5.8	0.203

Notes: \* significant difference of MTT assay or resazurin fluorescence results. QE = quercetin, EGCG = epigallocatechin gallate, data shows means ± SEM

The IC<sub>50</sub> of QE using MDA-MB-231 cells was 278  $\mu$ M after 24 hours of treatment [11] whilst the IC<sub>50</sub> value of EGCG using MDA-MB-231 cells was 87  $\mu$ M or 109  $\mu$ M for 24 or 48 hours of treatment, respectively [6]; these investigations using tea phenols and MDA-MB-231 cells employed MTT as cell proliferation assay [6,11]. Accordingly, past literature IC<sub>50</sub> values based on MTT cell proliferation assay were larger compared to IC<sub>50</sub> values in the present study probably because of the longer incubation time (72 hours) used (Table 1).

In Fig. 1, cell viability was calculated as described previously [11] using the relation; Cell viability (%) =  $(N_t / N_0) * 100$ , where  $N_t$  is the cell number with drug treatment and  $N_0$  is the cell number with media only (zero drug concentration) treatment. Fig. 1 shows that low concentrations of methotrexate, QE or EGCG were stimulatory compared to 100% viability observed with cells treated with culture media only. Cytotoxicity occurred at > 10  $\mu$ M and methotrexate was more effective when compared to QE and EGCG (Table 1). U-shaped hormesis drug dose-responses have been reported in breast cancer cell lines treated with QE, EGCG, daidzein, resveratrol, plant phytochemicals and drugs [15]. Interestingly, we found the IC<sub>50</sub> changed with the method for assessing residual viable cell number after drug treatment. The IC<sub>50</sub> value for methotrexate determined using the resazurin fluorescence assay for cell viability was 55.5±3.5  $\mu$ M and significantly different ( $P < 0.001$ ) from the IC<sub>50</sub> value based on the MTT assay (Table 1). For convenience, fluorescence instrumentation was adopted for investigations of methotrexate interactions with tea phenols (resazurin assay) as well as for monitoring changes in ROS (DCHF assay, Section 2.5).

### 3.2 Effect of Polyphenol Pre-treatment and Methotrexate on Breast Cancer Cell Viability

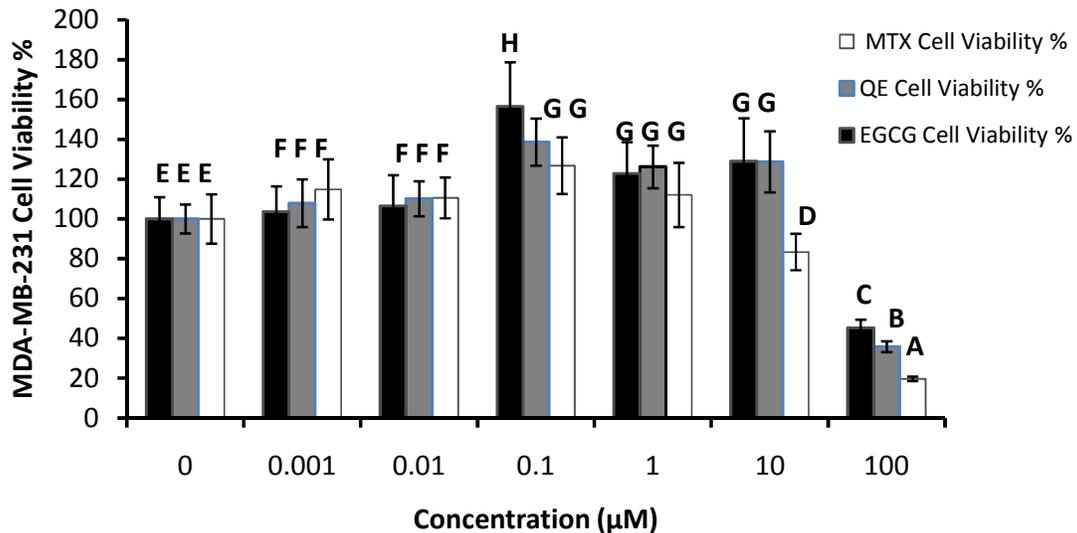
Previous investigations using MDA-MB-231 demonstrated that tea phenols increase the susceptibility of MDA-MB-231 cells to certain cancer drugs [8]. In this study, MDA-MB-231 cells were *pre-treated* with 10  $\mu\text{M}$  of QE or EGCG for 5 hours followed by methotrexate for 72 hours. The methotrexate dose-response curves were then analysed to determine IC<sub>50</sub> values (Table 1, lower half). In breast cancer cells pre-treated with QE there was a significant ( $P < 0.01$ ) decline in the IC<sub>50</sub> value for methotrexate by 28% (Table 1). For cells pre-treated with EGCG the IC<sub>50</sub> value for methotrexate declined by 16% but did not reach significance. These initial findings suggest tea phenols may enhance the effectiveness of methotrexate though more work will be needed to substantiate this effect.

### 3.3 Methotrexate and Polyphenol Effects on Breast Cancer Cell ROS

To get a better insight into how tea phenols affect breast cancer cells, we examined changes in intracellular levels of ROS with and without drug treatment. There were significant rises in intracellular ROS for MDA-MB-231 cells pre-

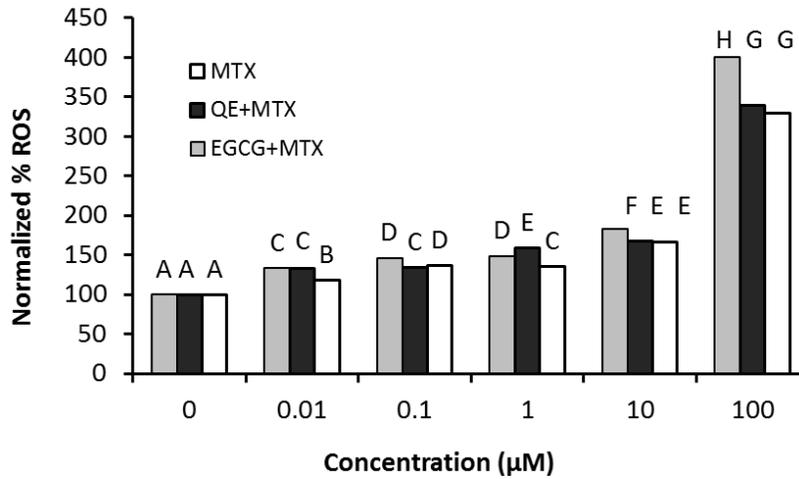
treated with 10  $\mu\text{M}$  concentrations of QE or EGCG for 5 hours, followed by cell washing to remove the external phenols, and 72 hour treatment with 0-100  $\mu\text{M}$  of methotrexate. The results in Fig. 2 show that the concentration of ROS increased by 50% and 300% following treatment with 10  $\mu\text{M}$  or 100  $\mu\text{M}$  methotrexate compared to a non-treated control. Pre-treatment with QE or EGCG seemed to produce slightly greater rises of ROS when cells were subsequently treated with methotrexate. Under the conditions of these tests, QE or EGCG seemed to act as pro-oxidants and there was no evidence for a direct antioxidant effect.

Fig. 3 shows the changes of intracellular ROS for MDA-MB-231 cells preloaded with DCFH-DA and then treated with non-toxic doses (1  $\mu\text{M}$ ) of methotrexate, QE, or EGCG and combinations of these agents for 60 minutes. Interestingly, low doses of QE, EGCG and methotrexate produced increases in intracellular ROS compared with the cells exposed to culture media only (control). According to Fig. 3, the ROS levels decreased in the sequence: media < MTX+EGCG = MTX+QE < MTX < QE < EGCG. The effect of EGCG alone was significantly higher than MTX+EGCG (Fig. 3). The results suggest that pre-treatment with QE or EGCG could ameliorate ROS formation in response to MTX but more research is needed to confirm this. There were no significant changes

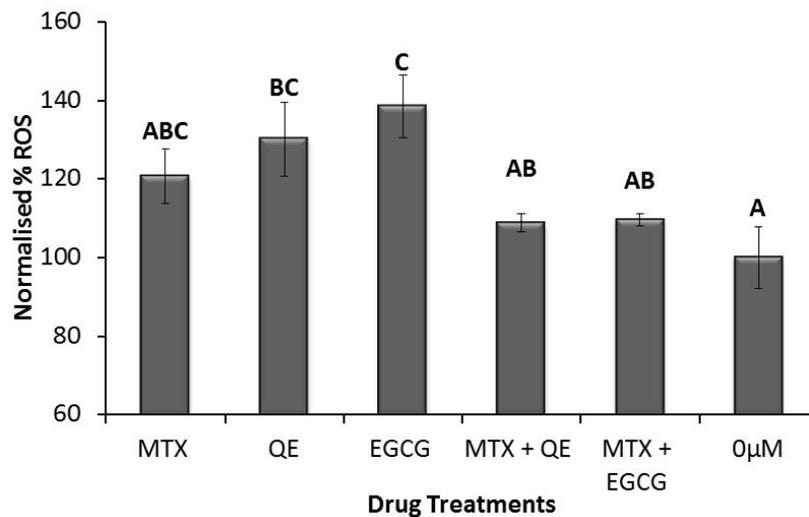


**Fig. 1. The effects of methotrexate (MTX), quercetin (QE) or epigallocatechin gallate (EGCG) on MDA-MB-231 breast cancer cell viability**

Bars show average values from three independent experiments with  $N = 18$  pooled observations with  $\pm$  standard error of the mean. Different letters at each concentration show significantly different results across all treatments in this study,  $n = 18$  for each treatment ( $P < 0.05$ ). Treatment time was 72 hrs



**Fig. 2. The effect of breast cancer cell treatment with quercetin (QE) or epigallocatechin gallate (EGCG) on methotrexate induced reactive oxygen species (ROS) measured using 2', 7'-dichlorofluorescin diacetate (DCFH-DA) as intracellular probe**  
 Cells were treated with 10 µM of QE and EGCG for 5 hours, followed by 72 hour methotrexate drug test n=18 for each treatment. Different letters show significantly different results across all treatments in this study (P<0.05)



**Fig. 3. The effect of combination treatment with quercetin (QE) and methotrexate or epigallocatechin gallate (EGCG) and methotrexate on intracellular reactive oxygen species (ROS) measured using 2', 7'-dichlorofluorescin diacetate (DCFH-DA) as intracellular probe**  
 MDA-MB-231 cells were treated for 60 min. bars show average values from three independent experiments with N = 18 pooled observations ± standard error of the mean. Different letters at each concentration show different subsets of data identified by SPSS post-hoc analysis (P<0.05)

in intracellular GSH for any treatments (data not shown). Decreases of GSH may occur in cells due to rising oxidative stress. However pro-oxidant agents such as QE and EGCG may also increase GSH and hence the overall response is less predictable. Though plant phytochemicals behave as antioxidants in-vitro, these agents

often show low levels of bioavailability and levels in the circulation or tissues are insufficient to account for a direct antioxidant effect. It has been suggested that low levels of phytochemicals and oxidation products may produce “indirect” adaptive response, .e.g. induction of antioxidant enzymes in cells [16].

#### 4. CONCLUSION

This study confirmed that methotrexate was cytotoxic for MDA-MB-231 cells and that this cell line is more resistant compared to the non-resistant breast cancer cell lines such as MCF-7 with a reported IC<sub>50</sub> value of 0.9 µM (14). QE and EGCG are also cytotoxic to MDA-MB-231 but with lower efficacies compared to methotrexate according to their IC<sub>50</sub> values (Table 1). A 5-hour pre-treatment with QE or EGCG prior to methotrexate decreased the IC<sub>50</sub> value for methotrexate by 28% (QE) or 16% (EGCG) indicating tea phenols may augment the effects of methotrexate. Measurements of ROS showed methotrexate, QE and EGCG had a pro-oxidant effect in the cells. However, the rise of ROS was lower following combination treatment using methotrexate with EGCG. Under the conditions of the present study, GSH levels were not significantly affected. The initial hypothesis appears to be supported by the current finding, i.e. tea polyphenols show an enhancing effect towards methotrexate cytotoxicity that has been reported for other systems [8,9].

In speculating on the wider implications of the current preclinical studies, consumption of tea is unlikely to adversely affect methotrexate therapy. However, more research is needed into the conditions required to optimize favourable interactions between tea polyphenols and methotrexate particularly since the former polyphenols are unstable under the conditions typically employed in culture [16]. In the longer term, enhancement of methotrexate by tea phenols would offer opportunities for revitalizing the use of an already established drug without further additional drug approval costs.

#### ETHICAL APPROVAL

MDA-MB-231 cells were cultured outside the human body; therefore the storage of cell lines does not require a human tissue authority (HTA) license. No ethical approval is required.

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#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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