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Methotrexate Combination Effects with Genistein and Daidzein on MDA-MB-231 Breast Cancer Cell Viability

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Authors' contributions

This work was carried out in collaboration between both authors. Author ROA designed the study, wrote the protocol, performed NLR and CompuSyn analysis and wrote the first draft of the manuscript. Author AM managed the literature searches, conducted data collection, performed the statistical analysis and wrote the MSc thesis from which this paper derives. Both authors read and approved the final manuscript.

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ABSTRACT

Aims: To investigate the effect of methotrexate and soy isoflavones genistein and daidzein on MDA-MB-231 breast cancer cell viability.
Study Design: *In-vitro* study using cultured cells
Place and Duration of Study: Nutrition Innovations Centre for Food & Health (NICHE), School of Biomedical Sciences, Ulster University, data collected September 2014-2015.
Methods: Human breast cancer MDA-MB-231 cells were cultured in DMEM (with 10% FBS, 1% Pen strep) and treated with methotrexate, genistein or daidzein for 72 hrs. Combinations treatments used non-fixed ratios of methotrexate (0-100 µM) and genistein or daidzein (30 µM) with cell viability monitored using the MTT assay.
Results: The 50% effect dose (EC50) was 44.7±6.4 µM for methotrexate, 55.8±3.9 µM for genistein or 67.4±12.2 µM for daidzein. Combination treatments with genistein or daidzein

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produced EC50 of 57.6±2.0 or 29.7±2.4 µM for methotrexate, respectively. The combination index (CI) was 1.9 for methotrexate-genistein whilst CI was 1.1 for methotrexate-daidzein near the median dose. Values for CI decreased from 5.0 towards 1.0 as the ratio of methotrexate: isoflavone increased. The results are discussed in terms of prevailing ideas concerning how phytochemicals affects drug adsorption, distribution, metabolism and excretion (ADME) and the expected consequences for cytotoxicity.

Conclusions: Treatment of MDA-MB-231 breast cancer cells with methotrexate and genistein or daidzein produces interactions consistent with antagonism (CI =1.1-5.0) but the effects are predicted to diminish with rising methotrexate to isoflavone ratio.

Keywords: Methotrexate; breast cancer; isoflavones; genistein; daidzein; interactions.

1. INTRODUCTION

Methotrexate is used frequently for treating leukemia, solid tumors and rheumatoid arthritis [1]. Acquired resistance to methotrexate [2] prompts high-dose therapy leading to a likelihood of toxic side-effects [3]. Combination therapy with antioxidant phytochemicals was proposed to mitigate the toxic side effects associated with methotrexate but the impact on drug effectiveness remains controversial [4,5,6]. Observational studies showed there were low rates of breast cancer incidence in Asian women linked to the consumption of soy products [7]. A variety of soybean products were found to prevent methotrexate gastro-intestinal toxicity, but the anti-apoptotic components appeared to be relatively high molecular weight (>10 kd) compounds [8]. Soy isoflavones possess anticancer activity [9,10]. Genistein produces synergisms with other anticancer agents due to a chemo-sensitizing effect [10,11].

To our knowledge no investigation of the effect of soy isoflavones on methotrexate cytotoxicity towards breast cancer cells has been published. The aim of this study was to investigate the effect of methotrexate combined with soy isoflavones genistein and daidzein on MDA-MB-231 breast cancer cell viability. The approach conforms to a variable ratio method for assessing interactions using the median effect model detailed by Chou and co-workers [12].

2. MATERIALS AND METHODS

Methotrexate, genistein, daidzein, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl Sulfoxide (DMSO) and other chemicals were obtained from Sigma-Aldrich (UK).Human breast cancer cell line MDA-MB-231 was from American Type Cell Culture Collection (LGC Standards Teddington, Middlesex, UK). Dulbecco's Modified Eagle's Medium (DMEM) type 31885 (with low glucose), Fetal bovine serum (FBS), Trypsin (1X) and consumables for cell culture were from Fischer Scientific- Invitrogen Ltd (UK).

2.1 Cell Culture

MDA-MB-231 breast cancer cells were cultured using DMEM medium supplemented with +10% FBS and 1% pen step. Culture flasks and 96-well micro-plates were incubated at 37℃ in a 5% CO_2 atmosphere (LEEC Research CO_2 Incubator, LEEC Ltd., Nottingham, UK). Cells were trypsinized, counted using a NucleoCounter (model NC-3000, ChemoMetec, Allerod, Denmark) and seeded at 10,000 cells/well in 96 well plates with 50 µl media per well. Cells were incubated overnight at 37℃ to allow attachment.

Stock solutions of methotrexate, genistein, and daidzein (100 mM) were prepared with DMSO, diluted 10 fold with DMEM and sterilized with 0.2 µm cellulose acetate filters before use. Sterilized solutions were serially diluted to achieve 2x target concentration (max; 200 µM with $\leq$0.05% DMSO final concentration). Cells were treated with methotrexate, genistein or daidzein alone and incubated for 72 hours. Cell viability was determined using the MTT assay (Section 2.2.). These tests were analyzed to find EC50 values and such data were used for the design of combination studies.

2.2 MTT Assay

Microwell plates were washed x 2-fold with ice cold PBS (100 µl) with a third wash remaining in the wells. MTT reagent 20 µl (5 mg/ ml in PBS buffer) was added per well and plates were incubated at 37℃ for 2 hours. Blue formazan crystals formed were dissolved by adding 100 µl isopropanol (with 0.04N HCl) and incubating for one hour. Absorbances were measured at 570 nm using a microplate reader (VersaMax™ ELISA microplate reader, Molecular Devices, Sunnyvale, CA, USA.).Data were expressed as

mean ± standard error of means (SEM) of 2-independent experiments with six-replicate (microwells) per treatment-concentration (n=12).

2.3 Combination Studies

Samples containing (4x target concentration) methotrexate (25 µl) were added to microplate wells containing 10,000 seeded cells and 50 µl of culture media. Then 25 µl of genistein or daidzein (4x target concentrations) were added to achieve a final concentration equal to one-half of EC50 for each isoflavone. The final range of methotrexate concentrations were 0, 1, 2, 5, 10, 20 and 100 µM. The cells were incubated for 72 hours and subjected to MTT assay as describe above. Combination test involved 3-independent experiments with 6 microwells per each treatment concentration (n=18).

2.4 Data and Statistical Analysis

Experimentally values for cell viability were fitted by non-linear regression (NLR) to the Logistics function shown below, where PRED is the predicted response, C = minimum response, and M= maximum response. E is 50% effect dose or drug-dose, which produces a 50% response (E50), and B is the steepness of the curve [13].

PRED =C+ ((M-C)/(1+EXP(B*Ln(D/E))))

NLR was implemented using SPSS software (IBM SPSS v21.). Estimates for EC50 were subjected to isobologram analysis. Dose-response data were also analyzed median effect model. The fraction of affected cells (Fa) and unaffected cells (Fu) are calculated for different doses of drug (Fa = 100-% viable cells/100 and Fu = 1-Fa). A plot of log (Fa/Fu) versus drug concentration was fitted to a straight-line graph (Y = mx +c) to determine the median effect dose (Dm) and slope (m) using CompuSyn™ software [12]. Values for the combination Index (CI) were calculated either manually or via CompuSyn™. To determine CI manually, we used the relations below, where d_{50}MTX and d_{50}Gen are the 50% effect dose from combination studies, and $EC50_1$ and $EC50_2$ are values for each agent alone.

$$CI = \frac{d_{50}MTX}{EC501} + \frac{d_{50}Gen}{EC502}$$

The size of CI is indication whether combination therapies produce synergism (CI<1.0), antagonism (CI>1.0) or additive behavior (CI=1.0) [13].

3. RESULTS

3.1 Dose-response Parameters

Table 1 and Fig. 1 show dose-effect parameters for MDA-MB-231 cells treated with methotrexate, genistein or daidzein determined using a logistic function to fit experimental data. There was a good fit for NLR predictions with observed points (R2 = 0.98-0.99). Table 2 shows dose-effect parameters arising from CompuSyn™ analysis of the same data. The median dose (Dm) corresponds to the 50% effect dose (EC50) and slope (m) is a measures of the steepness of the plot of log (Fa/Fu) versus drug concentration.

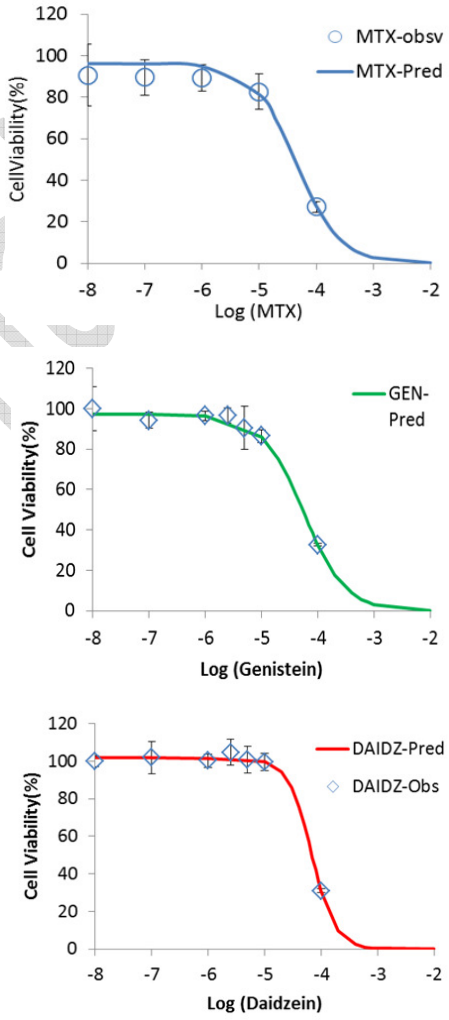

Fig. 1. Dose response curve for MDA-MB-231 breast cancer cells treated with methotrexate, genistein or daidzein as single treatments

Continuous lines shows response predicted by NLR. Experimental points are shown as means ± SD (n=12) from two independent experiments

Table 1. Dose-response parameters for MDA-MB-231 breast cancer cells treated using methotrexate and isoflavones determined by non-linear regression analysis (SPSS)

Agent/ NLR parameter	EC50 (E)µM	Slope (B)	Max (M)
Methotrexate	44.7±6.4	1.14±0.29	96.2±3.3
Genistein	55.8±3.9	1.17±0.11	97.4±1.7
Daidzein	67.4±12.2	2.1±2.20	102.0±1.0
Methotrexate (+30µM Genistein)	57.6+2.0	0.62±0.04	99.0±0.54
Methotrexate (+30µM Daidzein)	29.7±2.4	0.40±0.02	111±2.0

Data shows mean ±SEM as determined by NLR with bootstrap; Parameters are EC50 (E), response slope (B) and maximum cell viability (Max). The lower-limit for cell viability (C) was constrained as zero during NLR

Table 2. Dose-response parameters for MDA-MB-231 breast cancer cell treated with methotrexate, genistein or daidzein as determined by CompuSyn *

Agent/parameter	Dm (µM)	Slope (m)	r
Methotrexate	42.1±5.0	0.46±0.08	0.916±0.005
Genistein	50.4±2.6	0.88±0.31	0.996±0.064
Daidzein	64.1±1.5	1.90±0.18	0.911±0.005

**Mean ±SD, from two independent experiments with 6 replicates per drug dose, n=12. Dm = median dose (µM), m = response slope, r = regression coefficient*

Results for NLR analysis of treatments combining methotrexate and genistein or daidzein are shown in Fig. 2. Isobologram analysis predicted EC50 values for methotrexate of 20-22 µM in the presence 30 µM daidzein (~1/2 EC50 dose). The actual EC50 values observed by for methotrexate combinations with genistein or daidzein are shown Table 1.

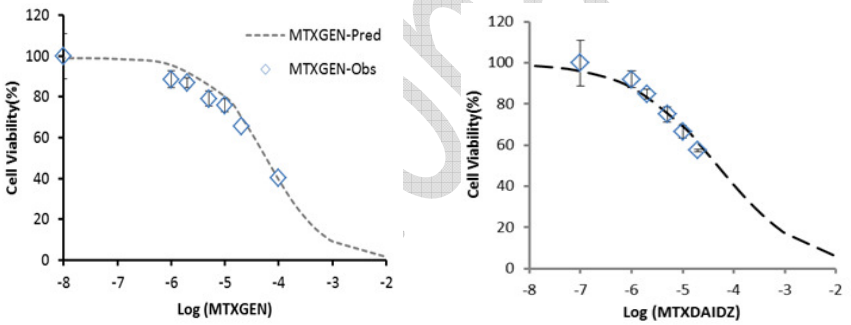

Fig. 2. Combination treatment of MDA-MB-231 breast cancer cells using methotrexate and isoflavones

Notes (left panel) methotrexate plus genistein (MTXGEN), (Right-panel) methotrexate plus daidzein (MTXDAIDZ)

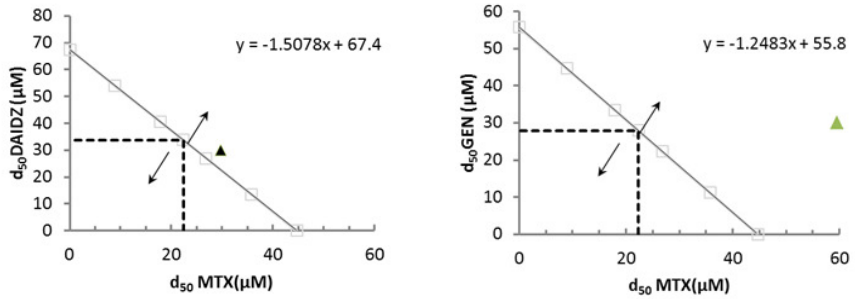

Fig. 3. Isobologram for methotrexate combination with daidzein or genistein

The iso-effective doses predicted to produce 50% effect when daidzein (DAIDZ) or genistein (GEN) is added with methotrexate (MTX) in a variety of doses; (♦) = observed EC50 for combination studies. *Dotted = predicted in this study for additive (no interaction) response between two agents*

The combination index (CI) value using data from Table 1 was CI = 1.9 for methotrexate-genistein and CI = 1.1 for methotrexate-daidzein combination at the concentrations corresponding to 50% effect. The values for CI were predicted to increase from 1.1 to 5.0 (Fig. 4) with decreasing methotrexate: isoflavone ratio (Fig. 4).

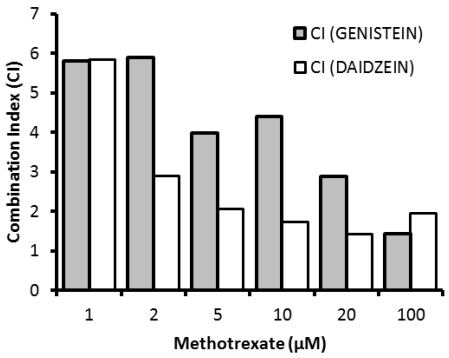

Fig. 4. A plot of combination index (CI) values for treatment of MDA-MB-231 breast cancer cells with methotrexate (1-100 μM) and 30-μM daidzein or genistein

Data generated using CompuSyn analysis

4. DISCUSSION

Methotrexate is an anticancer agent [1-3,14]. Consumption of soy isoflavones is thought to reduce the risk of breast cancer [8,9,10]. Methotrexate inhibits cell proliferation by blocking the enzyme dihydrofolate reductase (DHFR), depleting the intracellular pool of folate, and by restriction of methyl-group availability for DNA synthesis [1]. In addition, exposure to methotrexate increases intracellular oxidative stress due to the inhibition of NAD(P)H-liked reductases [14,15]. Soy isoflavones show estrogen receptor activation, tyrosine kinase inhibition, induction of cell-cycle arrest, anti-inflammatory action, and general antioxidant activity [10,16,17]. For the preceding reasons, we tested the hypothesis that combination of methotrexate with isoflavones would produce enhanced anti-cancer activity compared with each agent alone.

EC50 values from this study (Table 1) are in broad agreement with previous reports, taking account of differences in assay conditions, e.g. different culture medium, and drug exposure times. The EC50 was 80 μM for methotrexate treatment of MDA-MB-231 cultured with MEM media and a drug exposure time of 24 hrs [18]. By comparison, EC50 was 18.5 μM methotrexate with low protein (5% FBS) medium [19]. Tests using genistein and MDA-MB-231 cells showed that EC50 ranges from 46.8 μM [20] to 50-90 μM [21,22] depending on the exposure time and other assay conditions.

There was antagonism between methotrexate and soy isoflavones in this study. The EC50 for methotrexate increased from 44.7 μM to 57.6 μM in the presence of genistein with CI>1.0 indicating antagonism. The EC50 value for methotrexate with daidzein present (29.7 μM) was closer to values (20-22 μM) predicted by isobologram analysis (Fig. 3) for additive response meaning neither positive nor negative interaction (Table 1 and Fig. 3.) but here also CI >1.0. For both isoflavones CI> 1.0 indicating antagonism. The degree antagonism between methotrexate and genistein seem to be greater than those with daidzein. CompuSyn^{Tm} predictions showed decreasing antagonism (Fig. 4) with increasing methotrexate: isoflavones ratio.

Relating *in-vitro* data to human exposure conditions requires that EC50s are subjected to *in-vitro*, in vivo extrapolation (IVIVE) by stepwise correction for the effects of absorption, serum protein binding, liver metabolism and renal clearance [23,24]. Past literature, data may be useful also for addressing IVIVE issues. Briefly, it is known that methotrexate is bioavailable and oral doses of < 20 mg/m^2 (<0.54 mg/Kg) methotrexate are 50-95% absorbed leading to peak plasma concentrations of 300-2000 μM within 1.5-3 hours and a half-life of elimination of 4-6h [3]. Most of the circulating methotrexate is excreted via the kidneys in an intact form with <10% metabolism in the liver to form 7-OH methotrexate [3]. On the other hand, the plasma-concentration profile for soy isoflavones is influenced by a host of factors, e.g. relative proportion of aglycone and glycosylated forms, type of food matrix used for administration, and forms of processing [25]. Using the dose for genistein or daidzein frequently used in human trials (45-56 mg/ day), the fractional excretion rate (apparent bioavailability) was 20-50% with a peak plasma concentration of 2-5 μM some 4-8hrs after intake [26]; reviewed by [27]. On the basis of such data [3,26,27], a typical exposure to 20 mg methotrexate + 56mg genistein would produce plasma concentrations for methotrexate that are 60-400 times higher compared to the peak plasma concentration for isoflavone. Moreover, the peak concentrations for isoflavone would occur after the peak for methotrexate [3,26,27]. According to the present paper high

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methotrexate: isoflavone ratios are not conducive for antagonism.

Evidence is emerging that antioxidant phytochemicals can reduce toxicity to healthy cells, whilst not affecting efficacy [4-7] depending on changes to absorption, metabolism, distribution and excretion (ADME) characteristics [15,28,29,30]. Research using leukemic cells showed that genistein inhibits methotrexate uptake by the reduced folate transporter owing to its role as tyrosine kinase inhibitor [31,32,33]. Genistein was found to promote the transcription of efflux transporter (ABCC1/MRP1) protein for MDA-MB-231 cells with no net effect onmitoxantrone toxicity owing to the simultaneous inhibition of the same transporter [34]. Many polyphenols from beverages were also reported to inhibit methotrexate and folate uptake at low pH involving the proton coupled folate transporter [35-39]; genistein had no effect on the low-pH uptake of methotrexate and folate by CaCo2 cells [35]. Interestingly, genistein was reported to moderate genes from MDA-MB-231 cells suppressed by epigenetic mechanism [21] thereby increasing the cell sensitivity to therapeutic drugs. In general, methotrexate toxicity would be enhanced by phytochemicals that increase uptake, promote methotrexate modification to polyglutamated forms, and / or decrease methotrexate efflux [15]. For example, genistein (and its metabolites) were found to be inhibitors for breast cancer resistant protein (ABCG2/BCRP) efflux transporter [40,41]. Methotrexate and 7-OH methotrexate were also identified as substrates for ABCC2 (MRP2), ABCC3 (MRP3), and ABCG2/BCRP) and found to have an enormous impact on drug concentration profile [29].

5. CONCLUSION

In conclusion, a diverse range of potential interactions may occur between genistein and methotrexate that go to produce antagonism with regard to cytoxicity for MDA-MB-231 cells. The results from this study show a rising tolerance of breast cancer cells towards methotrexate in the presence of genistein. However, we speculate that antagonism is unlikely where concentrations for methotrexate are much higher than genistein. More research is needed also to consider methotrexate interactions with isoflavones in terms of changes to ADME and the consequences for other health outcomes. The different levels of antagonism observed for methotrexate and genistein or daidzein is interesting and worthy of further study. There is scope also to consider the possible role of soy isoflavone/ MTX therapy on immune responses or rheumatoid arthritis [1,3,8,14,35,36].

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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