Combination effects of different ratios of PCB 153 and the phytoestrogen genistein upon DNA synthesis in an MCF-7 breast cancer cell line.

Miriam N. Jacobs* Sue Starkey** and Richard Hoffman**

*School of Biological Sciences,** EIHMS, University of Surrey, Guildford, Surrey,GU2 5XH UK

Introduction

Organo-chlorine chemicals able to mimic oestrogens or anti-androgens may play a role in the worldwide rise in breast and testicular cancers and male reproductive disorders. Interest in phytoestrogens is increasing due to their possible cancer preventative effects for oestrogen dependent cancers, and their beneficial post menopausal effects. Both are available from the diet, and individually have low potencies compared to oestradiol. However human populations are exposed to complex mixtures of environmental agents which may act together or modulate one another to produce biological effects. PCBs are known to bind to oestrogen receptors (ERα and β)[1-3] some having oestrogenic effects across species by stimulating the transcriptional activity of the ER, others having antioestrogenic activity. They are also Ah receptor agonists with additional varied toxicological endpoints, which include developmental defects, neurotoxicity and immunotoxicity. Xenoestrogens such as the organochlorines are ubiquitous, and unlike phytoestrogens have long half lives. They are present at detectable levels in food and water. DDT and PCBs such as 2,2',4,4',5,5'-hexachlorobiphenyl (PCB 153) and PCB 118, are the predominant congeners in fatty foods such as fish, fish oils, meat and dairy products due to their lipophilicity and tendency to bioaccumulate. Phytoestrogens are also delivered through the diet, and are widely available from plant foods, e.g. in pulses such as soybeans, grains such as rye, oilseeds such as linseed,- and other plants with nitrogen fixing bacteria.

Epidemiological studies have shown that Asian women and vegetarians have lower breast cancer risk, together with a relatively higher excretion of urinary phytoestrogens[4-6] than Western populations. In the past, high dietary intakes of phytoestrogens have given cause for concern in animal husbandry and wild animals in captivity, due to the initiation of infertility syndromes. Phytoestrogens such as daidzein and genistein have been shown to bind to oestrogen receptors, at relatively low levels, over a concentration range of 0.3 to 10 μM[1, 7]. Genistein has also been shown to induce wide ranging anti-cancer effects in cell lines independent of any hormone related influence. Phytoestrogens have been proposed to influence carcinogenesis via their hormonal and antihormonal actions. Oestrogenic effects of phytoestrogens include binding to the ER or type II estradiol (E2)-binding site, promotion or proliferation of reproductive organs in animals, induction of pS2 protein expression, and stimulation of growth in oestrogen-dependent human breast cancer cells. Proposed antioestrogenic effects include competition with E2 for binding to the ER, reduction of oestrogen synthesis via inhibition of aromatase activity, downregulation of E2-induced expression of ER and pS2 protein in MCF-7 cells, and inhibition of E2-induced growth in breast cancer cells[10]. Genistein may also mediate the inhibition of tyrosine phosphorylation.

This study was designed to test directly the interactions between different dietary phytoestrogens and organochlorine xenoestrogens using the MCF-7 cell proliferation assay, based upon a standard protocol [2,11] at levels which may occur physiologically (based upon human dietary intakes and biological samples of the respective compounds as reported in the literature). MCF-7 cells require oestrogenic agents for growth, so the assay is an appropriate *in vitro* screen for compounds mimicking the endogenous hormone. Here we report the combined mitogenic effects

of two environmental oestrogenic agents, one a phytoestrogen, genistein and the other a xenoestrogen, PCB 153, chosen for their dietary predominance compared with other dietary oestrogens in their classes, at 20:1 and 40:1 ratios.

Materials and Methods

i) Cell Culture: The MCF-7 human breast cancer cell line was kindly provided by Prof. P. Smith, University of Cambridge, UK. The cells were maintained in cell culture flasks with DMEM/10% foetal calf serum containing antibiotics (100 units/ml penicillin and 100μg/ml streptomycin) at 37°C and incubated with 5% carbon dioxide after each procedure. The medium was changed once every other day, and the cells were passaged once each week.

ii) Cell proliferation assays Cells were cultured in 96-well plates (2×10^3 /well) in medium for 2 days. The media was removed from each well and the cells washed once with 200μl phenol red free DMEM, due to the potential oestrogenic effect of the phenol red. This was removed and 200μl phenol red free DMEM containing 10% charcoal stripped serum was added to each well. The plated cells were incubated for 2 days. On day 5 the media were removed and the test compounds were added with or without 10nM E2. DNA synthesis was determined by measuring incorporation of [methyl ^{3}H] thymidine 24h after adding the test compounds, using a liquid scintillation rack beta counter.[11]

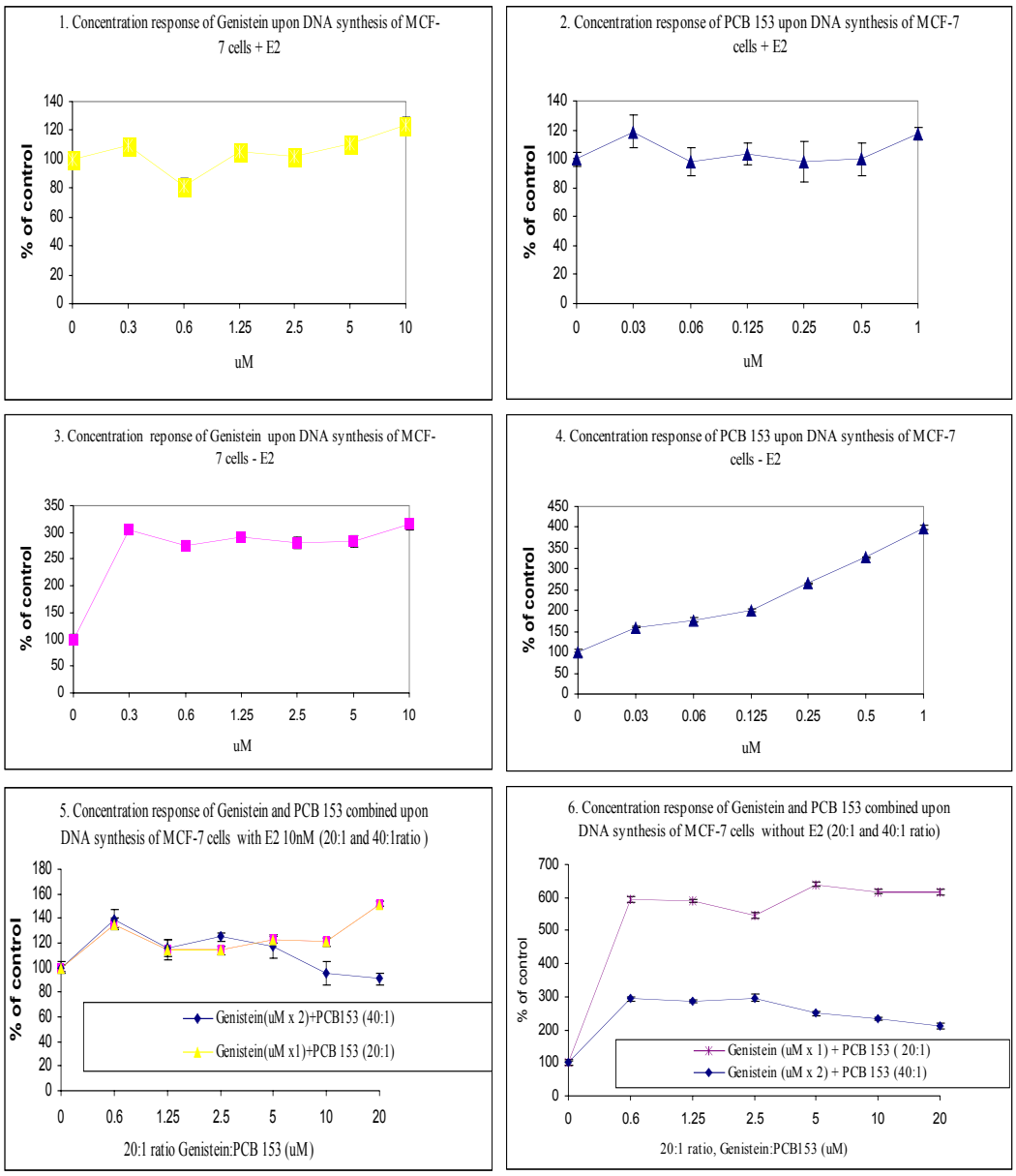
iii) The test compounds: The Genistein stock solutions were dissolved in methanol at concentrations of 10mM. They were stored in the dark at –20°C. The E_2 stock solution was prepared by dissolving E_2 in ethanol at a concentration of 1mM, and was stored in the dark at -20°C. The final concentration of ethanol in the test solution was less than 0.01%, below the concentration which affects the cells. The PCB compound was kindly provided and prepared in methanol by Dr. C. Wright, CSL., Norwich, UK, at a stock concentration of 1mM, and stored at 4°C. The concentration of 17β oestradiol required for maximum cell growth, was taken to be 10nM, which is reported to be optimum in the literature[8,10]. Each curve was supported by 10-18 observations over 6 different concentrations with the 10:1 and 20:1 combinations.

Treatment of results: The means and standard errors of the concentration-response of the thymidine incorporated DNA synthesis of MCF-7 cells were calculated and normalized with respect to each experimental control. The concentration-response relationships of genistein and PCB153 upon DNA synthesis of MCF-7 cells individually and in 10:1, 20:1, and 40:1 combinations with and without 10nM E2 were investigated. The top concentration for PCB153 was 1μM, and for genistein the top concentration was 40μM. The cells with no treatment were considered to be negative controls, and the cells pretreated with E2 were the positive controls. Data with standard errors above 15% were excluded. The stimulation of DNA synthesis in the presence of E2 was indicated by a minimal increase of at least twofold compared to cells in the absence of E2.

Results and discussion

The tests determined concentrations at which genistein and PCB 153 elicited an oestrogenic response, and examined the hypothesis that genistein was able to reduce the oestrogenic effect of a second molecule, namely PCB 153. Representative test results are presented in Figs 1-6. The PCB concentrations remained the same throughout. Where error bars are not shown they are smaller than the symbols. 10nM E2 stimulated a three to fourfold increase in DNA synthesis in the +E2 control compared with the -E2 control. Both PCB 153 and Genistein did not display significant enhanced DNA synthesis above the positive control (Figs 1 and 2). In the absence of E2, a threefold increase of DNA synthesis was observed at concentrations of 0.3 to 10μM for genistein

(Fig 3) and an enhanced concentration related DNA synthesis was observed for 0.03 to1μM PCB 153 (Fig 4). The top concentration of 1μM PCB 153 appears not to have reached peak oestrogenic



stimulation. The combinations of genistein and PCB 153, in the presence of 10nM E2 did not significantly enhance DNA synthesis at doses shown (Fig 5) . In the absence of E2, significantly enhanced DNA synthesis relative to the –E2 control was observed, and the effect appears to be additive. However the DNA synthesis of the 40:1 ratio of Genistein:PCB 153 was reduced

compared to the 20:1 ratio (Fig 6). This suggests that the higher ratio of Genistein:PCB 153 may reduce the oestrogenicity of PCB 153. Genistein may have an antioestrogenic or inhibitory effect in presence of PCB 153 at concentrations above 20μM. Further observations are required for the 40:1 test ratio. It would also be preferable to use hydroxy PCB 153, the active metabolite of PCB 153. The increased cell growth seen with the combinations could be also be due to nonspecific growth enhancement. The 40:1 genistein: PCB 153 ratio may be the dose that gives this effect for genistein. The dose response trends were clear, did not alter, were reproducible and are similar to recent results for genistein, reported in the literature[1, 9, 10]. Phytoestrogens have variable effects on DNA synthesis in MCF-7 cells. A concentration of 50μM genistein has been reported to inhibit DNA synthesis induced by a range of concentrations of E2[10]. Hsieh and coworkers[9] report that *in vitro* concentrations of genistein, above 20μM have an inhibitory effect upon the MCF-7 cell line, but an oestrogenic effect at lower concentrations. Genistein administered with 10nM E2 did not show the same stimulation of DNA synthesis.

In these tests, genistein (concentrations ranging from 0.3 to 20μM) did not reduce or have a synergistic effect upon the induction of DNA synthesis by PCB 153, at PCB 153 concentrations of 0.03, 0.06, 0.125, 0.25, 0.5 and 1μM. PCB 153 displayed oestrogenic activity at all concentrations tested. *In vitro* and *in vivo* evidence for the anticancer, inhibitory activity of phytoestrogens varies according to type, timing, and size of dose. Timing of the oestrogenic compound administration appears to be critical. If dietary phytoestrogens are administered before mammary gland maturation and initiation with a mammary carcinogen, then the numbers of tumors are likely to be reduced, due to the effect of the oestrogen on mammary gland maturation[12]. However if genistein (acting oestrogenically) is administered after the development of an oestrogen dependent tumour, tumour growth will be stimulated[9]. Although there are few indications of harmful effects at present, possible proliferative effects have been found in this study and reported by others[8].

The apparent multiplicity of action of certain hormonal carcinogens (i.e., oestrogenic and genotoxic) must be considered when examining the possible risk or benefits of exposure to phytoestrogens. At certain concentrations used in this proliferation study, genistein was not shown to be protective against the oestrogenic activity of PCB 153, but appeared to be protective at the 40:1 ratio. However in view of the contradictory evidence, more work needs to be done to elucidate possible protective or oestrogenically additive effects of genistein towards xenoestrogens and breast cancer.

References

1. Kuiper, G. C. J. M., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P.T., van der Burg, B., & Gustafsson, J-Å. *Endocrinology* **1998** 139: 10. 4252-4263
2. Soto, A.M., Sonnenschein, C., Chung, K.L., Fernandez, M.F., Olea, N., Serrano, F.O. *Environ Health Perspect* **1995** 103 (Suppl 7): 113-122.
3. Gierthy, J. F., Arcaro, K. F., & Floyd, M. *Chemosphere* **1997** 34: 1495-1505
4. Aldercrutz, H. *Proc. Soc. Exp. Biol. Med.***1998** 217**:**241-246
5. Bingham, S.A., Atkinson, C., Liggins, J., Bluck, L., Coward, A. *British Journal of Nutrition,***1998** 79**:** 393-406
6. Cassidy, A. *Proc. Nutrit. Soc.* **1996** 55**:** 399-417
7. Miksicek, R.J. *Proc. Soc. Exp. Biol. Med ,* **1995** 208, 44-50.
8. Zhou & Lee, A. S. *Journal of the Natl Cancer Institute,* **1998** 90**:** 381-388
9. Hsieh, C-Y., Santell, R. C., Haslam, S. Z., & Helferich, W. G. *Cancer Res.* **1998** 58: 3833-3838
10. Wang, C., & Kurzer, M. S. *Nutrition and Cancer* **1998** 31**:** 90-100
11. Pink, J. J., & Jordan, V. C. *Cancer Res.* **1996** 56: 2321-2330.
12. Lamartiniere, C. A., Moore, J., Holland, M. B., & Barnes, S. *Proc. Soc. Exp. Biol. Med.* **1995** 208:120-123