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Pleiotropic combinatorial transcriptomes of human breast cancer cells exposed to mixtures of dietary phytoestrogens

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ABSTRACT

Combinations of estrogen receptor agonists have been shown to exert more potent effects than single compounds in many single-endpoint bioassays. However, to our knowledge, it has never been tested how genome-wide expression programs are shaped by the interplay of multiple estrogenic stimuli. In view of the abundance of dietary phytoestrogens, we selected binary mixtures of these phytochemicals to determine their global impact using high-density DNA microarrays. MCF7 cells, a frequent *in vitro* model for molecular processes associated with breast cancer, were exposed to a sub-saturating concentration of coumestrol either alone or in combination with analogs that exhibit 1000-fold lower estrogen receptor activity. As expected, in the presence of coumestrol, the induction of many estrogen-sensitive genes was not further increased by the addition of resveratrol or enterolactone. However, it was surprising to find that these weak phytoestrogens, when combined with coumestrol in equal concentrations, were able to more than double the number of significantly regulated transcripts. Thus, phytoestrogens with low receptor affinity interact with other estrogenic agonists to generate more widespread expression fingerprints. This effect involving the number of susceptible transcripts instead of their amplitude of induction remains undetected if mixtures are evaluated with conventional bioassays.

1. Introduction

A variety of hormone-like chemicals that modulate essential endocrine functions have been associated with adverse health effects, including cancer, reproductive deficits and developmental disorders (Maffini et al., 2006; Markey et al., 2002). Particular attention has been given to compounds that mimic the action of the endogenous hormone 17β-estradiol (Swan, 2000; Kaufman and Adam, 2002). Such estrogenic chemicals, collectively referred to as xenoestrogens, display manifold biological effects by activating one of several known estrogen receptors (Kuiper et al., 1997; McDonnell and Norris, 2002). In most cases, the low level of these xenoestrogens in the diet or environment contrasts with the high doses needed to produce a response in experimental systems. However, the interplay of xenoestrogens provides an explanation for this apparent discrepancy. In fact, estrogenic mixtures yield a measurable outcome even in situations where each single component is present below the threshold of detectable effects (Payne et al., 2001; Silva et al., 2002; Charles et al., 2002; Brian et al., 2007). The joint activity of functionally related hormones or hormone-like chemicals is more severe because, in combination, they interact following the widely accepted dose addition model (Loewe and Muischnek, 1926; Kortenkamp, 2007). For example, mixtures of estrogen receptor agonists have been shown to stimulate the proliferation of breast cancer cells in a dose-additive manner (Payne et al., 2001; Rajapakse et al., 2004).

Mammalian systems challenged by natural or anthropogenic estrogens almost invariably respond by reprogramming their genome (Naciff et al., 2002; Buterin et al., 2006). Several studies demonstrated that, in principle, the concept of dose addition provides a valid predictive tool to assess combined effects on gene expression. In fact, estrogen-like chemicals cooperate in a dose-additive manner when their activity is assessed with exogenous reporter genes (Silva et al., 2002; Charles et al., 2002) or when the expression of endogenous markers is determined in breast cancer cells (van Meeuwen et al., 2007; Heneweer et al., 2005). The recent advent of large-scale technologies offers the opportunity to use gene expression profiling as a tool to monitor the complex biological activity of hormones and endocrine-like chemicals. Such genome-wide transcriptional analyses generate recognizable

Abbreviations: DCC-FBS, dextran/charcoal-stripped fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ER-CALUX, Estrogen Receptor-mediated Chemical-Activated LUciferase eXpression); FBS, fetal bovine serum.

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"molecular fingerprints" that could be used to predict the impact of new untested mixtures (Afshari et al., 1999; Burczynski et al., 2000; Pennie et al., 2000). However, it is of paramount importance to determine whether the concept of dose addition can be applied to global transcriptomes or, alternatively, if mixtures of hormones or chemicals yield different expression signatures than each individual component. The present study was instigated by the generally increased consumption, particularly in Western societies, of food supplements containing high levels of bioavailable phytoestrogens (Rice and Whitehead, 2006; McCarty, 2006). In fact, epidemiologic findings suggest that phytoestrogens may exert health-protective effects in preventing breast or prostate cancer, cardiovascular disorders or postmenopausal symptoms (reviewed by Branca and Lorenzetti, 2005). Conversely, phytoestrogens may also be responsible for potentially deleterious endpoints particularly in light of their ability to stimulate the growth of breast cancer cells in diverse experimental systems (Hsieh et al., 1998; Allred et al., 2001). This view is supported by the finding that a dietary phytoestrogen supplementation induces hyperplasia and biomarkers of cell proliferation in the breast of pre- and postmenopausal women (Petrakis et al., 1996; McMichael-Phillips et al., 1998; Hargreaves et al., 1999). Thus, establishing the consequences of phytoestrogen intake is an important public health issue (Messina et al., 2006).

In conjunction with regular dietary sources such as vegetables, fruits or diary products, phytoestrogen-rich food supplements give rise to systemically active mixtures of phytochemicals circulating in the body (Adlercreutz et al., 1982; van Meeuwen et al., 2007). Therefore, the purpose of this study was to examine the global transcriptional impact of xenoestrogen mixtures on breast cancer cells using, as an initial model system, binary combinations of phytoestrogens that differ >1000-fold in their biological potency. A key implication of our findings is that the true consequences of dietary phytoestrogens in modifying the risk of mammary cancer cannot be extrapolated from the activity of single major representatives but, instead, has to be evaluated in relation to the overall impact of complex mixtures of these natural xenoestrogens in food and food supplements.

2. Materials and methods

2.1. Chemicals

Resveratrol was purchased from Sigma–Aldrich (St. Louis, MO, USA); 17β-estradiol, coumestrol and enterolactone were from Fluka (Switzerland). The inhibitor ICI 182,780 was purchased from Tocris Bioscience (Avonmouth, UK). All solvents and reagents were of analytical grade quality.

2.2. Cell culture and treatments

All media for cell culture were from Invitrogen (CA, USA). Human T47D.Luc cells (BioDetection Systems, The Netherlands) were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with sodium bicarbonate, 1 mM L-glutamine and 7.5% fetal bovine serum (FBS; HyClone Laboratories, USA). The MCF7 cell line subtype BUS (Soto et al., 1995) was maintained in DMEM supplemented with 10% FBS. The antibiotics used were 0.1 U/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen). All cell lines were cultured at 37 °C in xenoestrogen-free plastic (Corning Inc., Grand Island, USA) under humidified air containing 5% CO_2. Before each experiment, the cells were transferred to phenol red-free medium and cultured for 48 h in the presence of 5% dextran/charcoal-stripped FBS (DCC-FBS). Dimethyl sulfoxide (DMSO) stocks of each test compound were added to the culture medium. Unless otherwise indicated, the final solvent concentration was adjusted to 0.1% (v/v).

2.3. Cytotoxicity assays

A commercial kit was used to measure intracellular ATP levels. Briefly, MCF7 cells were grown in multi-well plates as outlined before and exposed to increasing concentrations of each phytoestrogen. After 24 h, the CellTiter-Glo reagent (Promega) was added and the luminescent signal was recorded in a microplate reader following the manufacturer's instructions. Additionally, the CellTiter 96 and CytoTox 96 assays (Promega) were used to monitor the overall metabolic activity and the release of lactate dehydrogenase.

2.4. ER-CALUX test

The ER-CALUX (Estrogen Receptor-mediated Chemical-Activated LUciferase eXpression) assay was carried out following the instructions provided by BioDetection Systems (Amsterdam, NL). Briefly, T47D.Luc cells were seeded in microtiter plates at a density of 5000 cells per well in 0.1 ml phenol red-free medium containing 5% DCC-FBS. After 24 h, the medium was renewed and the cells were incubated for another 24 h followed by the addition of the indicated test compounds dissolved in DMSO. Solvent controls and a standard 17β-estradiol dose response were

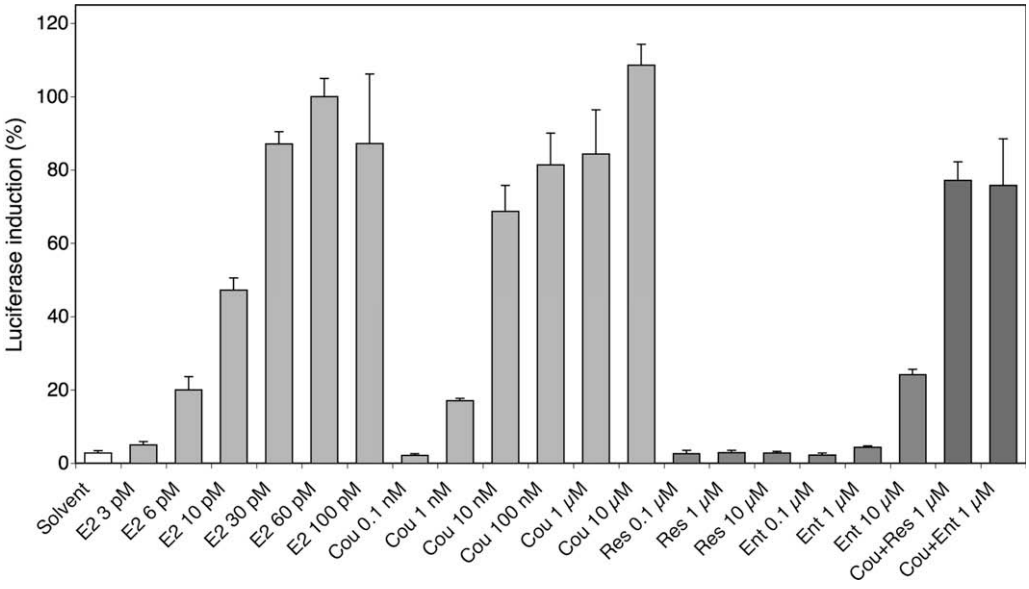

Fig. 1. Comparison of phytoestrogen activity in the luciferase reporter gene assay. Stably transfected T47D.Luc cells were incubated with 17β-estradiol (E2, 1–100 pM) as well as coumestrol (Cou), resveratrol (Res) or enterolactone (Ent) at concentrations of 0.1–10 μM. Binary mixtures were generated by combining coumestrol with either resveratrol or enterolactone (1 μM each). The final concentration of the DMSO solvent was 0.1% (v/v). Estrogen receptor activation was determined by measuring the luciferase induction from a minimal promoter containing repeats of estrogen response elements (mean values of 5–6 independent experiments). The results are shown in percentages of the induction observed with 60 pM 17β-estradiol.