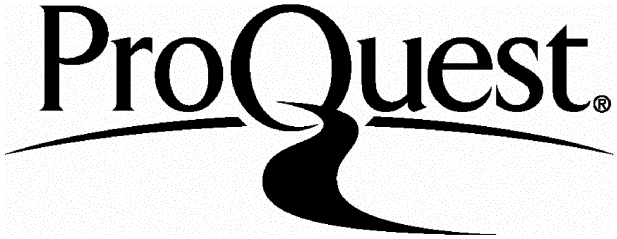
Xenoestrogens: Assessing the predictability of mixture effects

A thesis submitted to the University of London in fulfilment of the requirements for the degree of Doctor of Philosophy at the University of London

Joachim Peter Payne

Centre for Toxicology
School of Pharmacy
London WC1N 1AX

October 2000



Abstract

Recently there has been growing concern over the increasing incidence of endocrine related disorders in both humans and wildlife. This has prompted researchers to speculate about a common underlying environmental cause. A large number of natural and synthetic chemicals have been shown to interact with the estrogen receptor, and it is believed that these xenoestrogens are the agents responsible. We are constantly exposed to many such agents, however, the study of interactions between these chemicals within biological systems has often been hampered by the application of unsuitable models of mixture action. In this study we have employed, for the first time, the well-validated models of concentration addition and independent action to analyse xenoestrogen mixture effects in the MCF-7 cell proliferation assay and the yeast estrogen screen. Both models yield excellent predictions of mixture action in our test systems, and indicate that the xenoestrogens *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDE and β-HCH act additively in the MCF-7 assay. Similarly, we observe that interactions between *o,p'*-DDT, 4-octylphenol, 4-nonylphenol and genistein are additive in the yeast estrogen screen. Assuming that additivity holds true *in vivo*, we have attempted to estimate the human exposure to xenoestrogens which would be required to significantly modulate the activity of estradiol. Our calculations indicate that at current levels of exposure these chemicals could pose a risk to human health. Although this estimate is by no means conclusive, we feel that a large step forward has been made in understanding xenoestrogen mixture effects.

For Lisa, Elaine, Peter, Esther and Tim

List of figures

List of tables

List of abbreviations

AR	Androgen receptor
BBP	Benzylbutylphthalate
CA	Concentration addition
CDHus	Charcoal-dextran stripped human serum
CGH	Comparative genomic hybridisation
CI	Confidence interval
COC	Combined oral contraceptive
CPRG	Chlorophenol red-β-D-galactopyranoside
DBCP	Dibromochlorpropane
DEHP	Diethylhexylphthalate
DES	Diethylstilbestrol
DMEM	Dulbecco's modified eagle's medium
E2	17β-estradiol
EC50	Concentration eliciting a half-maximal effect
ED	Endocrine disrupter
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor (α)
ERE	Estrogen response element
ES	Effect summation
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
HRT	Hormone replacement therapy
IA	Independent action
MIS	Mullerian inhibiting substance
NOEC	No observable effect concentration
NP	4-nonylphenol
OP	4-octylphenol
PBS	Phosphate buffered saline
RTK	Receptor tyrosine kinase
ROS	Reactive oxygen species
SD	Standard deviation
SHBG	Sex hormone binding globulin
SRB	Sulphorhodamine B
TCA	Trichloroacetic acid
UHQ	Ultra high quality water
YES	Yeast estrogen screen

Acknowledgements

I would like to thank Andreas Kortenkamp, my supervisor, for his unending help and support in the completion of this thesis, and for being a good friend. Thanks also go to the BBSRC for providing the studentship which allowed me to carry out this work.

I would also like to thank John Sumpter (Brunel University, UK) for supplying the yeast cells; Nicky Beresford (Brunel University, UK) for her technical support with the yeast estrogen screen; John Masters (UCL, London, UK) and Dallas Swallow (UCL, London, UK) for providing the MCF-7 cell stocks; Martin Scholze (University of Bremen, Germany) for his help with the mathematics behind CA and IA; Rolf Altenburger (UFZ Centre for Environmental Research, Germany) and Thomas Backhaus (University of Bremen, Germany) for their informal discussions; Chris Jones (UCL, London, UK) for his advice on CGH; and finally my two BSc project students Jo McLean and Jocelyn Hasskamp for their help in developing the MCF-7 cell assay. Most particularly their contributions to the work on seeding density and culture time experiments.

The Centre for toxicology has been a wonderful place to work, made all the brighter by the presence of Rob, Dan, John, Niss, Elisabete, Naash and Montse, as well as many others who popped in for a visit. My time at the school would not have been the same without the likes of Dave, Chris, Steve, Paul, Annie, Glenda, Brian, Peggy and of course Stella.

Last but not least I give great thanks for the support, both emotional and financial, received from my family and from Lisa. Thanks a million.

Chapter 1

Estrogens in the environment:

A problem for us all ?

"Hey there farmer give up that DDT, it puts spots on the apples and its killing the birds and the bees"

Lyrics from Amy Grant's "Big yellow taxi"

1.1 Introduction

During the last forty years there has been mounting concern over increases in the incidence of endocrine related cancers in man, particularly those affecting breast and testicular tissues. In addition, over a similar time-period there has been a worrying decrease in male fertility, which is also reflected in many animal populations. These events have caused some researchers to speculate about a common underlying cause.

1.1.1 Breast cancer

Breast cancer is the most common malignancy affecting women in the western world, and accounts for 32% of all newly diagnosed female cancers in the US (Kelsey and Bernstein 1996). About 95% of these tumours are adenocarcinomas, mostly infiltrating ductal carcinomas, which can be treated via surgical intervention and chemotherapy. Due to improvements in detection and treatment, the mortality from breast cancer has fallen since the mid 1980s, however, the incidence of women developing these tumours is still increasing at a rate of 2% annually since 1980 (Wolff et al. 1996). Increases had been observed prior to this, with rates increasing from 82 per 100 000 to 109 per 100 000 between 1973 and 1990 in the US (Wolff et al. 1996). There are numerous, well documented risk factors for this disease, however, the etiological factors identified so far do not account for the majority of breast cancers or the increasing incidence.

The endogenous hormone 17β-estradiol (E2) plays a critical role in the development of the female breast both at puberty and during the menstrual cycle, and there is strong evidence that a woman's lifetime exposure to this hormone is an

important risk factor in the genesis of breast cancer (Toniolo et al. 1995, Berrino et al. 1996). Early menarche and late menopause increase the total number of ovulatory cycles, therefore increasing the cumulative estrogen "dose" to the breast epithelium (Feigelson and Henderson 1996), hence women who experience menopause before age 45 have only half the risk of those at 55 (Henderson and Bernstein 1996).

As with some other tumours, breast cancer has a familial element, with relatives of a sufferer having a higher chance of developing the malignancy themselves. This phenomenon has partly been explained by the discovery of the hereditary breast cancer genes BRCA1 and BRCA2, which may account for up to 90% of breast cancer in some families (Kelsey and Bernstein 1996). However, these genes are only thought to account for 5-10% of all cases, with about 30% of cases being attributed to lifetime exposure to the E2 (Davis et al. 1997). This indicates that other, as yet undiscovered genes or environmental factors are involved.

1.1.2 Testicular cancer

Testicular cancer is now the most common neoplasm affecting men between the ages of 25 and 34 in many parts of the world. Although far less prevalent than many of the cancers of old age, testicular cancer affects up to 1% of the population in some countries, and as incidence has been increasing for several decades (Toppari et al. 1996). An analysis of cancer registry data from the UK, USA, Scandinavia and Australia has shown an annual increase of approximately 3% in men under 50 years of age, over the last 25 years (Carlsen et al. 1992). Risk factors for this disease are far less obvious than those for breast cancer, however, a marginally significant increase

in risk is seen among men who's mothers were exposed to exogenous hormones, possibly linking this with the observed increase in breast cancer.

1.1.3 Male reproductive function

Reports on declining semen quality have been published since the early 1970s, but they received little attention until the early 1990s when Carlsen et al. (1992) attempted to draw together all the findings to date. Following an analysis of data on 15,000 men from 61 separate studies over a period of 50 years, it was concluded that mean sperm counts had fallen from 113 million/ml in 1940 to 66 million/ml in 1990, i.e. a reduction of nearly 50% in as many years. Despite some reports to the contrary and some criticisms of Carlsen's methods (Farrow 1994), the weight of evidence still points towards a real problem (Auger et al. 1995, Swann et al. 1997). This issue is not only related to the quality and quantity of sperm production, but also concerns general disorders of the male reproductive tract. The incidence of testicular maldescent (cryptorchidism) and urethral abnormalities (hypospadias) have also increased over the past 30-50 years (Carlsen et al. 1992, Toppari et al. 1996), yet there still remains no single obvious risk factor for these conditions.

1.1.4 A common cause

The relatively short time-frame in which the incidence of these disorders has increased points to an environmental rather than a genetic cause, and the observations of hormones as risk factors makes it tempting to speculate about a common underlying cause.

The idea that hormones can increase the incidence of cancers is not a new one. As far back as the 1940s, Bittner (1948) had demonstrated the influence of hormones on the genesis of mammary cancers in mice. However, it was some years later before the significance of his work was understood. A rekindling of his theories was brought about some forty years later with the concept of estrogen as a cause of human cancer (Henderson et al. 1988). Here, the idea was proposed that several human cancers (breast, testicular, ovarian and endometrial) may be attributable to lifetime exposure to bioavailable estradiol. The theory proposed was plausible, but did not fully explore or explain the increasing incidence of these tumours.

A few years later, while Carlsen et al. (1992) were investigating the fall in male sperm counts, other researchers were refining the estrogen hypothesis to include xenobiotic agents with estrogenic activity that may be responsible for the rise in rates of breast cancer (Davis et al. 1993). With the observation that lifetime exposure to estrogens was possibly the single most important risk factor for the development of breast cancer, it soon became apparent that exogenous agents which mimicked this hormone could exacerbate the problem. The additional observation that increases in breast cancer incidence coincided with the massive expansion in the chemical industry in the latter half of this century only served to strengthen the argument (Wolff et al. 1996). A survey of the existing literature revealed numerous, persistent environmental pollutants with the ability to act in a similar fashion to estradiol.

In 1969 Bitman et al. noted the ability of the environmental pollutant *o,p'*-DDT to bind to estrogen receptors (ER) of the avian oviduct, while Welch et al. (1969) were observing this chemical's ability to promote uterine growth in rats. Another chlorinated

insecticide, chlordecone, was revealed as an estrogenic agent in 1979 (Hammond et al.) when it too was shown to interact with ER and increase rat uterine weight. Throughout the 1980s more and more chemicals were found to possess estrogenic activity. Many were widely used pesticides, including methoxychlor, aldrin, dieldrin and toxaphene (Ramamoorthy et al. 1997), while other chemicals including alkylphenols (Nimrod and Benson 1996), some phthalate plasticisers (Harris et al. 1997), polychlorinated biphenyls (Coldham et al. 1997), and other chlorinated aromatic hydrocarbons were also shown to possess estrogen-like activity (Wiese and Kelce 1997). This led to the theory of organochlorides as a preventable cause of breast cancer (Colborn et al. 1993).

If these chemicals were affecting rates of breast cancer, then it seemed logical that they could play some role in the increases in cases of other endocrine related disorders. At the same time as the breast cancer hypothesis, Sharpe and Skakkebaek (1993) were formulating there own hypothesis regarding the role of these xenoestrogens in falling male fertility. Previously, Bullock et al. (1988) observed that women who had received the synthetic estrogen diethylstilbestrol (DES) were far more likely to have given birth to male offspring suffering cryptorchidism and hypospadias, with decreased semen volume and sperm counts. This raised the question as to whether reduced male reproductive function in the general population could be attributed to altered exposure to estrogens during fetal development. Sharpe and Skakkebaek also noted the changes in diet and lifestyle occurring during the 20th century, as well as the increase in chemical exposure, and proposed a mechanism for xenoestrogen involvement in male reproductive disorders. Briefly, exogenous

estrogens may upset the normal balance of estradiol and follicle-stimulating hormone (FSH) production in the Sertoli cells and the pituitary gland, respectively. This could result in an abnormal production of Müllerian inhibiting substance (MIS) by Sertoli cells. MIS is required for normal testicular development and descent, so alterations in MIS levels may adversely affect testis physiology. Despite some scepticism at the time (Thomas 1995), there was plenty of historical evidence to support their theory. As far back as 1949, aviation crop dusters who handled DDT were found to have reduced sperm counts (Singer 1949), while more recent studies such as that by Whorton et al. (1977) found increased rates of azoospermia and oligospermia in male pesticide workers.

1.1.5 Biological activity of E2

E2 is a steroid hormone and acts via binding to intracellular receptors. The estrogen receptor protein was first identified in the 1960s and was subsequently sequenced and cloned some 20 years later (Green et al. 1986). Estrogens are transported loosely bound to carrier proteins (albumin and sex hormone binding globulin) in the blood, to their target tissues. There, they diffuse into cells and specifically bind the inactive ER. This binding event activates ER, a process which includes receptor dimerisation, to form an activated receptor complex. This active form then binds to specific sequences of DNA called estrogen response elements (ERE). These are DNA regulatory sequences found within or flanking estrogen responsive genes, which when activated, promote the expression of that gene to produce one of a host of proteins under the control of E2 (Wiese and Kelce 1997), these include c-fos

and c-jun, which are known to drive the cell cycle (Parker 1995). Hence, one of the results of this gene activation is cell proliferation.

More recently, a second pathway of ER activation has been elucidated, which acts independently of E2. Here, a growth factor can indirectly activate ER via first activating a cell surface growth factor receptor tyrosine kinase (RTK). This then initiates a cascade of cellular signals (including ras, raf and MAP kinase) which ultimately results in the phosphorylation and activation of ER (Bunone et al. 1996). This too can result in expression of the cell cycle elements c-fos and c-jun.

It is therefore possible that a xenobiotic could act via both pathways, producing an exaggerated (synergistic) response.

To add a further level of complexity to this situation, Kuiper at al. (1996) have described a second distinct ER, termed estrogen receptor β (ERβ) to distinguish it from the existing ER, now termed ERα. Data already suggests that these two forms possess different binding affinities for E2 and xenoestrogens (Kuiper et al. 1998), however, for the purposes of this study, ER will refer to the original, α receptor.

1.1.6 The wildlife connection

Humans are not the only species to be affected, and there have been numerous observations of declining reproductive function in many animal species from diverse orders. Some marine molluscs have demonstrated morphological changes in the female reproductive system, giving rise to so-called "imposex" animals (Campbell and Hutchinson 1998). This has also been observed in fish (Purdom et al. 1994), where examples of hermaphroditism are becoming more common, and some males have

begun producing the normally, female-specific egg protein vitellogenin (Sumpter and Jobling 1995). Other aquatic species including reptiles such as alligators (Guillette Jr. et al. 1994) have also exhibited reduced fertility due to underdeveloped reproductive organs. Clutch sizes are becoming smaller in some avian species, while abnormal nesting behaviour in gulls has been seen, including more female-female pairing (Fry 1995). Mammals too are not exempt from this list, with disturbed male fertility in Florida panthers and several species of seal (Colborn et al. 1993).

Perhaps the most compelling evidence for the role of xenoestrogens in disease came with the observation of falling alligator populations in Lake Apopka, Florida between 1980 and 1987 (Guillette Jr et al. 1994). This event followed an extensive spill of halogenated pesticides from the nearby Tower Chemical Co. facility in 1980. It was documented that many juvenile alligators exhibited developmental abnormalities of the gonad and abnormal levels of sex hormones, disorders that were attributed to the high levels of DDT to which they had been exposed (Semenza et al. 1997).

Similar observations had been made previously by Fry and Toone (1981) who associated DDT contamination in the early 1970s with reproductive failure of western gulls off the coast of California. The poor breeding success was due to reduced numbers of adult males, as well as feminisation of some males. They also note similar problems in other avian species, including brown pelicans and double-crested cormorants.

Taken together, the weight of evidence seems to indicate that xenoestrogens are involved in a large number endocrine related disorders in man and wildlife. However, if this is the case, then why have so many human epidemiological studies failed to

conclusively correlate serum levels of xenoestrogens with these disease states ?

1.1.7 Lack of conclusive evidence from epidemiological studies

The use of epidemiology has been crucial in confirming the role played by endogenous estradiol in the etiology of breast cancer. However, the story could have been very different. Due to the poor design of many of the early epidemiogical studies, the consensus of opinion in the early 1990s was that endogenous estrogens did not reflect breast cancer risk (Toniolo 1997). Yet, with emerging data from several prospective studies in the mid 1990s (Helzlsouer et al. 1994, Dorgan et al. 1996), the picture began to change.

In a large 5 year prospective study covering over 14 000 women, subjects who developed breast cancer showed higher levels of estrone, total estradiol and free estradiol (Toniolo et al. 1995). Of these factors, the one most strongly linked to breast cancer risk appeared to be the level of free estradiol.

However, these observations have not been mirrored with xenoestrogens. Despite the finding that the levels of organochlorides in women today are up to 10-fold higher than that of endogenous estradiol (Hansen and Jansen 1994), many epidemiological studies have failed to find any strong correlation between serum xenoestrogen levels and breast cancer. For every report of a link between agent and disease, there is another which shows no statistical relationship, which has lead to a great deal of controversy in this field.

Perhaps the most frequently studied pollutant, DDT, is the one to have caused the most controversy. Despite being more strongly associated with breast cancer in

greater numbers of studies (Wolff and Toniolo 1995) than any other environmental contaminant, other reports appear to contradict this. Firstly, African-Americans tend to have a higher body burden of DDT than Caucasians, yet a lower rate of breast cancer. Moreover, DDT exposure is associated with lower socioeconomic classes, whereas breast cancer rates increase with socioeconomic group (Ahlborg et al. 1995). The picture is no clearer with other compounds, Falck et al. (1992) found elevated levels of PCBs, *p,p'*-DDT and *p,p'*-DDE in fat samples from women with breast cancer compared to those with benign breast disease, however the study, as with many others, was relatively small, involving only forty subjects. In a much larger analysis (14290 samples) a year later Wolff et al. (1993) confirmed an association with *p,p'*-DDE but found no correlation with PCBs. However in 1994 in an even larger study (57040 subjects) found no increased breast cancer risk for either PCBs or *p,p'*-DDE (Krieger et al. 1994). The findings of the 1994 study have been supported more recently by additional findings that DDT and PCBs are not associated with breast cancer (Hunter et al. 1997, van't Veer et al. 1997).

These are by no means the only studies of this type, but they do highlight the rapid changes in opinion during the last few years.

1.1.8 Can mixture effects explain the problems encountered in epidemiology ?

Despite the lack of epidemiological support for a link between xenoestrogens and breast cancer, it remains true that people are constantly exposed to potentially hundreds of estrogenic agents.

Ever since it became apparent that estrogenic environmental contaminants may

play a part in the genesis of a number of human diseases, hundreds of chemicals have been tested for their activity in numerous laboratories throughout the world using a variety of *in vivo* and *in vitro* assays (Jobling et al. 1995, Soto et al. 1995 and Zava et al. 1997). However, in *in vitro* assays, the potency of these compounds was so much lower than estradiol, that unphysiologically high concentrations were required to elicit effects. This caused researchers to question the impact of these agents as endocrine disrupters (Safe 1995).

The solution to this dilemma seemed to present itself when a number of groups began to report synergistic interactions between xenoestrogens tested in combination. The concept that chemicals may interact to modify each other's effects is a familiar one to pharmacologists. One agent may affect another's absorption, metabolism or excretion, or compete with it for binding to a receptor. Consequently chemical A may act to enhance or reduce the response normally elicited by chemical B, or have no influence on B's action. This concept gives rise to three broad classes of mixture effect, zero interaction (or additivity), synergism and antagonism. Additivity is inferred when the response to two agents is what is "expected", while if the response is greater than expectation there is a positive interaction or synergy. Oppositely, antagonism is when the response is less than expected (Berenbaum 1989).

Soto et al. (1994) observed greater than additive responses to aldrin, endosulfan and toxaphene combinations in their MCF-7 breast cancer cell proliferation assay, while Sumpter and Jobling (1995) obtained similar results using cultured fish hepatocytes. At the same time, *in vivo* studies were also producing results suggesting synergistic combination effects with binary mixtures of PCBs on sex-reversal of male-determined

turtle eggs (Bergeron et al. 1994). Possibly the most startling finding came when Arnold et al. (1996a) published a ground-breaking paper claiming that combinations of weak environmental estrogens such as dieldrin, endosulfan or toxaphene, were 1000 times as potent in ER-mediated transactivation as any chemical alone. This seemed to provide evidence favouring the link between xenoestrogens and breast cancer, that weak estrogens acting in combination synergised, i.e. produced a greater response than would be thought based on the potencies of the individual agents. If such a marked synergy could be observed with only binary mixtures, then how much more could be expected from a mixture of ten or twenty chemicals ? However, the excitement was short-lived.

Other groups tried to reproduce the Arnold experiments with little success (Ramamoorthy et al. 1997, Ashby et al. 1997, Gaido et al. 1997a,1997b). Following similar disappointments in his own laboratory, a full retraction of the initial report was published a year later (McLachlan 1997). This led to a great deal of debate regarding combination effects in this field, with many groups taking a closer look at their own data. This once again cast a shadow of doubt over the importance of xenoestrogens, leaving a number of questions still unanswered.

A reason for the number of discrepancies in studies of combination effects eventually became apparent. The determination of synergy relies on measuring deviations from the predicted behaviour of a mixture, so the critical step lies in generating of a sound predictor of "expected" mixture effects based on the individual potency of each agent (Kortenkamp and Altenburger 1999). As Berenbaum (1989) states, expectation is not to be derived from an understanding of mechanisms of action,

since given enough information, the effects of all combinations would eventually be shown to be what is "expected" and would therefore be deemed to show zero interaction. Hence we require a method of comparing test outcomes with single agents and mixtures which functions irrespective of the agent's mechanism of action. As Berenbaum shows (1989) this can be achieved by using the one feature of a chemical's action which is not the subject of mechanistic considerations, its concentration-response curve. By applying various mathematical concepts of mixture effects to concentration-response data, it is possible to determine whether the interaction of a mixture of chemicals is additive, synergistic or antagonistic for any given test system. Despite the wealth of knowledge regarding mixture action developed in pharmacology over the last hundred years, studies in this area have not made full use of the models available. Instead, many researchers have implicitly adopted a model others may refer to as effect summation, which may not be the most suitable in this situation.

1.2 Is effect summation the best model for assessing mixture effects of xenostrogens ?

With the model of effect summation, the expectation is that the effect of a mixture (E_{1+2}) is equal to the sum of the effects of the single compounds in that mixture (E_1 and E_2). This assumes that the two agents act additively, while mixtures producing a greater than expected effect are considered to act synergistically. Using this model, a number of groups have concluded that xenoestrogens may act synergistically in a range of test systems. Unfortunately, inappropriate use of this model can lead us to an interesting paradox.

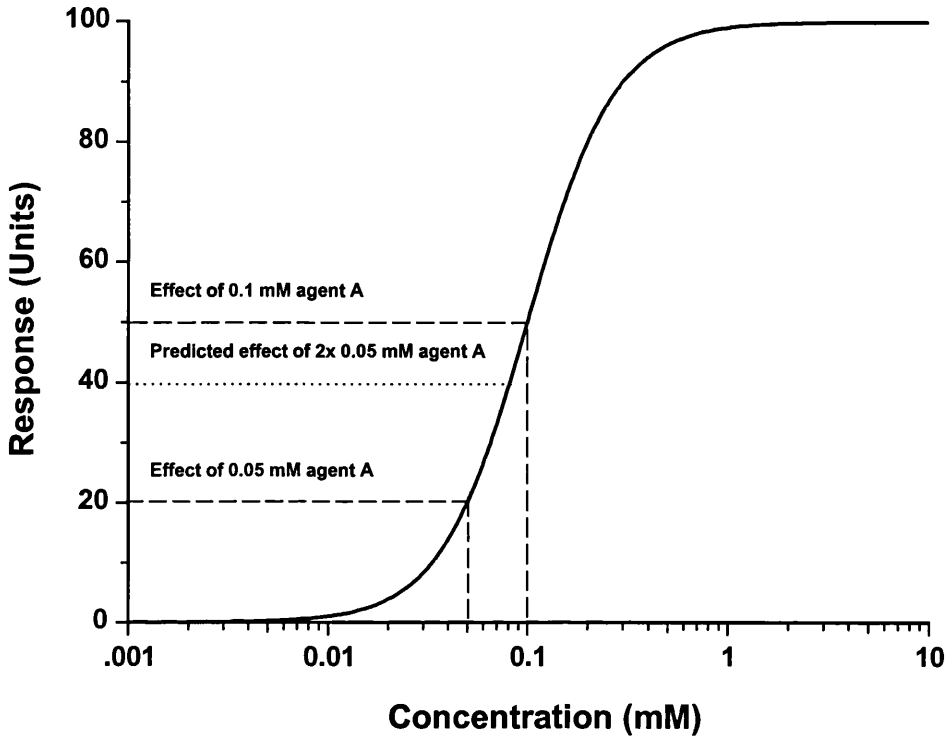


Figure 1.1 Hypothetical concentration-response curve for an estrogenic agent A, demonstrating the logical inconsistency of effect summation. For explanation see text.

Consider the hypothetical concentration-response curve for chemical A (Figure 1.1). This agent elicits a response of 20 when present at 0.05 mM and a response of 50 when present at 0.1 mM. If a sham mixture of A with itself, such that the final concentration was double the initial concentration (2 x 0.05 mM = 0.1 mM) was tested experimentally, it would produce an effect of 50. However, the predicted response using effect summation for this mixture would be a response of 40, clearly lower than observed and leading the experimenter to incorrectly conclude synergy. This is illogical as a chemical cannot synergise with itself.

As Berenbaum (1989) has demonstrated, this paradox is easily overcome. The

model is only applicable to combinations of agents which produce linear dose-response curves that pass through the origin of the graph, a situation rarely seen in toxicology, and not necessarily the case with the estrogen-like chemicals (Arnold et al. 1996a, Ashby et al. 1997). Hence the analysis of data in a number of studies led to the incorrect assumption of synergy. There is therefore a need for models which can be applied to combinations of agents independent of the shape of their dose response curves. Such models have been developed within pharmacology and toxicology, and include the models termed concentration addition and independent action.

1.2.1 Alternative models to effect summation

The model of concentration addition (CA) can be traced back to Loewe and Muischneck (1926) more than half a century ago, and is based on the concept of "similarly" acting chemicals. In this model it is said that chemicals in a mixture act as a dilution of each other, meaning that any effect can be obtained by replacing one chemical wholly, or in part, by an equi-effective quantity of another. Each individual component of a mixture is assumed to contribute to the observed overall effect by acting in proportion to its concentration, regardless of any effect thresholds.

The validity of this model for agents binding to an identical receptor site has been confirmed *in vitro* in a number of studies (Pöch 1993), but this does not mean it will necessarily hold true in an intact organism. Here, the interplay between pharmacodynamics and pharmacokinetics may modulate the exact combination effect observed. However, this model has gained acceptance in many areas due to its plausible pharmacological basis, so much so that Berenbaum has proposed it to be a

"general solution" to the analysis of combination effects (1989). One of the features of this model is its ability to predict combination effects even when individual agents are present below their no observable effect concentrations (NOEC), a case that may well occur in nature.

The model of independent action (also called response addition) can be traced back to the work of Bliss (1939) and was developed on the basis of stochastic considerations. It is thought to be more applicable to chemicals that have "different" sites or modes of action within an organism or test system. Although, as with CA, the understanding of "different" is not always clear, especially with reference to endpoints such as cell death.

The concepts of CA and IA were developed independently to suit different experimental contexts and there are no rational criteria for choosing between them. Nevertheless, both models are currently regarded as equally valid reference points for predicting the effects of mixtures of chemicals (Greco et al. 1995). In addition, since they function independently of the profile of the individual curves, these models do not suffer the constraints of effect summation. One major benefit of these concepts is that they can be adapted to plot predictions based upon additivity as a traditional log concentration-response curve, allowing a direct comparison with observed mixture data (Faust et al. In press).

1.3 Research objectives

It is the objective of this research to assess whether mixture effects with xenoestrogens can be predicted on the basis of data for the individual agents. In

addition, we are interested in assessing the nature of these effects in terms of additivity, synergy and antagonism. From these observations we also hope to investigate the potential breast cancer risk associated with exposure to realistic levels of endocrine disrupters.

Since our analysis of combination effects will rely heavily on the generation of accurate concentration-response relationships, this study will involve the development of sensitive and highly reproducible assay systems. This is essential if we are to be able to differentiate weakly synergistic interactions from additive ones.

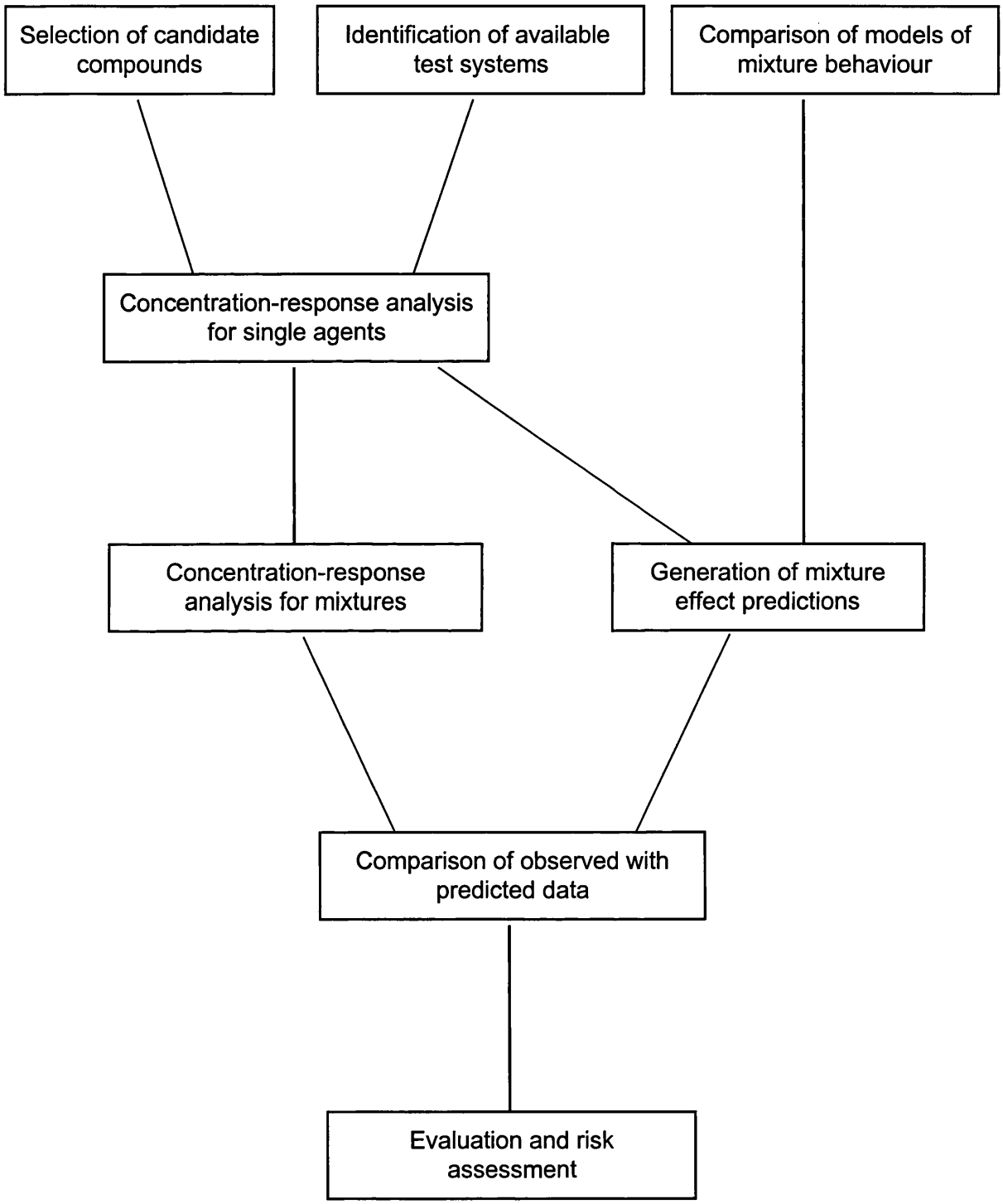
1.3.1 Research overview

Figure 1.2 shows an outline of the rationale behind this research. In order to proceed we need to select a number of estrogenic chemicals and also suitable assay systems in which to test them. For each agent we will generate concentration-response curves and use these to predict mixture behaviour with appropriate models of mixture action. The validity of these theoretical predictions can then be tested against experimentally generated mixture data. With a knowledge of mixture behaviour, we will then attempt to determine the human health risks associated with xenoestrogen exposure.

1.3.2 Selection of candidate compounds

A large number of structurally diverse, natural and man-made chemicals have been shown to produce estrogen-like effects both *in vivo* and *in vitro*. For the purposes of this investigation, we are interested in those most likely to play a role in the etiology

Research plan



of endocrine-related disorders. Ideally, our chosen test agents would fulfill the criteria:

- Mass produced synthetic chemicals
- High environmental prevalence
- Available in high purity for experimentation
- Previously shown to possess estrogen-like activity

Hence the following representative chemicals were selected:

Organochloride pesticides

DDT isomers 1,1-Bis (4-chlorophenyl)2,2,2-trichloroethane (*o,p*'-DDT, Lancaster, UK) and 2,2-Bis (4-chlorophenyl)-1,1,1-trichloroethane (*p,p*'-DDT, Sigma, UK) and the breakdown product 2,2-Bis (4-chlorophenyl)-1,1-dichloroethylene (*p,p*'-DDE, Sigma, UK).

The byproduct of lindane manufacture, β-Hexachlorocyclohexane (β-HCH, J.T. Baker, UK).

Phthalates

Diethylhexylphthalate (DEHP, Sigma, UK) and benzylbutylphthalate (BBP, Aldrich, UK)

Alkylphenols

4-octylphenol (OP, Aldrich, UK) and 4-nonylphenol (NP, Aldrich, UK)

For comparison we also selected the endogenous hormones 17β-estradiol (E2, Sigma, UK) and testosterone (Sigma, UK), as well as the antiestrogenic drug tamoxifen (Sigma, UK) and the plant estrogen genistein (Sigma, UK). We acknowledge that this list does not cover the full range of xenoestrogens, however, this study is not intended

to be a mass screening exercise.

1.3.3 Identification of suitable test systems

Numerous test systems exist for the identification and characterisation of estrogenic agents. Ideally we would like to investigate xenoestrogen activity at a number of organisational levels, from the molecular to the cellular. A list of possible candidates was drawn up and subjected to a selection procedure.

Selection criteria:

- High sensitivity and specificity
- Effects measurable with minimal variability and maximal reproducibility
- High throughput possible
- Availability of relevant facilities within the laboratory

Radioligand binding studies

Radioligand binding studies have been a mainstay of receptor pharmacology for many years, with much of the early work on xenoestrogens using this technique. Although these are sensitive assays, they are unable to distinguish ER agonists from antagonists, and binding to ER is not sufficient to determine the estrogenicity of a compound. Also, the need for consistent, purified receptor preparations preclude high throughput, while the use of radioisotopes is always of concern (Zacharewski 1997).

Breast cancer cell proliferation assay

Human breast cancer cells, such as the MCF-7 line, are estrogen-responsive, and concentration related increases in cell proliferation can be easily measured. This has allowed their use as a tool for investigating the activity of numerous estrogenic compounds. In addition, it is recognised that not all xenoestrogens act via ER, and MCF-7 cells offer a range of other molecular targets for agents to act upon (androgen-, aryl hydrocarbon- and growth factor receptors)

In comparison to in vivo assays, it is relatively rapid (duration approximately nine days) and sensitive, and MCF-7 cells are both metabolically active and of human origin. The system has been well validated and is considered by many to be equivalent to the increase of mitotic activity of the rodent endometrium (Soto et al.1995). In addition, there have been few reports of false positives, and with a reported E2 detection limit of 30 pM it is one of the most sensitive *in vitro* assays for assessing estrogenicity.

However, this assay is susceptible to variability depending on differences in cell lines used, culture conditions and serum batch, thus complicating interlaboratory data comparisons.

MCF-7 ERE-luciferase reporter system

In order to facilitate more rapid analyses of MCF-7 cell proliferative responses, a modified cell line bearing a reporter gene was created. Briefly, MCF-7 cells are transiently transfected with a plasmid containing two estrogen-responsive elements linked to the luciferase gene, the estrogenic potency of agents then being assessed by increases in luciferase activity. There are concerns over the long-term reproducibility of transiently transfected cells, so the use of stable transfectants would give more

consistent results.

Although this assay possesses all the benefits of the standard proliferation assay, as well as a more easily measured endpoint, there is the requirement for an accurate luminometer, not currently available within the laboratory (Zacharewski 1997).

Yeast estrogen screen

This assay originated in the Genetics Department at Glaxo and was established to identify compounds which interact with the human estrogen receptor. The yeast (*Saccharomyces Cerevisiae*) has human ER DNA stably integrated into its main chromosome and a reporter plasmid bearing the *lac-Z* gene to allow rapid spectophotometric analysis of results. The test is simple and well characterised and has a relatively rapid time-course (96 hours), allowing high throughput (Routledge and Sumpter 1996).

On the negative side, yeast cell structure differs significantly from that of mammalian cells, and is unlikely to possess the same metabolic capabilities. Although it is a sensitive assay, able to respond to low levels of weak estrogens, it detects only agents which interact with ER, and will not distinguish agonists from antagonists. Other groups have recently reported the ability of the potent anti-estrogens tamoxifen and ICI 182.780 to test positive in YES. In addition, it should also be remembered that not all estrogen-like agents act via ER (Andersen et. al. 1999).

Rodent uterotrophic assay

17β-estradiol stimulates uterine growth by inducing a measurable cellular hypertrophy and hyperplasia in rodents. This forms the basis of the rodent uterotrophic assay, where increases in uterine weight in estrogen-treated animals can be used to generate dose-response relationships. Since the 1930s this has remained the most commonly used method for detecting and studying the efficacy of estrogenic compounds, and it is still considered to be a "gold-standard" within the field.

Being an *in vivo* assay, it offers some significant benefits over other systems as it is able to model absorbtion, distribution, metabolism and excretion of test compounds, providing greater information on xenoestrogen behaviour. However, as with all *in vivo* assays there is likely to be considerable inter-animal variability.

In addition, our need to generate numerous well defined concentration-response curves calls for a technique which can generate many datapoints. This could not be realistically met with such an expensive and labour-intensive method (Schatz et. al. 1984)

Consequently, for reasons of speed and reproducibility it was decided to use the MCF-7 cell proliferation assay and yeast estrogen screen to generate concentration-response relationships for the chemicals selected both singly and in combination.

1.3.4 Models of mixture effects:

Two appropriate and well-validated models of mixture effects were also selected, namely:

- Concentration addition (including the method of isoboles)

- Independent action

1.3.5 Generation and analysis of data

Using the two assay systems chosen (MCF-7 proliferation and yeast estrogen screen), comprehensive concentration-response relationships will be generated for each of the individual agents selected, along with appropriate positive and negative controls. From this we can compare the relative estrogenic potencies of the chemicals in question, and by comparing the results from the separate assays gain some insight into their possible mechanisms of action.

Firstly, by applying the data generated for the single agents to the two models (CA and IA), we can generate predictions of the effect of a mixture for any number of compounds at any given mixture ratio. From this we can compare the different profiles of the curves generated by the two models.

Secondly, we will generate experimental concentration-response data in the MCF-7 and yeast assays for given mixtures of xenoestrogens for comparison with the model predictions.

From the experimental mixture data we will analyse the quality of the two model predictions and determine whether the mixture effect is additive, synergistic or antagonistic. From information on mixture behaviour and human exposure to xenoestrogens, we should further the knowledge of the risk posed to man by these chemicals.

1.4 Overview of Chapters

Following this introduction to the field of combination effects of xenoestrogens, Chapter 2 contains details of a thorough optimisation of the MCF-7 cell proliferation assay for the purpose of mixture studies, and highlights the need for good reproducibility of results. Having established that effect summation may not be the most appropriate concept for studying mixture effects, Chapter 3 explores the usefulness of the method of concentration with binary mixtures. However, human exposure involves numerous agents, and as a result Chapter 4 will compare the predictions generated by the models CA and IA for four component mixtures.

So far the only test system studied has been the MCF-7 cell proliferation assay, so Chapter 5 will explore the use of CA and IA in an alternative system, the yeast estrogen screen. The information obtained regarding mixture effects will then be used in Chapter 6 to investigate the possibility of estimating the breast cancer risks associated with xenoestrogen exposure. Chapter 7 will then discuss the possible implications of this study.

Chapter 2

Improving the reproducibility of the MCF-7 cell proliferation assay for the detection of xenoestrogens

"An estrogen is a substance that can elicit the mitotic stimulation of tissues of the female genital tract; therefore measuring cell proliferation is of key importance in assessing estrogenicity. To determine whether chemical "X" is an estrogen, one must test its ability to induce proliferation of estrogen responsive target cells..."

Hertz 1985

2.1 Abstract

The MCF-7 cell proliferation assay is potentially a simple and highly reproducible tool for the identification of estrogenic compounds. However, its widespread use has been complicated by the lack of a standardised protocol, resulting in considerable inter-laboratory variability. We have explored the sources of variability both in relation to cell lines and test regimens and report an optimised procedure for the identification of estrogenic agents. Two supposedly identical MCF-7 parent cell lines (designated UCL and SOP), and the BUS subline were cultured according to an existing protocol, and responses to E2 assessed. Despite yielding almost identical EC_{50} values, the proliferative response varied widely between cell lines from 0.98-fold over controls (UCL) to 8.9-fold (BUS), indicating major differences between them. Selecting the MCF-7/SOP line for further work, we carried out a thorough and systematic optimisation of the MCF-7 cell proliferation assay, finding that a 72 hr period in estrogen-free medium before treatment strongly influenced the cell's response to E2. 1 nM E2 increased proliferation from 1.5-fold to 6.5-fold relative to vehicle-treated controls, a response similar to that seen with MCF-7/BUS cells in the E-SCREEN protocol devised by Soto and her colleagues. With parent MCF-7 cells, other laboratories have reported only 4.5-fold increases as maximal. Here we present evidence that the choices of cell line and culture conditions are crucial in determining test outcomes, and once chosen and adhered to, the assay yields reproducible results.

2.2 Introduction

Improvements in industrial and agricultural productivity over the last 50 years have meant that large quantities of structurally diverse, persistent chemicals have been released into the environment. Since the 1960's there has been growing concern that a number of these chemicals are weakly estrogenic and may be disrupting endocrine functions in wildlife and man (Bitman et al. 1968, Colborn et al. 1993). More recently, scientists have linked these "endocrine disrupters" to fertility problems and the increasing incidence of cancer in estrogen-sensitive tissues such as the testis and the breast (Sharpe and Skakkebaek 1993, Forman and Moller 1994, Wolff and Weston 1997).

Identification of the causative agents has been slow, since it is difficult to predict the estrogenicity of xenobiotics purely on the basis of their chemical structures (Katzenellenbogen et al. 1995). The classical rodent uterotrophic assay, which measures the proliferation of uterine tissue in response to estrogens, is time-consuming, expensive and not always conclusive. It has therefore been proposed that a battery of *in vitro* tests be used to screen the growing number of potential chemicals (Klotz et al. 1996).

Recently, the MCF-7 cell proliferation assay (E-Screen) has grown in popularity as a rapid and straight-forward test for detecting weakly estrogenic compounds. In this assay, estrogen-dependent cells are grown in the presence of test compounds. A dose-related increase in cell numbers in treated cultures is taken as evidence of the estrogenicity of the test compound (Soto et al. 1995). This assay is now widely used by a number of groups (Klotz et al. 1996, Ramamoorthy et al. 1997 and Zava et al.

1997) and has been recognised as being biologically equivalent to the increase in mitotic activity of the rodent endometrium (Soto et al. 1995). However, one problem associated with the growing popularity of this assay is the absence of a standardised protocol, which has lead to differing test results being reported by a number of laboratories.

Widely varying test regimens and numerous cell sublines have been used which may explain much of the variability in test results (Zacharewski 1997). Jones et al. (1997) have recently reported that changes in culture conditions, such as the numbers of cells plated and the duration of incubation in estrogen-free medium before treatment with test compounds, can have striking effects on the responses to estradiol, while Villalobos et al. (1995) have demonstrated the impact of different MCF-7 cell sublines on test outcomes. In a recent, large inter-laboratory study, Andersen et al (1999) report that by using a standardised cell line (MCF-7/BUS) with similar protocols, good agreement can be achieved with most test compounds. However, they acknowledge a lack of consistency with agents such as benzyl butyl phthalate and *p,p'*-DDE.

Originally, the MCF-7 breast cancer cell line was derived from a pleural effusion taken from a woman with metastatic breast carcinoma who was previously treated with radiation and hormone therapy (Soule et al. 1973). Since that time however, the cell line has undergone numerous changes, and studies have shown that MCF-7 cell line variants show intrinsic differences in features such as estrogen-dependent proliferation rate and population doubling time (Villalobos et al. 1995), and susceptibility to apoptosis (Burrow et al. 1998). It is conceivable that these differences are the result of genetic changes, and verification of this is beginning to emerge (Kallioneimi 1994). Jones et

al. (2000) have recently employed comparative genomic hybridisation (CGH) to analyse genetic changes in the MCF-7 cell lines BUS, UCL and SOP. CGH is a powerful molecular cytogenetic tool for the identification of DNA sequence copy number changes (gains and losses) across the entire genome in a single hybridisation experiment. They report considerable genetic variations between these three cell lines, which may partly explain the differences observed between individual laboratories.

The use of proliferation rates as a form of cell characterisation is therefore potentially unsatisfactory since similar responses to E2 do not necessarily indicate similar reponses to other weak estrogens. This is especially the case with agents such as *p,p'*-DDE and β-hexachlorocyclohexane (β-HCH), which induce proliferation via mechanisms unlike that of E2 (Kelce et al. 1995, Steinmetz et al. 1996) and so may induce different response in different cell lines.

2.2.1 Aims of this study

Here we will thoroughly explore the impact of cell line and culture conditions on the outcome of the MCF-7 cell proliferation assay, and assess this assay's usefulness for the characterisation of weak estrogens. To achieve this we will study two parent MCF-7 cell lines from different sources which we have designated SOP and UCL, as well as the frequently used MCF-7/BUS line.

2.3 The E:Screen assay, sources of variability

2.3.1 Cell line

Since, the initiation of this cell line 25 years ago (Soule et al. 1973), several MCF-7 stocks with differing estrogen-sensitivities have been developed. Sublines with

the postscript BB, BUS, ATCC and BB104 have all been used for estrogenicity assays (Villalobos et al. 1995), although population doubling times vary from 27 to 49 hours (in DMEM with 10% FBS). Another confounding factor is the gradual reduction in estrogen receptor numbers which affects cells of high passage number, thereby limiting their responsiveness in this assay.

At the chromosomal level, investigations of genetic changes in MCF-7 cells have recently highlighted major differences between supposedly identical cell stocks (Jones et al. 2000). In addition, Gooch and Yee (1999) have observed marked differences in susceptibility to apoptosis between different MCF-7 lines.

2.3.2 Seeding density

For reasons of personal preference, different groups have selected different cell culture plates for experimentation. Six-, twelve-, twenty four- and ninety six-well plates have all been used, and the number of cells seeded has varied accordingly. Too few cells will give a poor proliferation, whereas too many cells will rapidly reach confluence and thus underestimate the maximal proliferative response. Other groups have found that 10 000 cells seeded to a 12-well plate (density 2500 cells/cm^2) will give a good response after 6 days in culture (Villalobos et al. 1995).

2.3.3 Growth periods in estrogen-free media

It has been known for some time that the estrogen receptor is inducible, so by depriving MCF-7 cells of E2, they upregulate production of the receptor to compensate for the lack of the steroid hormone (Katzenellenbogen et al. 1987). Hence, some

groups incubate cells in the absence of estradiol for a period of time after seeding, prior to the administration of test compounds. Typically, this step lasts for 24 or 48 hours, but it is not carried out in all laboratories. This procedure is further complicated by different cell line sensitivities to the G_0/G_1 arrest estrogen deprivation causes (Villalobos et al. 1995).

2.3.4 Choice of serum

Fetal bovine serum is the most commonly used serum supplement in modern cell culture techniques and has been used in this assay for routine culture of MCF-7 cells. However, this assay relies on periods of cell growth in the absence of estrogenic agents, and bovine serum, like human serum, contains E2. A charcoal-dextran method can be employed to remove almost all of the hormone and some groups use this estrogen-depleted bovine serum for the assay. The one major concern with this method is the widespread use of growth promoters in animals today, which could influence the proliferation of sensitive cells. As a result we have opted to use estrogen-depleted pooled human serum for experimental work (ie, from male and female donors).

2.3.5 Serum concentration

In the adult human female, only about 2% of the circulating E2 occurs in its free form in the serum. The remaining 98% is transported bound to serum proteins such as albumin and SHBG (sex hormone binding globulin). This means that the quantity of serum in the culture medium will strongly influence the amount of hormone available for receptor binding in the assay. This will modify the apparent maximal response to

estradiol. Many xenoestrogens do not bind serum proteins and so will remain relatively unaffected by variations in serum concentration. Altering serum concentrations will also influence levels of other factors such as insulin and EGF, which both have an effect on MCF-7 cell proliferation.

2.3.6 Choice of solvents for test compounds

The test agents to be used in this assay show high lipid solubility, which limits the preparation of aqueous solutions considerably. To overcome this, a wide number of different solvents have been utilised by various groups (Hexane, DMSO and PEG). We elected to use ethanol (1% in media) as our routine solvent due to its ease of handling, low cellular toxicity and good solvent action with our chosen agents.

2.3.7 Frequency of exposure to test compounds

The most commonly used regime described in the literature is to administer test compounds only once, and to assess proliferation after a defined time-period. However, it is possible that over the course of the incubation, a test compound may be metabolised, or degraded, thereby underestimating its potency. To avoid this, media containing test agents would be replaced every day during culture. To us, these extra media changes seemed unnecessary on account of the inherent persistence of our compounds, and the relatively rapid onset of estrogenic action (Soto et al. 1995).

2.3.8 Duration of culture

After the administration of test compounds, it is necessary to allow the cells a

period of proliferation, although the duration remains a matter of personal choice. Anything less than 72 hours, and the cells will not have had time to respond fully to the chemical. However, with durations of more than 7 days it is likely that elements of the growth medium will become depleted or the cells reach confluence. Consequently we decided to investigate the influence of treatment time over a period of 7 days in culture.

2.3.9 Additional media supplements

The exact composition of MCF-7 cell culture medium still remains a matter of personal preference. As well as DMEM and FBS, many groups add factors such as epidermal growth factor and insulin. Also routine in some laboratories is the addition of antibiotics such as penicillin and streptomycin. However, we felt that in order to keep the number of exogenous chemicals to a minimum we would forego any non-essential media supplements.

2.4 Methods

2.4.1 Routine culture

Cells were maintained in 75 cm^2 cell culture flasks (Greiner) in full medium (Dulbecco's modified Eagle's medium, Gibco BRL Cat.# 31966-021, supplemented with 5% heat inactivated fetal calf serum, Gibco BRL). These were kept in a humidified incubator, 37°C, 5% CO_2 over a maximum of 20 passages, and routinely tested for mycoplasma.

2.4.2 Removal of endogenous estradiol from human serum

E2 was removed from pooled human serum (National Blood Transfusion Service, London) by treatment with charcoal and dextran, using a modification of an existing protocol (Soto et al. 1995). Activated charcoal (Sigma) was washed twice in ultra high quality (UHQ) water before use. A suspension of 5% charcoal and 0.5% Dextran T70 (Pharmacia) was prepared in a volume of UHQ water equivalent to that of the serum to be stripped. The suspension was pelleted by centrifugation (1000 g, 10 min), and the pellet combined with the serum and mixed by rolling (10 cycles/min, room temperature, 60 min). The mixture was then centrifuged for 20 min at 50 000 g and the charcoal-dextran stripped human serum (CDHus) filtered (0.2µm, Nalgene) and stored at -20°C for up to 6 months.

2.4.3 Measurement of cell proliferation in response to 17β-estradiol

In accordance with the protocol of Soto et al. (1995), one 70% confluent 25 cm^2 flask of MCF-7 cells (SOP, UCL or BUS) was washed with 5 ml phosphate buffered saline (PBS, Sigma) before the addition of 660 µL 0.25% trypsin-EDTA (Sigma). The flask was left for three minutes, after which the cells were detached, resuspended in 20 mL full medium, counted using an improved Neubauer counting chamber and seeded to 12-well plates (Corning) at a density of 2 $x10^4$ cells per well in 1 mL full medium. After 24 hr the cells were washed with 1 mL PBS. The medium was changed to 1 mL estrogen-free medium (phenol red-free DMEM, Sigma, UK, Cat.# D-5921 with 5% CDHus) before the addition of a range of concentrations of E2 (Sigma). Each plate contained three ethanol controls and was semi-randomised to counter systematic errors

induced by plate layout. Cell proliferation was assessed after 7 days in culture using the method of Skehan et al. (1990). Briefly, cells were fixed in cold 10% (w/v) trichloroacetic acid (TCA, BDH) for 30 min, washed 5 times with water and stained with 0.4% (w/v) sulphorhodamine B (SRB, Sigma) in 1% acetic acid for 10 min. Unbound SRB was removed by washing in 1% acetic acid, and bound SRB solubilised with 10 mM Tris pH 10.4 before being transferred to 96-well microtitre plates (Greiner). Dye intensity was measured at 510 nm on a plate reader (Labsystems Multiskan) and data expressed relative to controls using the formula below:

Fold increase over controls = [(Test Abs - 0.04) / (Control Abs - 0.04)] - 1

Where Test Abs is the absorbance at 510 nm of solubilised dye from drug treated cells and Control abs is the absorbance of the corresponding vehicle (ethanol) treated controls. Both values are corrected for the absorbance of the microtitre plate using values taken from an empty well (typically 0.04). The result was then reduced by one in order to yield a proliferation scale starting at 0.

Control experiments using known cell numbers for this assay had already demonstrated that cell numbers were directly related to staining intensity, and the plot was linear over the range of cell numbers observed in our experiments.

Phenol red-free DMEM was chosen as it has been shown that phenol red is itself estrogenic (Berthois et al. 1986), and pooled human serum was selected due to the widespread use of growth hormones in cattle which may interfere with responses. Dilutions of E2 were made up in absolute ethanol and administered so that the final

ethanol concentration was 1% in the media. Ethanol alone, at 1%, was found to have no influence on cell proliferation.

2.4.4 Modified cell proliferation assays

In a series of systematic modifications of the above protocol, MCF-7/SOP cells were seeded to 12-well plates (Corning) at a density of 1 or 2 x 10^4 cells per well in 1 mL full medium. After 24, 48 or 72 hr the cells were washed with 1 mL PBS. Then medium was changed to 1 mL estrogen-free medium, with E2 being added either immediately or 24 - 72 hr later with a further media change. Cell proliferation was assessed 7 days after administration of estradiol using the SRB assay outlined.

2.4.5 Data processing and presentation

Data are plotted as concentration of test agent in the culture medium vs proliferation relative to controls. So, a relative proliferation of 0 means no difference from control proliferation, while 1.0 equals a 100% increase in absorbance over controls (i.e. a doubling).

2.4.6 Measurement of total ER content

Determinations of the total ER content of cells subjected to varying treatment regimens were carried out using a modification of an existing method (Zava et al. 1997). Briefly, MCF-7/SOP cells were grown in full or estrogen-free medium for 72 hr before treatment. Cells were exposed to 10 nM [2,4,6,7-^{3}H]-17β-estradiol (Amersham, specific activity 72.0 Ci / mmol) for 1 hr in culture and then washed thoroughly with PBS

before trypsinising and counting. Non-specific binding was determined via competition with a 1000-fold molar excess of unlabelled E2. Aliquots, of 10^6 cells, were lysed with ice-cold TPSG buffer (0.2% Triton X-100 in PBS containing 0.1M sucrose and 10 % glycerol) for 5 min and vortexed vigorously to yield cell nuclei. These were pelleted by centrifugation (2000g for 5 min) and washed several times in PBS, before activities within the nuclear and cytoplasmc fractions were determined by scintillation counting (Beckman LS 6000IC) in a volume of 5 ml Ecoscint O (National Diagnostics).

2.4.7 MCF-7 cell proliferation with weak estrogens

Solutions of *o,p'*-DDT (purity 99+%, Lancaster), *p,p'*-DDE (purity 99%, Sigma) and β-HCH (purity 98%, J.T.Baker) were prepared and diluted in absolute ethanol, and added to MCF-7/SOP cultures at a final concentration of 1% in the medium. Proliferation assays were carried out according to our optimised protocol (see results for details). All plates contained three ethanol controls, and three wells containing E2 positive controls at either 10 nM or 10 pM to examine reproducibility. Layouts were semi-randomised to avoid systematic effects induced by the positioning of wells.

2.5 Results

2.5.1 Proliferative responses to estradiol in MCF-7 cells

In order to thoroughly characterise test outcomes to E2, we carried out comprehensive dose-response analyses for the three MCF-7 cell lines (Fig. 2.1). Cells were treated with a range of concentrations of E2 according to a protocol originally devised by Soto et al. (1995) and described here.

The concentrations of E2 that elicited half-maximal effects were similar in each case (13.3, 11.2 and 9.6 pM for BUS, UCL and SOP, respectively) and agreed well with data reported in the literature (Olea et al. 1996). However, there were marked differences in terms of the maximal proliferative effect of the hormone on the 3 cell lines (Fig.2.1). MCF-7/BUS produced the greatest maximal response (8.9-fold over controls), while variants UCL (0.98) and SOP (1.45) showed only small increases in cell numbers.

The results for MCF-7/BUS agreed well with the data reported by Villalobos et al. (1995) and Soto and her colleagues (1997), however, the other two lines produced far lower responses than had been previously reported with this protocol, unsuitable for confident concentration-response analysis of weak estrogens.

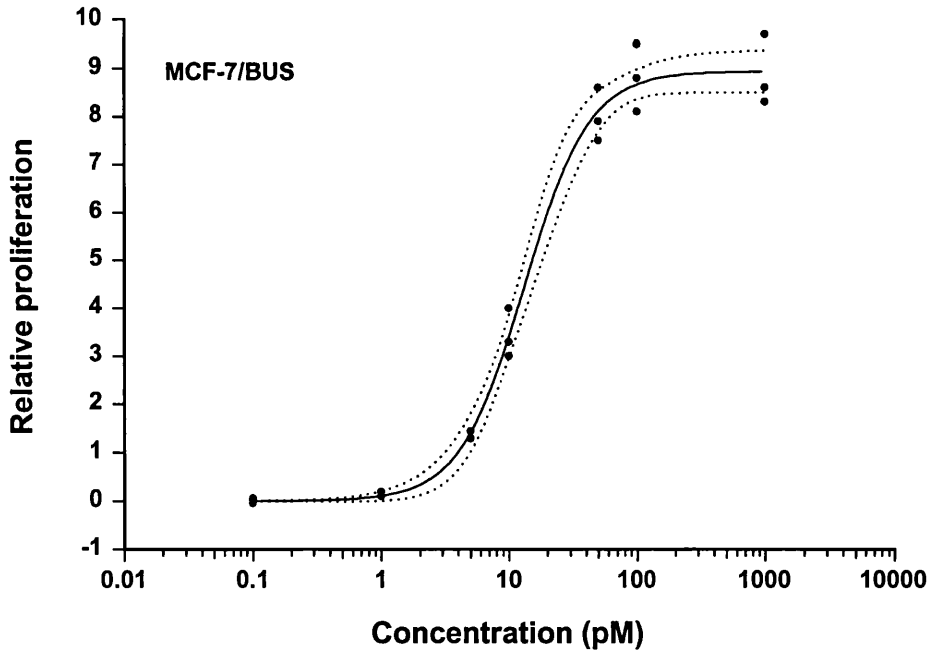
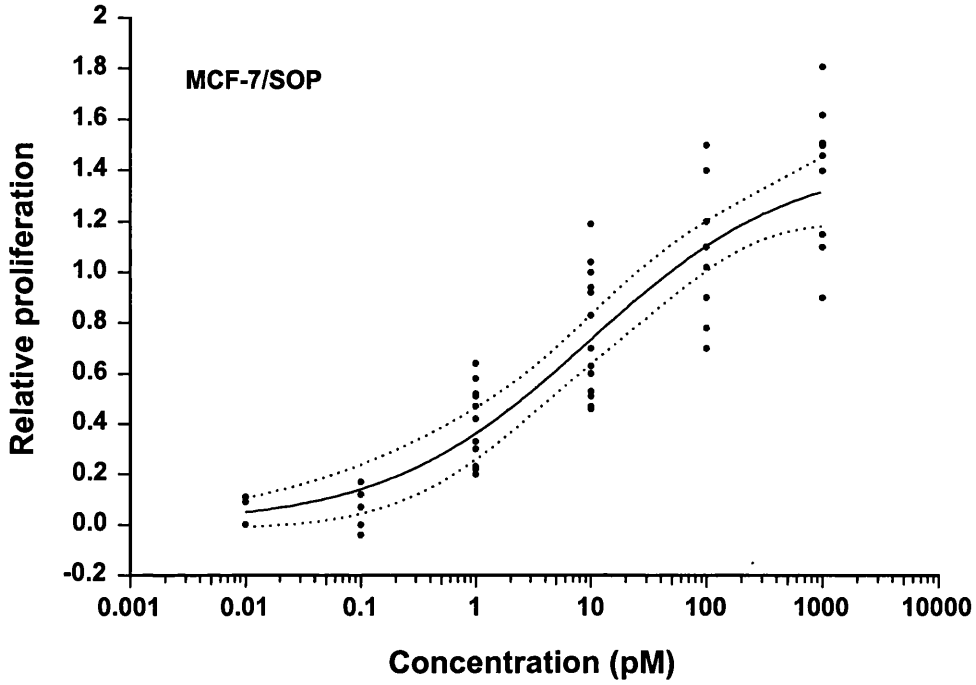
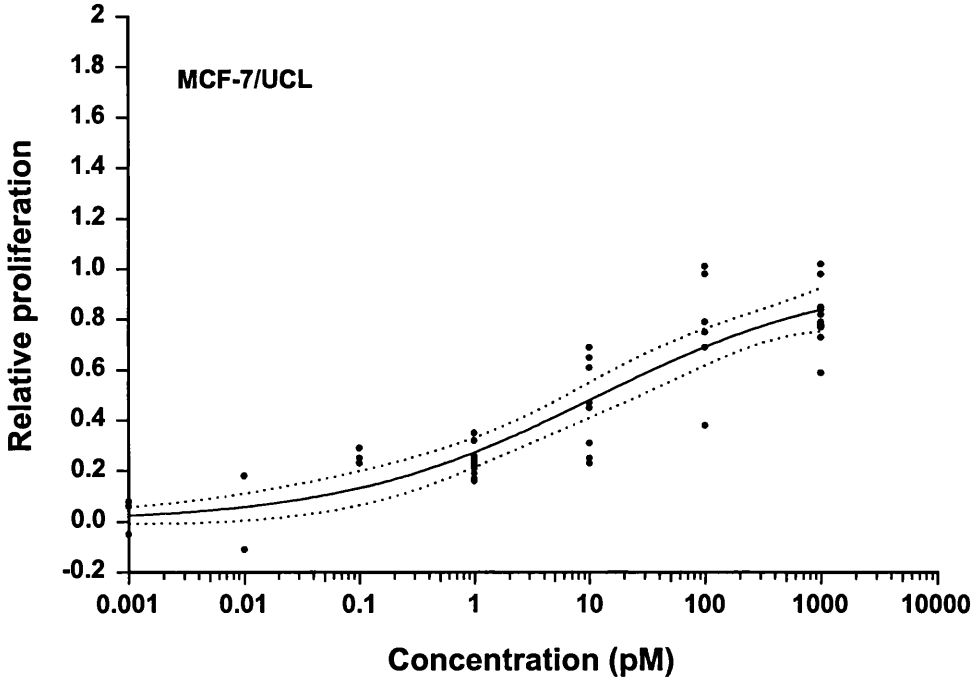


Figure 2.1 Concentration-response relationships for MCF-7 variants to E2. MCF-7/BUS, SOP and UCL cells were treated with E2 (0.1, 1, 5, 10, 50, 100, 1000 pM) according to the protocol of Soto et al. (1995). Points represent data from individual wells from two independent experiments, with proliferation given as fold increase over ethanol controls. Curves were generated by non-linear fitting to the asymmetric Hill function, solid lines represent the best fit, dotted lines are 95% confidence intervals.


MCF-7/SOP
Relative proliferation
2
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0.001
0.01
0.1
1
10
100
1000
10000
Concentration (pM)


MCF-7/UCL
Relative proliferation
2
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0.001
0.01
0.1
1
10
100
1000
10000
Concentration (pM)

2.5.2 Effect of seeding density on proliferative response

The influence of varying seeding densities on the proliferative response of MCF-7/SOP to E2 was investigated. We also studied the effect of varying the cultivation time in full medium between plating and treating with test compounds. Figure 2.2 shows the effect of these two factors on cell proliferation in response to 1 nM E2, the concentration which yields a maximal response. As described in the protocol by Soto (Soto et al. 1995), 20 000 cells were plated per well followed by administration of 1 nM E2 24 hr later. After 7 days of culture time, this regimen yielded a maximal proliferative response of only 1.5-fold over untreated controls, considerably less than the responses reported previously (Villalobos et al. 1995). This prompted us to vary plating density and duration of growth in full medium before addition of test compounds.

By extending the period of cultivation in full medium before dosing to 48 hr, we achieved a 2.95 fold increase in cell proliferation. However, a further extension to 72 hr led to a reduction in proliferation to 1-fold over controls. This diminished response was not due to reduced growth of the treated cells but to an increase in cell numbers in untreated controls, thus giving rise to a reduced relative proliferation of the cells.

In an attempt to increase the relative proliferative response of treated cells, we explored the effect of lower plating densities and seeded only 10 000 cells per well (Figure 2.2). Following cultivation for 24 hr in full medium, cells treated with E2 produced cell numbers 1.4-fold in excess of untreated controls, no different to the response seen with 20 000 cells per well. This was in excellent agreement with previously reported increases of 1 to 1.5 fold (Jones et al. 1997). However, when the growth in full medium was extended to 48 hr, a 4.1 fold increase was observed.

Cultures kept for 72 hr in full medium before treatment yielded only a 1.5 fold increase, again not significantly different from that with 20 000 cells.

It would therefore appear that a seeding density of 10 000 cells per well, combined with 48 hr cultivation in full medium before treatment, produced an optimal response.

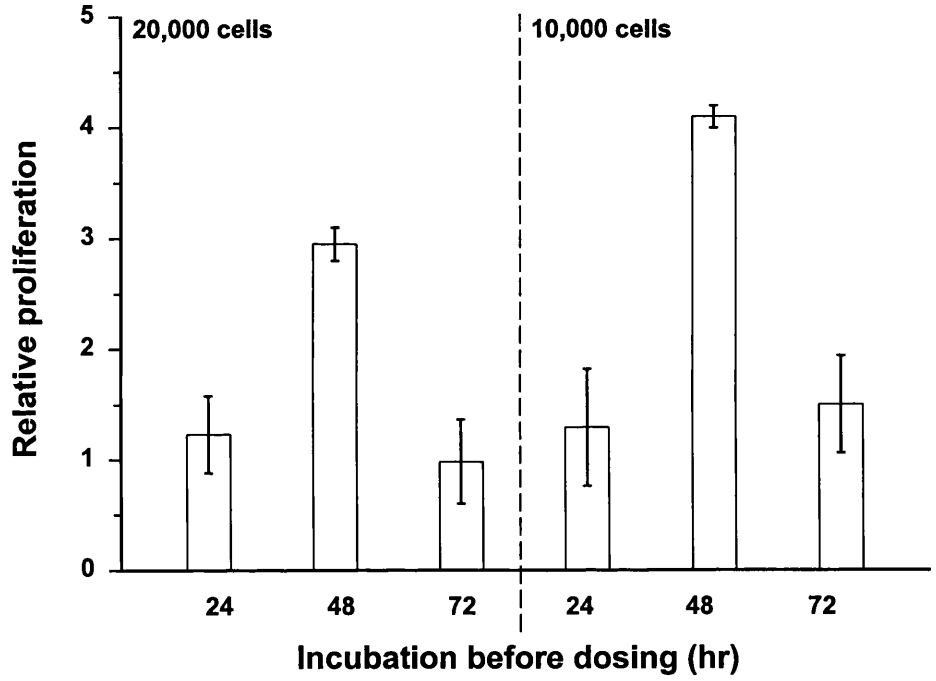


Figure 2.2 The influence of seeding density and incubation time before treatment on MCF-7 cell proliferation. Proliferation is given as fold increase in cell number over ethanol treated controls, data are mean ± SD, n ≥ 6, from at least two independent experiments.

2.5.3 Duration of growth in estrogen-free medium before treatment

We next examined how the proliferative response was influenced by growing the cells for varying periods of time in estrogen-free medium before dosing (Fig. 2.3).

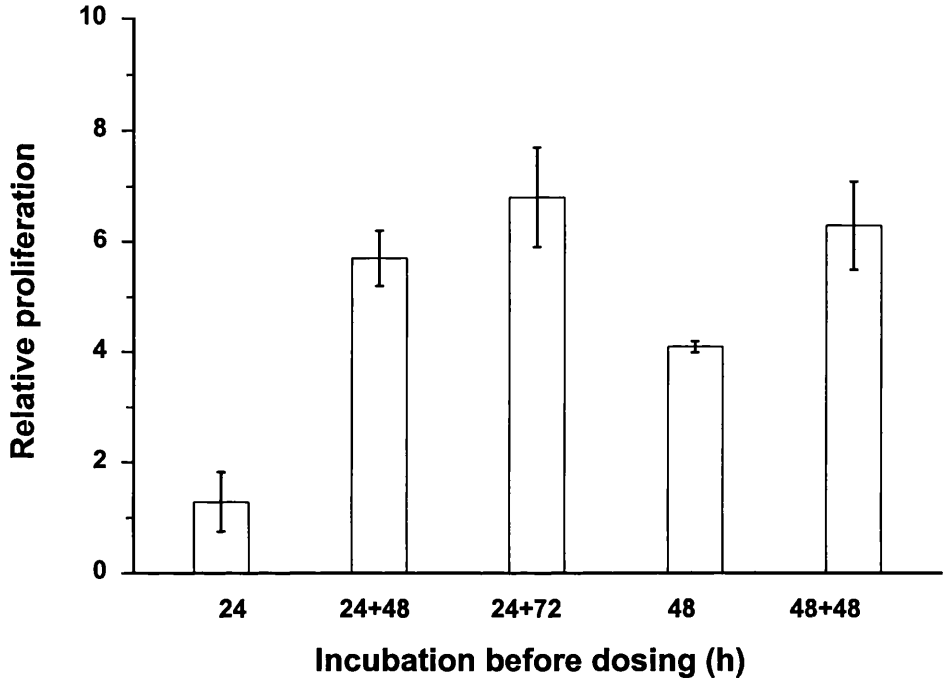


Figure 2.3 The influence of culture time in estrogen-free medium prior to treatment on MCF-7 cell proliferation. 10 000 cells per well were seeded and incubated in full medium for 24 or 48 hr then treated or given a further incubation in estrogen-free medium for 48 or 72 hr before treatment. All cultures were treated with 1 nM E2. Proliferation is given as fold increase in cell number over ethanol treated controls, data are mean ± SD, $n \geq 6$, from at least two independent experiments. All responses are significantly different to that obtained after a 24 h incubation ($p \leq 0.05$).

10 000 cells per well were plated in full medium and left for 24 or 48 hr before the medium was changed to phenol red-free DMEM with 5% CDHus. The cells were maintained under these conditions for 48 or 72 hr before adding fresh media and 1nM

E2. Cells grown in full medium for 24 hr followed by 48 hr in estrogen-free medium showed a proliferation of 5.7 fold over controls, slightly greater than the 4.5-fold increase observed by Jones et al. (1997). When left in estrogen-free medium for 72 hr rather than 48 hr, the response increased to 6.5-fold. A similar result was obtained with a regimen of 48 hr in full medium followed by 48 hr in estrogen-free medium.

2.5.4 Measurement of estrogen receptor (ER) content

The apparent increase in proliferative response of MCF-7 cells following a period of growth in estrogen free medium could be due to an increase in ER production. ER upregulation in response to deprivation of E2 has already been documented (Katzenellenbogen et al. 1987). Table 2.1 shows the distribution of radiolabelled E2 in MCF-7 cells following a 1 hr incubation with the hormone. It can be seen that under the new protocol there is an 88 % increase in total cellular ER accompanied by a 226 % increase in nuclear ER (73 % when corrected for total cellular ER).

	Total Cellular	Nuclear	Nuclear as % total
"Old" protocol	61.1 ± 9.5	9.4 ± 2.7	15.4
"New" protocol	114.6 ± 8.6 *	30.7 ± 6.1 *	26.7

Table 2.1 Distribution of radioactivity in MCF-7 cells treated with 1 nM ^{3}H-E2. Results are fmoles ER per 10^{6} cells ± SD, $n \geq 6$. Old protocol refers to a 24 hr incubation in full medium before treatment, new, a further 72 hr in estrogen-free medium. * values for "new" protocol differ significantly from "old" protocol ($p \leq 0.05$)

2.5.5 MCF-7/BUS vs MCF-7/SOP: a comparison

Concentration-response relationships were compared for MCF-7/BUS cells

under the Soto protocol with MCF-7/SOP cells under our optimised protocol for E2 (Fig.2.4). Although the SOP cells did not obtain the same maximum as the BUS cells (6.5 compared with 8.9), there was good agreement between EC_{50} values (13.3 and 9.6 pM respectively) both within the experiment and with literature values.

The dose-response curve produced by MCF-7/BUS cells (Soto protocol) was steeper than the one observed with parent MCF-7 cells (our protocol) (Fig. 2.4). This may be expected, given that MCF-7/BUS cells are a more homogenous population than the MCF-7 parent line.

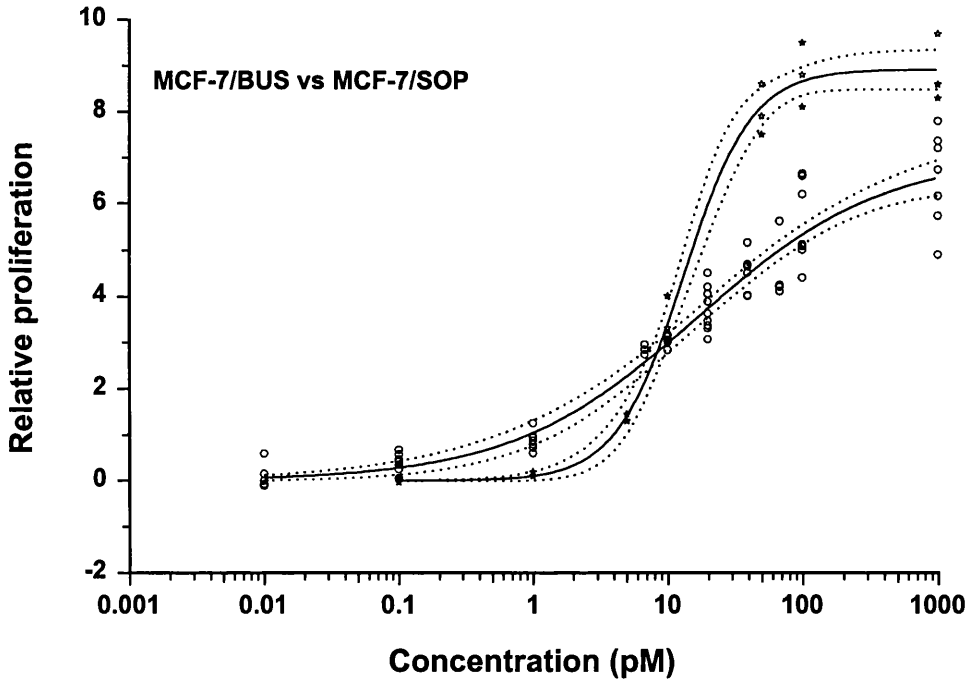


Figure 2.4 Concentration-response analysis for E2 in the MCF-7 cell proliferation assay. MCF-7/SOP cells were treated according to our improved protocol (○), and MCF-7/BUS cells were treated according to the regimen described by Soto et al. (1995) (☆). Points represent data from individual wells, curves were generated by non-linear fitting to the asymmetric Hill function. Solid lines represent the best fit, dotted lines are 95% confidence intervals. Proliferation is given as fold increase in cell number over ethanol treated controls.

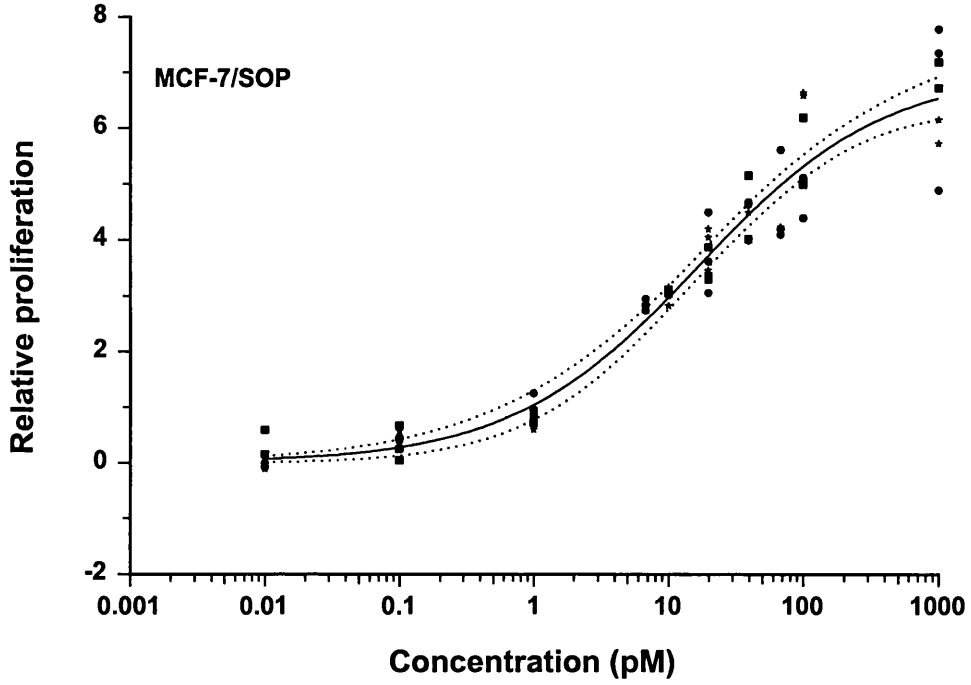


Figure 2.5 Concentration-response analysis for E2 in the MCF-7 cell proliferation assay. MCF-7/SOP cells were treated according to our improved protocol, in triplicate from three separate experiments (★, ●, ■). Points represent data from individual wells, curves were generated by non-linear fitting to the asymmetric Hill function. Solid lines represent the best fit, dotted lines are 95% confidence intervals. Proliferation is given as fold increase in cell number over ethanol treated controls.

In order to examine the inter- and intra-experimental variability, data from our experiments with MCF-7/SOP cells (new protocol) were plotted (Fig. 2.5). From this it can be observed that there was reasonably good agreement both within and between experiments. Throughout the mid-range of the graph, inter- and intra-experimental variability were no greater than 25 %, however, at the lowest and highest concentrations this value rose to about 50 % in some cases. At low concentrations this may be due to problems of handling highly diluted solutions, while at the higher ones this may be reflecting cell toxicity. Some researchers may argue that these outlying should have been omitted, however, we decided to retain them for completeness.

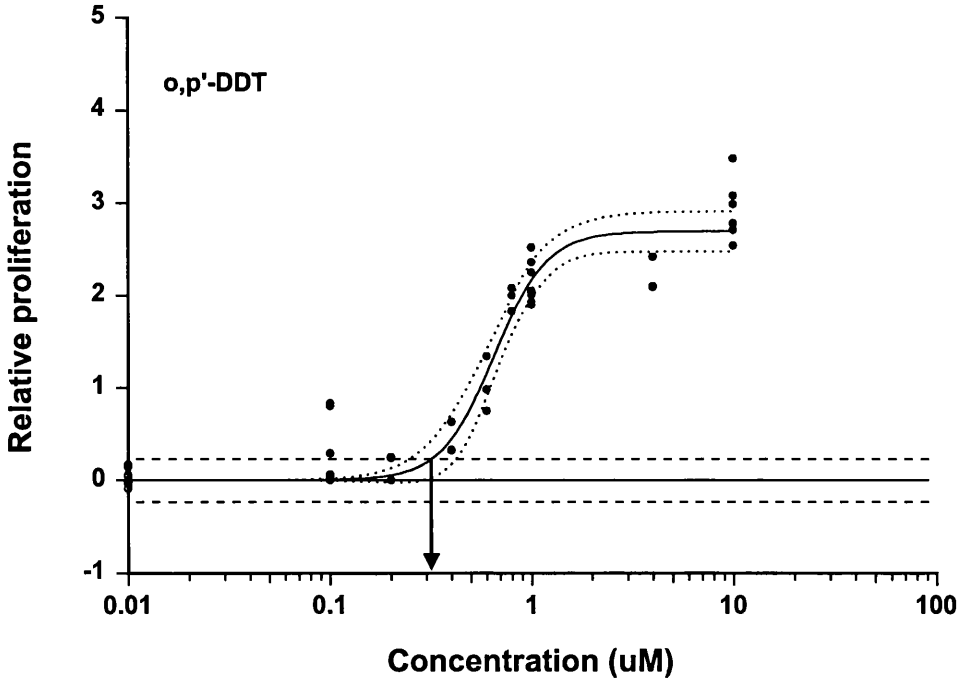
2.5.6 Responses to other agents

One of the primary uses of the MCF-7 cell proliferation assay is for the detection and investigation of weakly estrogenic environmental pollutants. Using our optimised protocol, we carried out thorough concentration-response analyses (Figure 2.6) for the recognised xenoestrogen *o,p*'-DDT and the MCF-7 proliferaters *p,p*'-DDE and β-HCH. Experiments were performed in triplicate and repeated twice more over a period of several months to determine the reproducibility of the data. In each case, excellent agreement was found. These agents produced concentration-response curves with maximal responses of 2.7, 4.4 and 5.9-fold over controls with EC_{50} values of 0.66, 3.3 and 3.9 μM respectively. From these data we were also able to calculate the lowest concentration at which a proliferation increased significantly over background values (Table 2.2). In view of the criticism levelled at no observable effect concentrations (NOEC) by the EPA, we counted as detectable effect levels that were clearly distinguishable from background noise, i.e. the variability of control values. For this assay, background was taken to be three times the standard deviation of ethanol-treated controls about the control mean (0 ± 0.23). The point at which the concentration-response curves bisected the upper band of this interval was considered to be the limit of detection. From pooled data, we determined that for *o,p*'-DDT and *p,p*'-DDE this was at 0.31μM and 0.9 μM respectively, while β-HCH was first detectable at 0.25 μM. This indicates that the system is useful for detecting low concentrations of weakly estrogenic agents. In light of concerns over the activity of agents that do not act like E2, we investigated the ability of tamoxifen to prevent proliferation induced by E2 and β-HCH. 1 nM Tamoxifen caused a 70% reduction in the proliferative response to

1nM E2, yet it reduced the response to 10 μM β-HCH by only 20%. This is probably a reflection of the differing modes of action of the two chemicals. E2 is by definition an ER agonist and so can be antagonised by tamoxifen, whereas β-HCH act via a non-ER mechanism and so remains relatively unaffected by tamoxifen. Testosterone was also tested at a range of concentrations (1 pM - 100 nM), but yielded no measurable proliferation of MCF-7 cells.

Compound	*o,p'*-DDT	*p,p'*-DDE	β-HCH
Conc (μM)	0.31	0.90	0.25

Table 2.2 Detection limits for selected test compounds in the MCF-7 cell proliferation assay, based on graphs shown in Fig.2.6 Given are concentrations required to produce proliferative responses exceeding 3 x SD for controls. For further explanation see text.



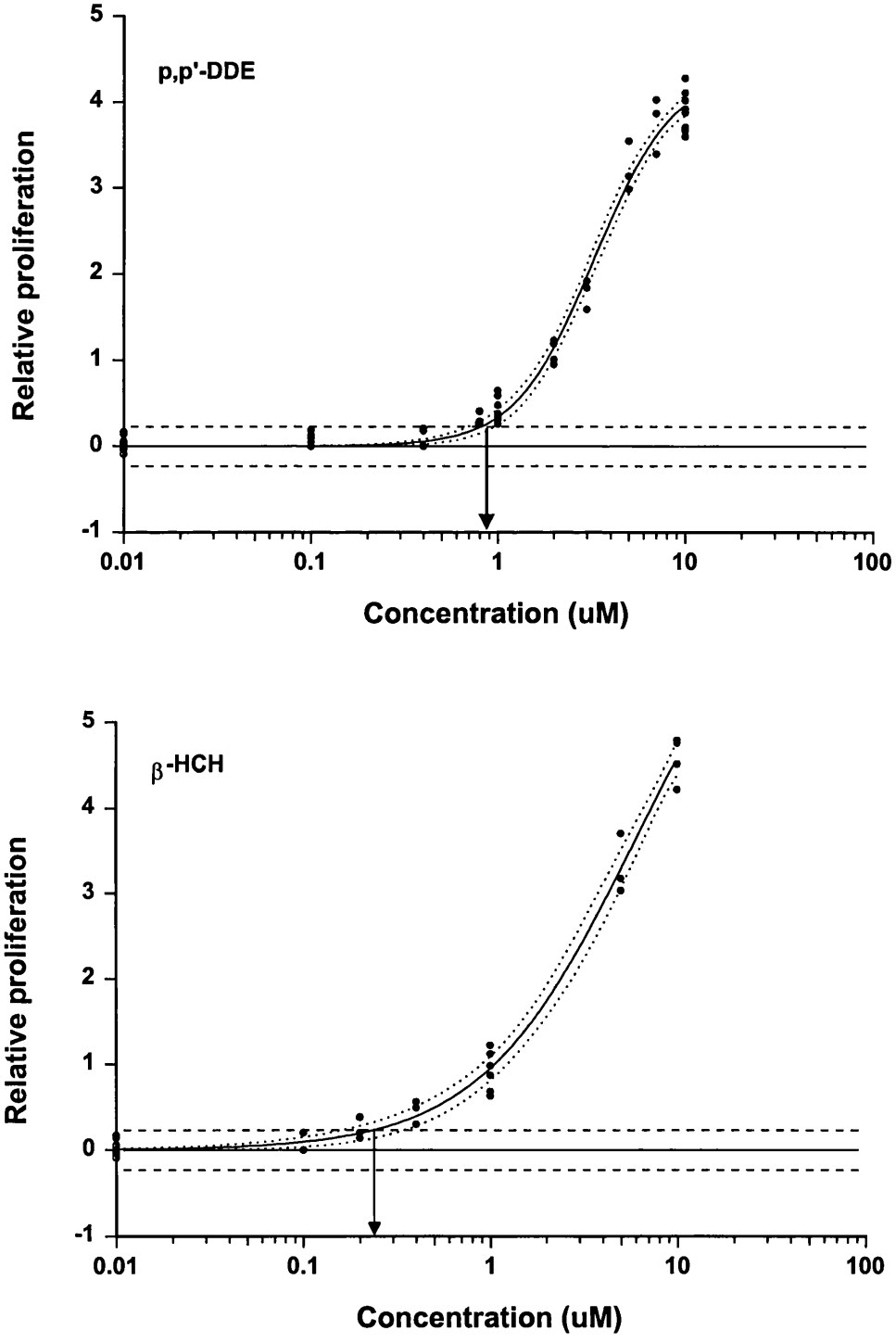


Figure 2.6 Concentration-response analyses for weak estrogens. Cells were treated according to our improved protocol and exposed to *o,p'*-DDT, *p,p'*-DDE and β-HCH. Points represent data from individual wells, curves were generated by non-linear fitting to the asymmetric Hill function. Solid lines represent the best fit, dotted lines are 95% CI. Controls are shown as mean ± 3 x S.D, arrows indicate points at which proliferation exceeds the upper background interval. Proliferation is given as fold increase in cell number over ethanol treated controls, data shown from 2 separate experiments.

2.6 Discussion

Since the instigation of the MCF-7 cell line 25 years ago, a number of recognised sublines have come into use and are well characterised in terms of their proliferative capacities, receptor levels and estrogen responsiveness.

It has previously been reported (Jones et al. 2000) that significant genetic differences exist between the three cell stocks tested. Using comparative genomic hybridisation, the profiles of copy number changes were determined, and changes common to all three lines were observed. These comprised of gains at 3p,5p,7q,8q,17q and 20q, and losses at 1p, 8p, 18q and 22q. These are in agreement with the most commonly observed alterations in genetically and phenotypically advanced breast cancers (Tirkonen 1998).

As well as these DNA copy changes which were common to all three cell lines, there were a number of striking differences, as confirmed by cell line : cell line co-hybridisation experiments. The MCF-7/SOP exhibited losses at 1q31-q32 and Xq12-q25, and gains at 9q31-q34 and 16q21-q23 that were not seen in MCF-7/BUS. While, MCF-7/UCL cells did not exhibit the loss of chromosome X or the gains at 7q, 9q and 16q seen with MCF-7/SOP cells. However, MCF-7/UCL showed numerous copy changes not seen in the other cells. These were losses at 2q, 4p, 10q, 11q, 17p, 17q 19 and 2, and gains at 2q, 4q and 11q (Jones et al.2000).

The large number of differences in copy number changes detected by CGH between the three cell lines may be of critical importance in relation to the observed differences in proliferative and estrogenic responses amongst different MCF-7 cell stocks. Whilst clues as to the underlying molecular genetics of these differences

cannot be provided by CGH, it is interesting to note that the greatest degree of cytogenetic variation is seen with the cell stock (UCL) which proliferates least well in the presence of E2.

Having selected an MCF-7 cell line, it is essential to utilise an assay with high sensitivity to detect the activity of weak estrogens. In our laboratory, we began using the cell line we designated MCF-7/SOP using a 24 hr full medium incubation before dosing as described by Soto et al. (1995) for MCF-7/BUS cells. This yielded results that were lower than had been previously reported (Villalobos et al. 1995). With the maximal response to E2 being only a doubling in cell number relative to controls, we were unlikely to detect less potent estrogens with any degree of confidence. By a succession of alterations to the culture conditions we were able to produce a 6.5-fold increase in cell numbers in response to E2, a proliferation which has not previously been reported with the MCF-7 parent cell line (Jones et al. 1997) and is more akin to that seen with the MCF-7/BUS cells (Villalobos et al. 1995). The previously reported maximal response observed with MCF-7 parent cell lines was a 4.5-fold increase over vehicle treated controls (Jones et al. 1997).

The increase in sensitivity to xenoestrogens we observed with our optimised protocol is most likely due to an upregulation of ER during the period of growth in estrogen-free medium. This hypothesis is supported by the observation that following a 72 hr deprivation of estradiol, cells exhibited a marked increase in both nuclear and total cellular ER.

In the course of our experiments we have been able to demonstrate the reproducibility of the MCF-7 cell proliferation assay using the BUS cells and the protocol

set out by Soto et al. (1995). Our data also support the findings of Jones et al. (1997) using the MCF-7 parent cell line and a regime of 24 hr in full medium followed by 48 hr in estrogen-free medium. Under these conditions we obtained a maximal proliferation of 4.7-fold, in good agreement with the 4.5-fold increase reported by Jones and her colleagues (1997).

A comparison of dose-response curves using our cells combined with our improved protocol with those produced when using MCF-7/BUS cells and Soto's protocol revealed only marginal differences in terms of EC_{50} and maximal response. With the MCF-7/BUS cells and the Soto regime we achieve a slightly higher maximal response and a lower EC_{50} than with our system. However, the most notable difference is in the shape of the curve. Using our cells and protocol we obtained a curve that is shallower at low concentrations of test compound, i.e. low doses of agents produce a greater response in our system than in the Soto system. This is probably due to the clonal nature of MCF-7/BUS cells yielding a more homogenous cell population compared with the parent cell line. Therefore, the parent MCF-7/SOP cells may offer advantages for the analysis of effects of chemicals at low concentrations.

Although Andersen et al. (1999) found the MCF-7 cell proliferation assay to be sensitive and reproducible in most cases, there was poor reproducibility with agents such as BBP, and those which act via a mechanism distinct from that of E2, such as *p,p'*-DDE. Other potentially problematic agents may include β-HCH, which is a potent MCF-7 proliferater, but not a classical ER agonist (Steimnetz et al. 1996).

Taken together, our findings show that the details of the regimen employed influence test outcomes just as strongly as the nature of the cell lines chosen for

experiments. However, provided there is consistency in choice of cell line and protocol, the MCF-7 cell proliferation assay is robust and highly reproducible. Our study provides a rationale for optimising the assay for any MCF-7 cell stock, but despite this there is still a need to establish a basis for comparing test outcomes from different laboratories.

2.7 Conclusions

The MCF-7 cell proliferation assay is a highly sensitive tool for the identification of estrogenic agents and is highly suitable for the analysis of combination effects with weak estrogens. However, there is still the need to adopt a universally approved cell line and protocol, in order to eliminate interlaboratory variability.

Chapter 3

Assessing the effects of binary mixtures of xenoestrogens using the model of concentration addition

3.1 Abstract

In this chapter we have investigated the usefulness of the model of concentration addition for assessing mixture effects. One application of this model is the method of isoboles, which is applicable for assessing the effects of binary mixtures. Here we present data from the MCF-7 cell proliferation assay with two well characterised endocrine disrupters, *o,p'*-DDT and *p,p'*-DDE. The effects of these agents were tested both when present singly and in binary mixtures. Observations were compared with an additivity prediction using the method of isoboles. Experimental mixture data deviated slightly from the line of additivity at low effect levels, leaving the possibility of a weak synergism. By rearranging the formula for CA it is possible to generate predictions for entire concentration-response curves, enabling us to observe deviations from additivity for the entire range of effect levels. This allows us to visualise more clearly the rightward shift of the mixture curves away from the additivity prediction, indicating weak synergy. Consequently we propose that analysis of whole concentration-response curves may be more informative than the method of isoboles. It will also be especially useful for analysing mixtures containing more than two components.

3.2 Introduction

In recent years the existence of synergy between xenoestrogens has been the subject of considerable debate, not helped by the publication and subsequent retraction of a paper by Arnold et al. claiming marked synergy between xenoestrogens. Many other claims of synergy have also been recently discredited (Kortenkamp and Altenburger 1998), due to the application of unsuitable model predictions. However, some genuinely synergistic interactions that had previously been overlooked have also come to light (Kortenkamp and Altenburger 1998), keeping alive the ongoing concern.

Various models have been proposed for the assessment of combination effects, including the longstanding and well validated model of concentration addition. In this model expectation of mixture effects can be calculated from the concentration-response relationships of the individual agents assuming that they act additivity. These predictions can then be compared with experimental observations to determine the nature of the mixture effect.

Here we examine the application of CA, particularly the method of isoboles, to analysing combination effects with a binary mixture of estrogen-like agents.

3.3.1 Concentration addition

The model of concentration addition (CA) can be traced back to Loewe and Muischneck (1926) more than half a century ago, and is based on the concept of "similarly" acting chemicals. In this model it is said that chemicals in a mixture act as a dilution of each other, meaning that any effect can be obtained by replacing one chemical wholly, or in part, by an equi-effective quantity of another. Each individual

component of a mixture is assumed to contribute to the observed overall effect by acting in proportion to its concentration, regardless of any effect thresholds. The relationship between concentrations and effects is given by the formula derived by Loewe and Muischneck (1926):

$$\sum_{i} \frac{X_i}{EC_{yi}} = 1$$

Here, X_i denotes the concentration of agent i in the mixture and EC is the concentration of that single agent required to elicit the same effect (y) as the mixture.

A final sum of 1 indicates that the combined effect of the chemicals in the mixture is additive, whereas values significantly less than this indicate synergy (i.e. a smaller amount than predicted of the mixture is required to elicit the given effect). Similarly, values above 1 indicate antagonism between the mixture components. This formula holds true for any number of constituents in a mixture, and rearrangement of the equation allows us to plot predictions of mixture response on a traditional log-concentration effect curve for comparison with observed mixture effects.

Recently this method has been employed by Altenburger et al. (2000) to investigate the predictability of multiple chemical mixture toxicity in the marine bacterium *vibrio fischeri*. Using a mixture composed of 16 similarly and specifically acting chemicals, CA demonstrated an excellent predictive power when compared with the observed data.

The validity of this model for agents binding to an identical receptor site has been confirmed *in vitro* in a number of studies (Pöch 1993), and this model has gained acceptance in many areas due to its plausible pharmacological basis, so much so that

Berenbaum has proposed it to be a "general solution" to the analysis of combination effects (1989). One of the features of this model is the ability to predict combination effects even when individual agents are present below their no observable effect concentrations (NOEC), a case that may well occur in environmental exposure scenarios.

One problem is that it is difficult to decide how the term "similar" action should be applied, and this may well vary between assay systems, even with the same set of compounds. At one extreme the term can be taken to mean interaction of agents with an identical substructure of an acceptor molecule (e.g. specific amino acid sequence of the estrogen receptor). Alternatively we may be content to define similarity purely in terms of endpoint achieved (e.g. cell proliferation). It is therefore necessary to test experimentally whether the method of isoboles is applicable to MCF-7 cell proliferation. To this end a combination of compounds that produce similar effects by differing molecular mechanisms was chosen. *o,p'*-DDT acts via binding to ER while *p,p'*-DDE is thought to act via the androgen receptor (AR) (Bitman et. al. 1968, Kelce et. al. 1995).

3.3.2 The method of isoboles

One application of CA is via the method of isoboles. We can trace the roots of this method as far back as the end of the 19th century with experiments, interestingly on the combined toxic effects of binary mixtures of pesticides (Fraser 1872). As demonstrated a century later by Berenbaum (1989), this model overcomes the problems associated with ES by comparing doses of agents which produce equi-

effective effects, that is, responses of equal magnitude (Figure 3.2). The method itself is purely descriptive and requires no prior knowledge of the underlying mechanisms involved. Nor does it produce the logical inconsistency with sham mixtures of a single compound seen with effect summation.

The one major drawback with this method is the two-dimensional, graphical nature of the analysis, which naturally makes it difficult to represent anything other than binary mixtures. However, it can be extended for the analysis of mixtures of three agents by constructing three-dimensional plots.

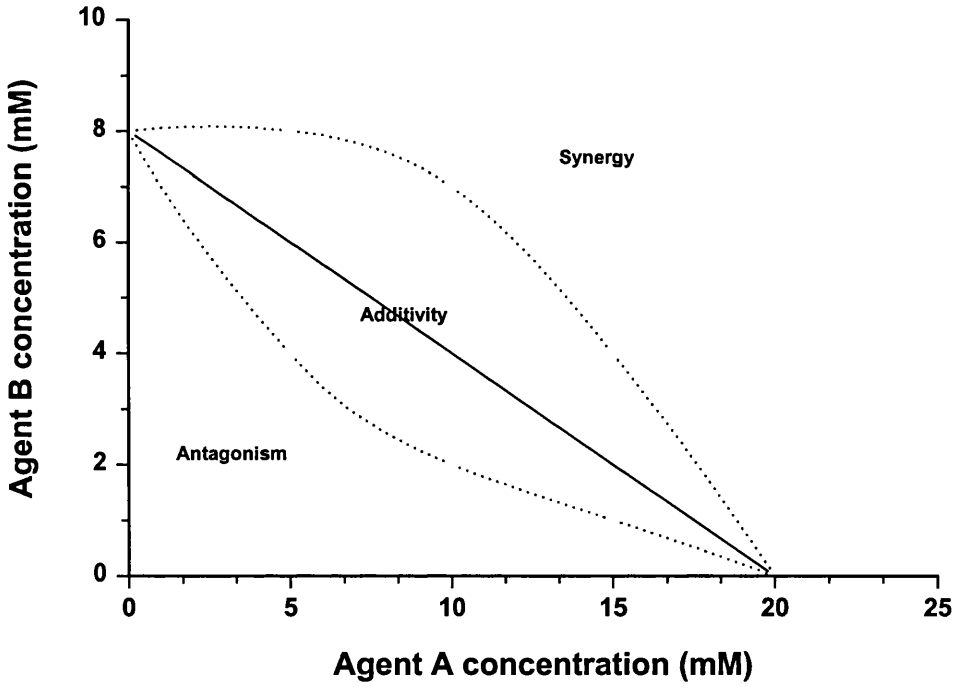


Figure 3.1 Hypothetical isobologram for two agents, A and B. The solid line indicates the quantities of each agent in a mixture of the two that would also produce the predetermined effect, assuming additivity. If the observed data for a mixture of the two deviates from this line then one would conclude synergy or antagonism (as indicated). For details see text.

For any binary mixture of chemicals this model relies on the availability of at least some data on concentration-response relationships of the two agents singly as well as in combination. From this, for any response level selected one can plot the concentrations of the two single agents that are required to produce that effect graphically (Fig.3.1). A straight line drawn between these two points gives the expectation of the mixture response assuming additivity. For example, two agents are found to induce proliferation in MCF-7 cells. Agent A causes a 100% increase in proliferation when present at 20 mM, while the more potent agent B elicits the same effect at 8 mM. The line of additivity is then defined by joining the the values of 20 and 8 mM in this case. If we now produce a mixture of the two in the ratio 2.5:1 (A:B) and apply it to the cells, let us assume that the mixture causes a 100 % proliferation at a concentration of 14 mM. Because we know the mixture ratio, we can determine that A is present at 10 mM and B at 4 mM in the mixture. Plotting this on the isobole yields a point on the line of additivity, indicating that the combined effect fo A and B is additive. Had we required e.g. 21 mM of the mixture (15 mM A and 6 mM B) to produce 100% proliferation, the point plotted would lie above the line of additivity and indicate antagonism (concentrations higher than predicted elicit the effect), while if we had obtained 100 % increases in proliferation with e.g. 7 mM of the mixture (5 mM A and 2 mM B) the point would lie below the line and we would conclude synergy.

By replicating this exercise for a several different ratios of the mixture, and analysing at a number of effect levels we can construct a response envelope on the isobole, in order to strengthen the validity of the observations. This is important since deviations from additivity may be dependent upon the mixture ratio or effect level

selected.

3.3.3 Why are all additivity isoboles linear ?

A proof of the general validity of the method (i.e. the linearity of additivity isoboles) is required. Here we present the proof proposed by Berenbaum (1989), and illustrate his argument with mixtures of alcoholic drinks.

Let us assume that we wish to assess the combination effect of a mixture of three alcoholic drinks: White wine (A), strong Belgian Beer (B) and German lager (C). Using an appropriately sensitive measure of drunkenness, it is found that the following doses (D) of the drinks, when enjoyed on their own, all produce an effect of, say, 37%:

White wine D_A = 50 ml

Belgian beer D_B = 100 ml

German lager D_C = 150 ml

A mixture of 10 ml white wine (d_A), 5 ml Belgian beer (d_B) and 7.5 ml German lager (d_C) is found to also produce 37% drunkenness. In order to detect whether these three drinks interact additively, antagonistically or synergistically, we need to compare the effect of the mixture with a combination of drinks that by definition is considered to act additively ("zero interaction"). The only possible reference point for a combination showing zero interaction is the sham combination of an agent with itself, since to expect white wine to act synergistically with itself would be absurd. In quite a different sense, most agents do interact with themselves, e.g. at their target sites, and assist or hinder each others action. However, as Berenbaum (1989) has pointed out, such cooperative effects, which give rise to sigmoid dose-response curves, are not the issue here. In the

context of combination effects, it is important only to analyse whether there is any advantage (or danger) in using white wine alone or in combination with continental beers.

A sham combination of alcoholic drinks can be made using any of the three, but we will select white wine. The aim is to prepare a mixture, which, on the basis of the effectiveness of white wine alone would be expected to produce the same effect as the combination under investigation. This is achieved by considering the individual potency of the three drinks, but only at a single chosen effect level (here 37%). We have no information regarding doses of each drink which are required to produce smaller or larger effects, nor is this information necessary.

Because white wine alone is twice as potent as Belgian beer in producing the predetermined effect level (37%), the 5 ml dose of Belgian beer in the combination has to be substituted with D_A / D_B x 5 ml = (50ml / 100 ml) x 5 ml = 2.5 ml white wine. Similarly, the 7.5 ml of German lager is replaced with D_A / D_C x 7.5 ml = (50 ml / 150 ml) x 7.5 ml = 2.5 ml white wine.

The sham combination which according to our *a priori* assumption shows zero interaction consists of 10 ml + 2.5 ml + 2.5 ml white wine. We now need to assess whether the zero interactive sham is more, less or as potent as the real mixture, in order to decide on the combination effect. We already know that D_A = 50 ml white wine is as effective as the combination of 10 ml white wine, 5 ml Belgian beer and 7.5 ml German lager. However, our sham combination only contains 15 ml white wine, not enough to produce the effect of the combination. We do not need information about the effect produced by 15 ml white wine, as we observe that the sham effect differs from that of

the real mixture. In this case we would conclude that a mixture of the three acts synergystically.

To choose a second example, let us consider a mixture containing 25 ml white wine (d_A), 1.25 ml vodka (d_D) and 1.875 ml whisky (d_E) which is assumed to also produce 37% drunkenness. The doses of vodka and whisky which alone produce this effect are:

Vodka	D_D = 5 ml
Whisky	D_E = 7.5 ml

The sham combination which should mimic the effect of the real mixture, if there is zero interaction (additivity) is prepared as follows:

25 ml white wine	
+ (50 ml / 5 ml) x 1.25 ml =	12.5 ml white wine
+ (50 ml / 7.5 ml) x 1.875 ml =	12.5 ml white wine

The total dose of white wine in the sham is 50 ml (equal to D_A) which in turn produces the same effect as the real mixture. The combination effect of our second cocktail is therefore additive.

This relationship can be formulated algebraically:

$$d_A + (D_A / D_D).d_D + (D_A / D_E).d_E = D_A$$

Dividing by D_A gives:

$$d_A / D_A + d_D / D_D + d_E / D_E = 1$$

which is the equation describing linear isoboles for additivity. These equations are easily generalised for any number of constituents in a mixture.

In validating our criterion for zero interaction we used quantitative factors that are general among agents and their combinations, and did not require information about the dose-response relationships of the individual components or their combinations. What matters is the quantitative relationship between the amounts of the individual constituents present in a mixture (d_A, d_B, etc.), and the amounts of the single agents (D_A, D_B, etc.) that are required to produce the same effect as the mixture. Therefore, additivity isoboles are linear independent of the shapes of the dose-response relationships of the mixtures and single agents.

3.4 Objectives

In this chapter we will examine the strengths and limitations of the method of isoboles and explore possible alternatives such as generating whole curve predictions of mixture effects. This will be carried out using the MCF-7 cell proliferation assay with the well characterised endocrine disrupters *o,p*'-DDT and *p,p*'-DDE.

3.4 Methods

3.4.1 Routine culture

Routine culture was carried out as described in Chapter 2.

3.4.2 Measurement of cell proliferation in response to test compounds and mixtures

According to our previously optimised protocol (Payne et al. 2000), one 70% confluent 25 cm^2 flask of MCF-7 cells was washed with 5 ml phosphate buffered saline (PBS, Sigma) before the addition of 660 µL 0.25% trypsin-EDTA (Sigma). The flask was left for three minutes after which the cells were detached, resuspended in 20 mL DMEM + 5% FCS, counted using an improved Neubauer counting chamber and seeded to 12-well plates (Corning) at a density of 10^4 cells per well in 1 mL DMEM with 5% FCS (full medium). After 24 hr the cells were washed with 1 mL PBS. The medium was changed to 1 mL estrogen-free medium (phenol red-free DMEM with 5% CDHus) and left for a further 72 hr. Again the medium was changed to 1 mL estrogen-free medium, with test compounds being added at a range of concentrations (0.01-50 µM). Cell proliferation was assessed after 7 days in culture using the method of Skehan *et. al.* (1990). Experiments were carried out in triplicate and repeated at least once.

o,p'-DDT (Lancaster) and *p,p'*-DDE (Sigma) were dissolved in absolute ethanol at stock concentrations of 1 mM and serially diluted. Agents were administered so that the final ethanol concentration was 1% in the media. Binary mixtures were produced from 1 mM stocks and serially diluted before administration.

3.4.3 Curve fitting and generation of predictions

For experimentally-generated concentration-response relationships for single agents and mixtures, data were fitted to the asymmetric Hill function using the graphical package Fig. P (Biosoft, Cambridge) using the following formula:

$$\text{Effect} = \text{Min} + (\text{Max} - \text{Min}) / [1 + (X/X_{50})\exp(-p)]$$

Where Min and Max are the minimal and maximal observed effects, X the concentration of the test agent, X50 the concentration of the test agent which yields half-maximal effects, and p the slope parameter. The 95% confidence intervals of mean effects were also estimated.

This model was selected due to its pharmacological usage for ligand-receptor interactions, and was found to yield better fits compared to logistic sigmoid or statistical sigmoid dosimetric models.

3.5 Results

3.5.1 Concentration-response relationships for *o,p'*-DDT and *p,p'*-DDE

Comprehensive concentration-response analysis was carried out for two well recognised endocrine disrupters, *o,p'*-DDT and p,p'-DDE, as shown in chapter 2. With these curves it was then possible to calculate the concentration of either agent needed to produce any predetermined effect level. We selected effect levels 0.5, 1.5 and 2.5 to cover a wide range of effect levels.

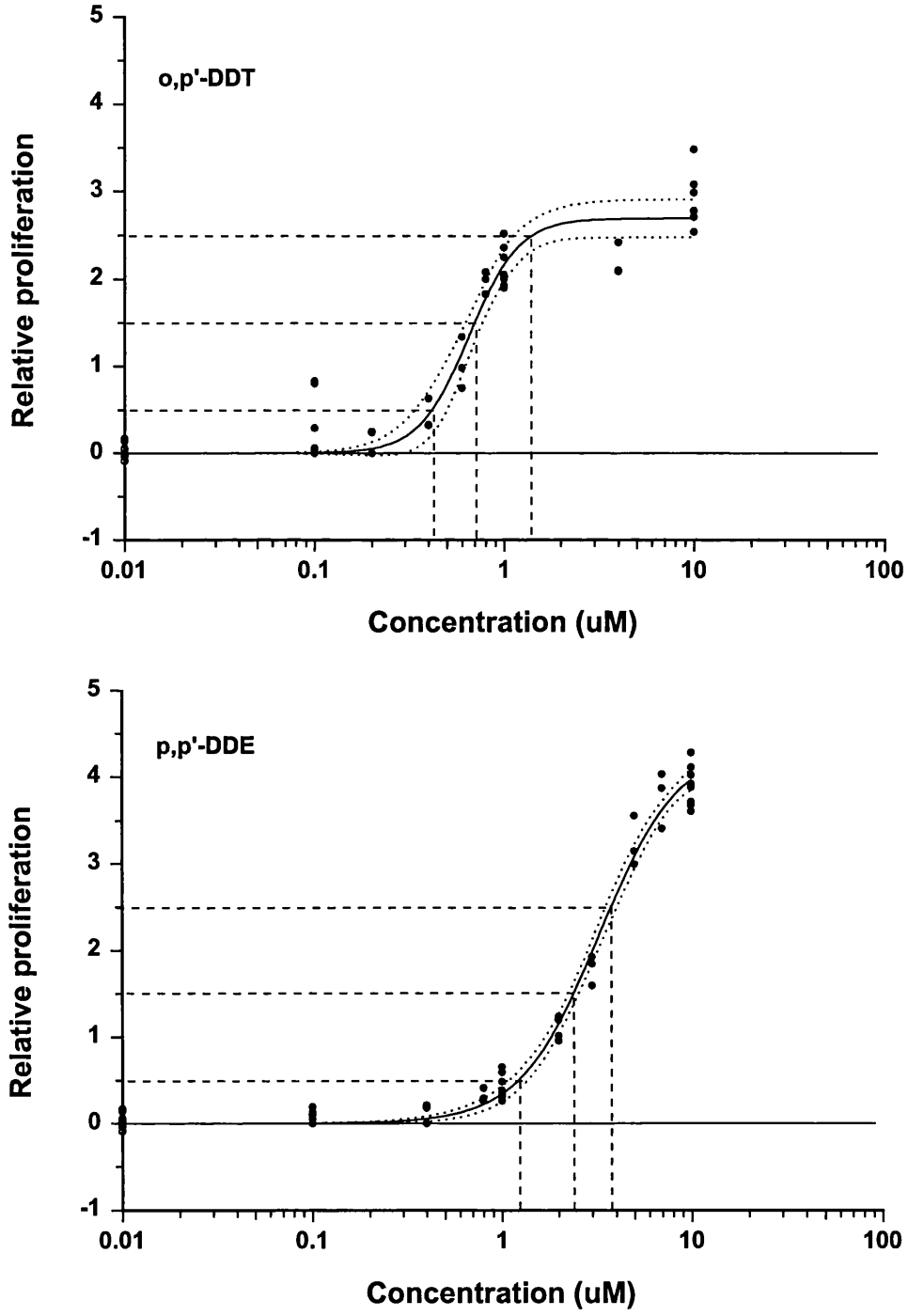
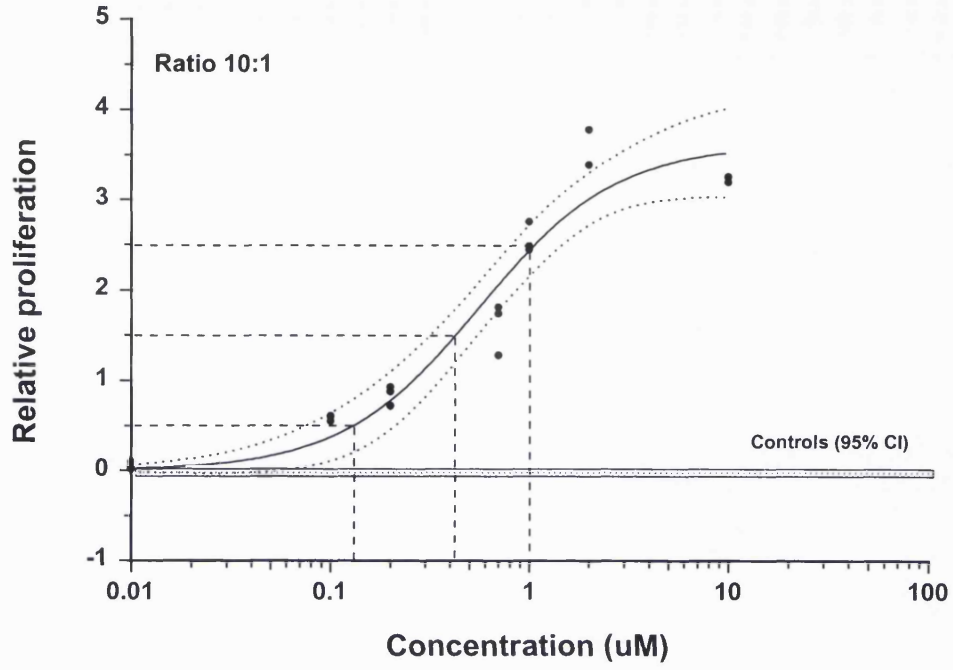


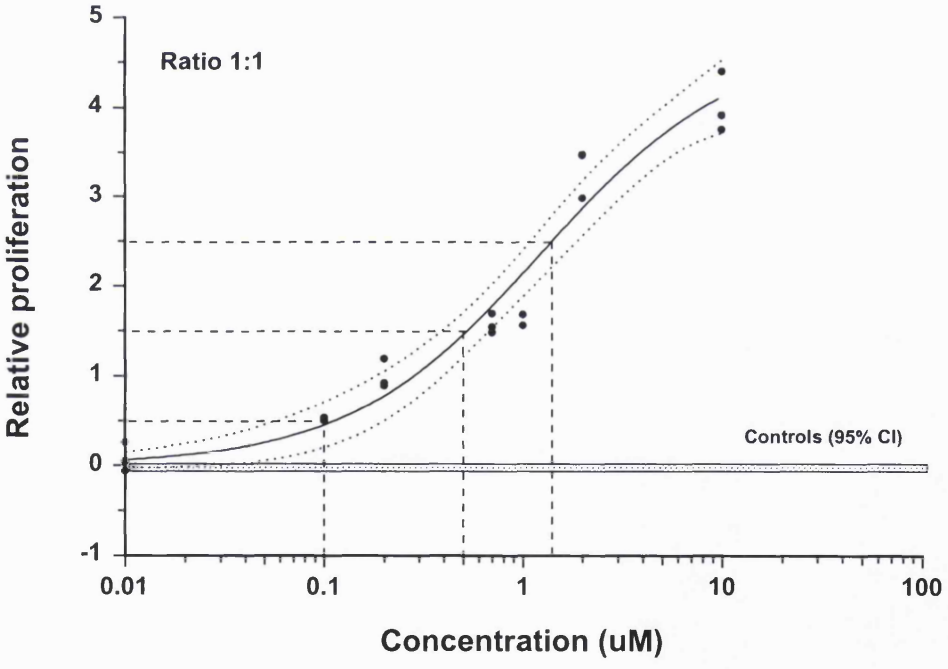
Figure 3.2 Concentration response relationships for *o,p*'-DDT and *p,p*'-DDE. Cells were exposed to *o,p*'-DDT or *p,p*'-DDE as detailed in chapter 2. Dotted lines indicate 95% CI, dashed lines indicate the effect levels selected for the analysis of combination effects and the corresponding concentrations.

Levels above 2.5 could not be analysed as they exceed the maximal response of *o,p*'-DDT. Effect level 0.5 was met by 0.42 µM *o,p*'-DDT or 1.23 µM *p,p*'-DDE, while effect levels 1.5 and 2.5 corresponded to 0.70 µM *o,p*'-DDT or 2.41 µM *p,p*'-DDE and 1.43 µM *o,p*'-DDT or 3.79 µM *p,p*'-DDE, respectively (Fig 3.2). Using these datapoints we are able to construct isoboles for the three effect levels selected.

3.5.2 Concentration-response relationships for binary mixtures

In order to characterise any deviations from additivity, we carried out concentration-response analyses on three mixtures of these two agents. The chemicals were combined in the ratios 10:1, 1:1 and 1:10 (*o,p*'-DDT:*p,p*'-DDE, respectively) and applied to the MCF-7 proliferation assay (Figure 3.3). Each mixture yielded curves with differing slopes and maximal responses (3.6, 4.8 and 5.6-fold over controls, respectively), as well as differing EC_{50} values (0.56, 1.26 and 7.48 µM). It was then possible to determine the relevant concentrations of the single agents required to elicit effect levels 0.5, 1.5 and 2.5.


Ratio 10:1
Relative proliferation
Controls (95% CI)
Concentration (uM)
5
4
3
2
1
0
-1
0.01
0.1
1
10
100


Ratio 1:1
Relative proliferation
Controls (95% CI)
Concentration (uM)
5
4
3
2
1
0
-1
0.01
0.1
1
10
100

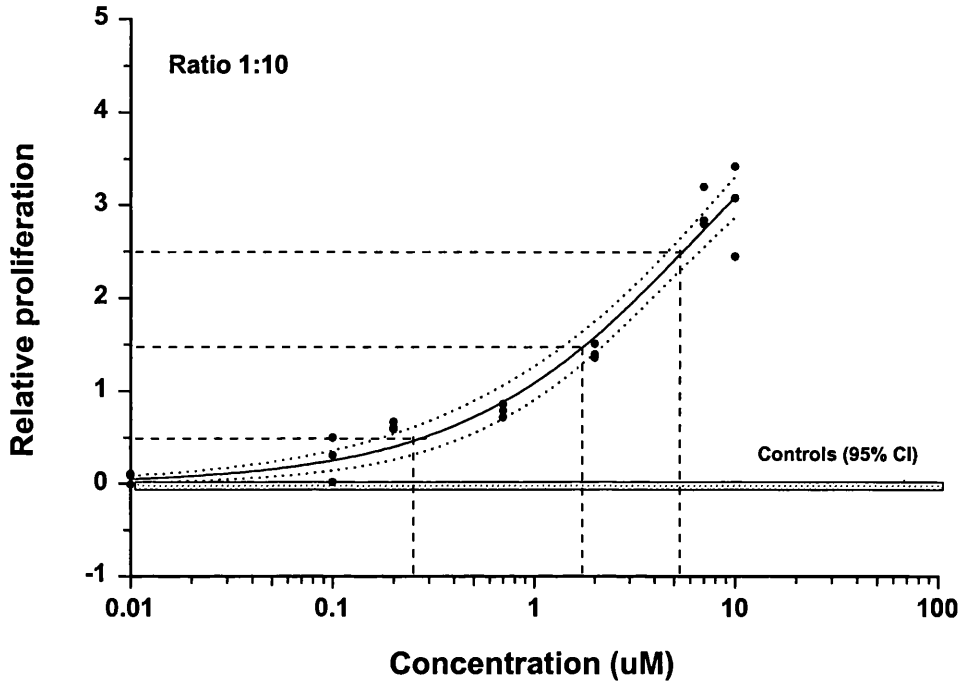


Figure 3.3 Concentration-response relationships for binary mixtures. Cells were treated according to our improved protocol and exposed to *o,p'*-DDT and *p,p'*-DDE in the ratios 10:1, 1:1 and 1:10. Points represent data from individual wells and curves generated by non-linear fitting to the asymmetric hill function (best fit solid, 95% CI dotted). Proliferation is given as fold increase over controls, experiments were carried out once in triplicate. Dashed lines indicate effect levels and corresponding concentrations. Horizontal bar indicates the 95% CI for controls about the mean (zero).

With a knowledge of the ratio of the two components it was also possible to calculate the concentration of each agent that was present in the mixture. However, we are still left with a critical dilemma, how much deviation from the line of additivity is needed before valid conclusions as to synergy or antagonism can be drawn?

3.6.3 What constitutes a significant deviation from additivity ?

The method of isoboles does not lend itself easily to statistical analysis, although

to overcome this problem, Altenburger et al. (1990) proposed the construction of confidence belts around the line of additivity. Mixture datapoints which then lie outside this interval can be considered to be statistically significant. By further adding a confidence interval to the mixture datapoints themselves we can more certainly determine the significance of any deviations from additivity.

When plotting the line of additivity it is possible to include a 95% confidence interval simply by obtaining the 95% CI for the two single agents directly from their respective single agent concentration-response curves at the relevant effect level. However, it is impossible to determine the 95% CI for the two mixture components individually, based solely on the CI of the mixture.

For example, the concentration of an equimolar mixture of DDT and DDE required to elicit an effect level of 1.0 might be 2 mM , with a standard deviation of 0.5 mM. From a knowledge of the mixture ratio (1:1) we can easily determine that 1 mM DDT and 1 mM DDE was present in that mixture. However, to what degree is the SD of 0.5 mM attributable to each compound ? It is impossible to completely deconstruct this value to yield SDs for the constituent parts, however, we are able to estimate these values using the following relationship.

Statistically, the standard deviation of the mixture concentration yielding a given response is equal to the square root of the sum of variances for the two components:

$$SD_{mix} = \sqrt{(s^2_{DDT} + s^2_{DDE})} = \sqrt{2} \times s \approx 1.4 \times s$$

For any effect level of the mixture, the corresponding concentration and 95% CI

can be read from the relevant curve. Since the 95% CI is equal to 1.96 x SD, we can calculate the corresponding standard deviation. Assuming equal variance for the two components, their individual SDs can then be estimated using the formula above. Given that the mixture SD equals 1.4 x the sum of the individual SDs, we were able to estimate SD values for *o,p*'-DDT and *p,p*'-DDE in the mixture. From these values we could then calculate their respective 95% CIs. In order to determine the significance of any deviations from additivity, we plotted mean and 95% CI for both the additivity line and the single agent datapoints. As shown in Figure 3.4, deviations from additivity were deemed to be significant when the confidence intervals for the experimental mixture datapoints did not overlap the confidence belt of the additivity line. Points within the confidence belt of the line of additivity were considered to represent an additive interaction, while points significantly below or above the line indicated synergy and antagonism, respectively.

3.5.4 Analysis using the method of isoboles

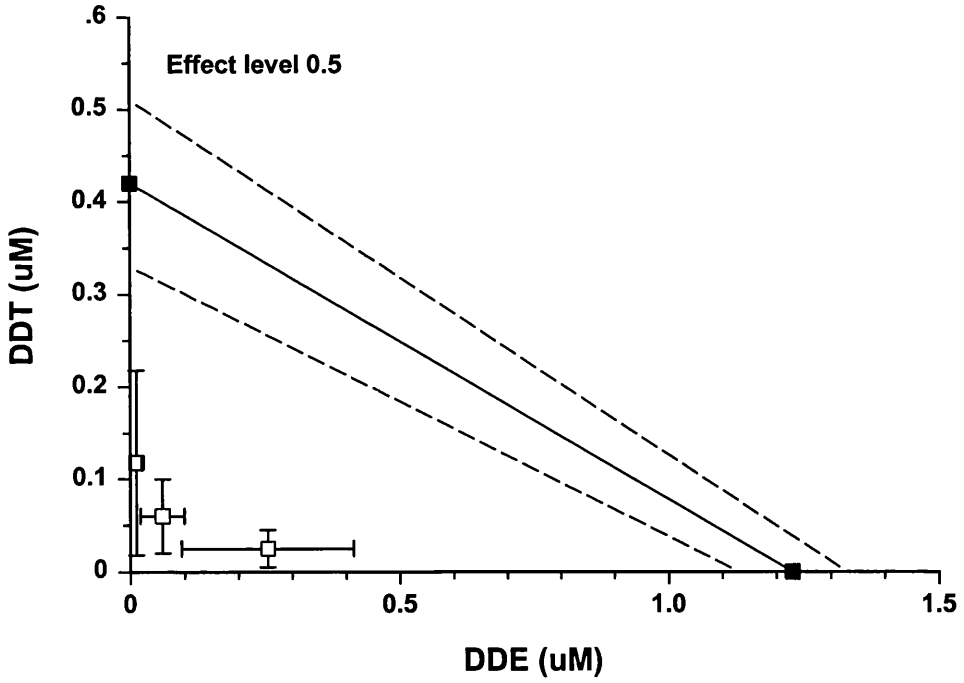
For each effect level set we calculated the corresponding concentration of the mixture for each mixture ratio, and using the ratios were able to determine the concentrations of each single agent present in the mixture. From the positions of these points on the respective graphs we were able to determine if any interaction had occurred between the two agents.

At effect level 2.5 (Figure 3.4), the mixture points for the 10:1 and 1:1 ratios lie within the additive confidence belt and so can be considered to show additivity. However, the 1:10 mixture point lies above the additivity line and is significantly

displaced from it, indicating a weak antagonism.

However, for effect level 1.5 (Figure 3.4), the datapoints for the mixture ratios10:1 and 1:1 lie slightly, but significantly below the additivity line, which can be interpreted as a weak synergy. This interaction is not observed with the 1:10 mixture which overlaps the additivity belt. At this mixture ratio, the CIs for individual agents are smaller than at effect level 0.5 or 2.5 as effect level 1.5 lies on the linear, exponential part of the mixture curves where the CI is narrowest.

As with effect level 1.5, for effect level 0.5 (Figure 3.4) all three mixture points lie below the line of additivity, however, there is no overlap of confidence intervals at any mixture ratio. This would suggest that there is another potential weak synergy at this effect level.



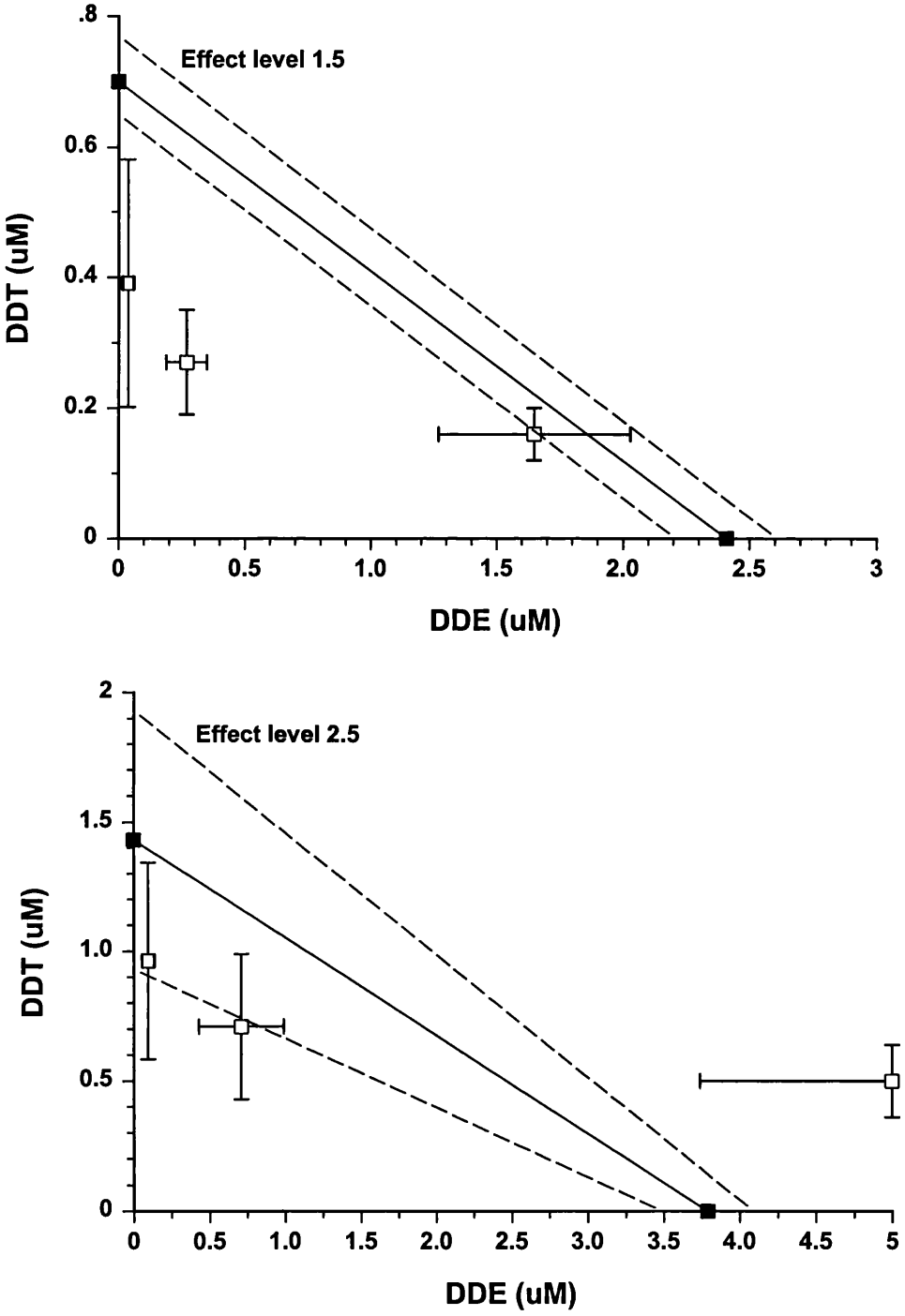


Figure 3.4 Construction of isoboles. Isobolograms were plotted for effect levels 0.5, 1.5 and 2.5 utilising concentration-response data from figure 3.3. Isobole shown as solid line with 95% CI (dashed). Mixture points (□) shown with 95% CI estimates.

It becomes apparent from the above that utilising the method of isoboles requires the construction of separate figures for every effect level studied. Ideally, we need a way of directly comparing observed mixture concentration-response relationships with an additivity prediction also in the form of a concentration-response relationship. By re-arranging the equation for CA, we would be able to represent complete concentration-response relationships for the observed and predicted data on the same graph. By adopting a new approach to the analysis, we reduce the number of graphs that need generating. With information on the concentration-response relationships of individual agents it is theoretically possible to generate predictions of the concentration-response relationship for a mixture containing any number of agents at any mixture ratio. Such predictions can then be compared with experimental observations of that mixture.

3.6 CA can be used to generate predictions of full concentration-response relationships

Assuming that a mixture acts additively, then, for a given effect level the following formula for binary mixture action applies:

$$\frac{X_1}{EC_{y1}} + \frac{X_2}{EC_{y2}} = 1 \qquad (1)$$

where X_i denotes the concentration of an agent (i) in the mixture and EC, the concentration of an agent producing the same effect, y, as the mixture. To utilise this formula for computing concentration-response curves, we must express X_2 in terms of X_1.

Cross multiplying gives:

$$\frac{X_1.EC_{y1} + X_2.EC_{y2}}{EC_{y1}.EC_{y2}} = 1 \qquad (2)$$

In order to express X_2 in terms of X_1 we define X_K , the total concentration of the mixture:

$$X_1 + X_2 = X_K \qquad (3)$$

The fractions of X_1 and X_2 in the mixture can now be defined as:

$$\frac{X_1}{X_K} = a_1 \quad \text{and} \quad \frac{X_2}{X_K} = a_2 \qquad (4)$$

Resolving for X_2 and substituting X_K gives :

$$X_2 = a_2 . X_K = a_2 . \frac{X_1}{a_1} \qquad (5)$$

Substituting into equation (2) yields:

$$\frac{X_1.EC_{y2} + X_1(a_2/a_1).EC_{y1}}{EC_{y1}.EC_{y2}} = 1 \qquad (6)$$

Factoring out X_1:

$$\frac{X_1 (EC_{y2} + (a_2/a_1. EC_{y1}))}{EC_{y1} . EC_{y2}} = 1 \qquad (7)$$

Therefore:

$$X_1 = \frac{EC_{y1} \cdot EC_{y2}}{EC_{y2} + (a_2/a_1).EC_{y1}} \quad (8)$$

Since $X_K = X_1 / a_1$

$$X_K = \frac{EC_{y1} \cdot EC_{y2}}{a_1.EC_{y2} + a_2.EC_{y1}} = \frac{1}{(a_1/EC_{y1})+(a_2/EC_{y2})} \quad (9)$$

This can then be generalised for any number (i) of components

$$X_K = \frac{1}{\sum_i (a_i / EC_{yi})} \quad (10)$$

Where: X_K = Mixture concentration producing effect y

a_i = Fraction of agent (i) in the mixture

EC_{yi} = Concentration of single agent producing predetermined effect y

Expression (7) allows us to calculate the total concentration of agents in a given mixture that is needed to elicit an effect y, provided the mixture composition and the concentrations of the single agents required to produce effect y are known. Iterative calculations of effect concentration for a range of effect levels then yields concentration-response curves.

In order to facilitate rapid calculation of CA predictions, spreadsheets were constructed (Figure 3.5). Predicted relationships could then be plotted as log concentration-response curves and could be compared with responses measured experimentally.

For CA, twenty evenly-spaced effect levels were selected, and the corresponding

mixture concentrations were predicted. For each level we determined the concentration of each single agent which individually produced that effect. These values were then expressed relative to that agent's prevalence in the mixture (fraction of mixture / effect concentration) for each agent. The sum of these fractional concentrations was then inverted to yield the desired mixture concentration (see equation 10).

For example, to determine the concentration of the equimolar mixture which elicited an effect level of 2.68, we first calculated the concentrations of the single agents which alone elicited that effect (EC). For *o,p'*-DDT and *p,p'*-DDE these values were 3.5670 and 4.1171. The next step was to divide each agent's mixture fraction by its corresponding EC and add these values together. For set effect 2.68 this gave us a value of 0.2616. By then inverting this sum we obtained the total mixture concentration (in μM) which elicited the predetermined effect, 3.822. By repeating this procedure for a number of effect levels we were able to construct a full concentration-response relationship for a mixture assuming that the constituents act additively. This can then be compared with observed mixture responses to determine the nature of the combination effect. If the observed data closely matches the prediction then additivity can be assumed, while a shift to the left in the observed data would indicate synergy. ie. effects are elicited with lower concentrations of the mixture than predicted. Similarly, a shift to the right in the observations would lead us to conclude antagonism, with lower effects being produced with a given mixture concentration.

DR-Model
E=Min+(Max-Min)/(1+((X/X50)^-P))

C=X50*(1/((Max-Min)/(E-Min)-1))^(1/p)

Parameters	Agent 1	Agent 2
Substance	**o,p'-DDT**	**p,p'-DDE**
X50	0.655	3.284
P	3.30	2.08
Max	2.69	4.36
Min	0	0
Fraction in the mix	0.5	0.5

modifying factor

0.13

set effect	**total conc**	sum of fract/EC	Concentration for single agent with that effect (EC)	
2.68	3.822	0.261617121	3.5670	4.1171
2.55	2.244	0.445544576	1.5792	3.8778
2.42	1.890	0.52922471	1.2739	3.6570
2.29	1.682	0.594472609	1.1121	3.4515
2.16	1.534	0.651815833	1.0032	3.2587
2.03	1.418	0.705271814	0.9212	3.0765
1.9	1.321	0.75697964	0.8551	2.9031
1.77	1.237	0.808352879	0.7991	2.7370
1.64	1.162	0.860504826	0.7502	2.5769
1.51	1.094	0.914456012	0.7063	2.4214
1.38	1.030	0.971269857	0.6658	2.2695
1.25	0.969	1.032175594	0.6279	2.1199
1.12	0.910	1.098717295	0.5916	1.9715
0.99	0.853	1.172974692	0.5564	1.8231
0.86	0.795	1.257936143	0.5213	1.6729
0.73	0.736	1.35819683	0.4859	1.5192
0.6	0.675	1.48141196	0.4490	1.3592
0.47	0.609	1.641749971	0.4094	1.1889
0.34	0.535	1.869801944	0.3648	1.0014
0.21	0.444	2.251312515	0.3102	0.7822
0.08	0.310	3.225534066	0.2280	0.4844

Figure 3.5 Spreadsheet layout for generating predictions using concentration addition.

3.6.1 Predictions of binary mixtures: A re-evaluation of the method of isoboles

With the ability to generate predictions of complete concentration-response relationships using the formula for CA, deviations from this prediction were compared with observations for the binary mixtures of *o,p'*-DDT and *p,p'*-DDE which had previously been assessed using the method of isoboles (Figure 3.6).

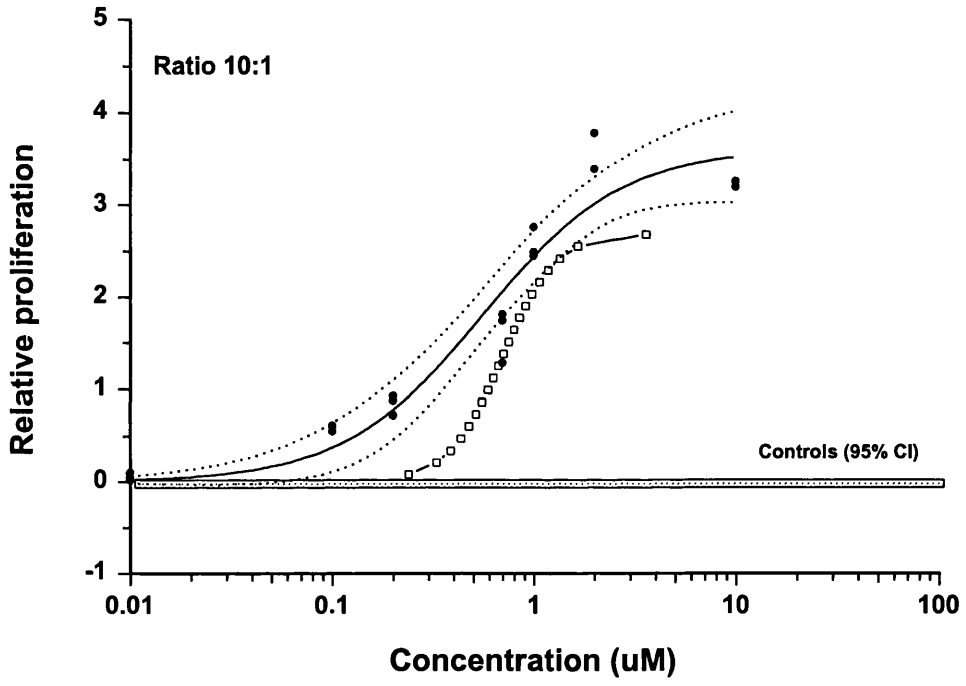
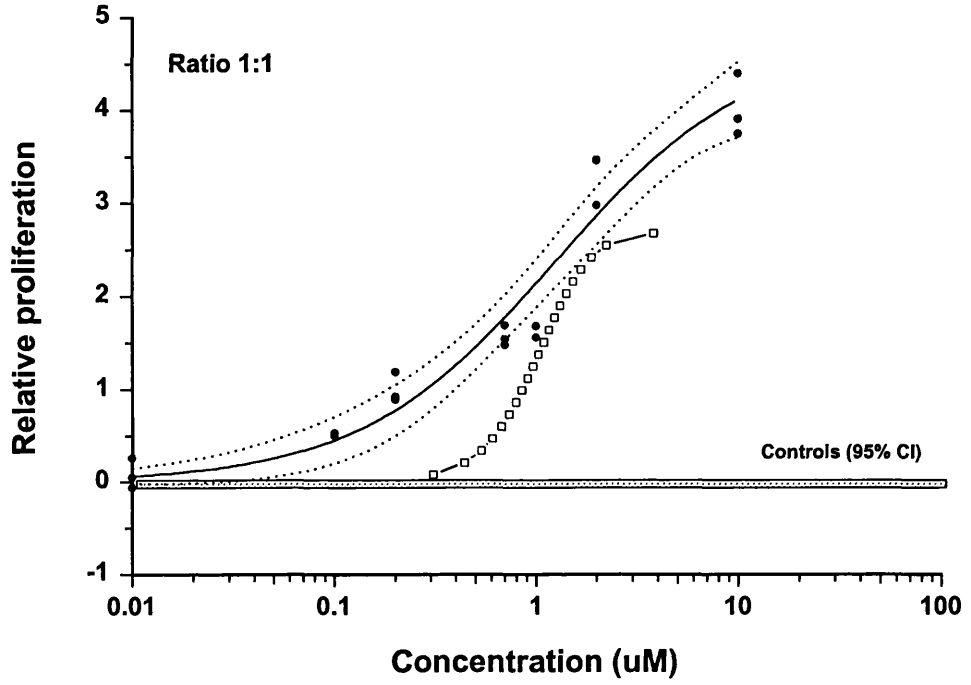
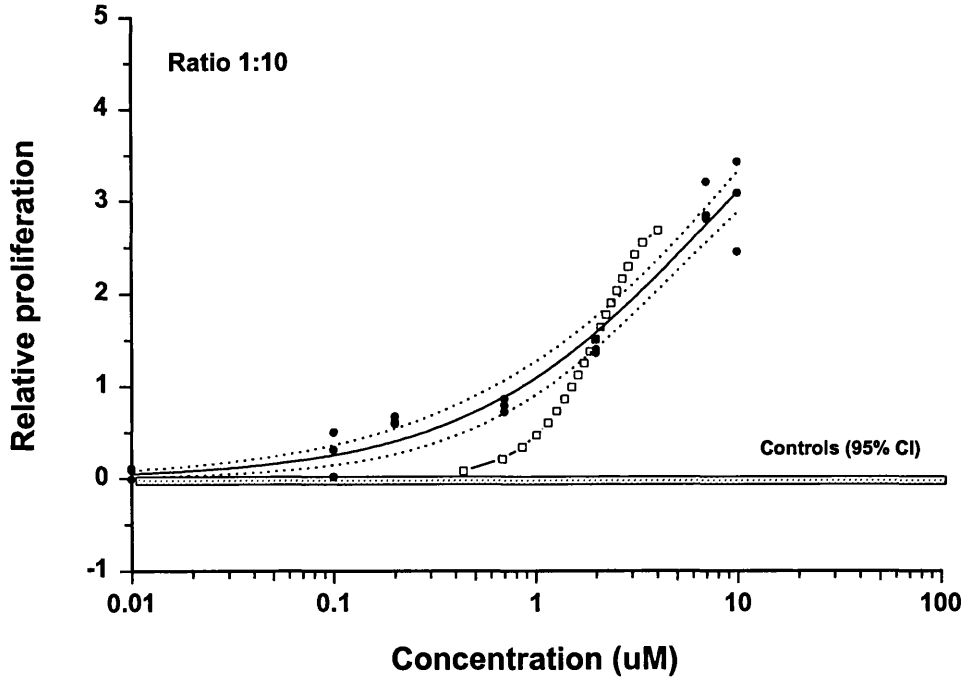


Figure 3.6 A comparison of observed data with model predictions for binary mixtures of *o,p'*-DDT and *p,p'*-DDE. Experimental data (●) for mixture ratios 10:1, 1:1 and 1:10 was compared with predictions calculated for using the model of CA (□).


Ratio 1:1
Relative proliferation
Controls (95% CI)
Concentration (uM)
5
4
3
2
1
0
-1
0.01
0.1
1
10
100


Ratio 1:10
Relative proliferation
Controls (95% CI)
Concentration (uM)
5
4
3
2
1
0
-1
0.01
0.1
1
10
100

From the parameters of the single agent concentration-response curves we computed prediction curves based upon the models of concentration addition for each of the three mixtures. These were then compared with the original mixture curves, which allowed us to examine the reason that synergy had been observed (Figure 3.6). It is clear that at low concentrations, all three experimental mixture curves exhibit a shift to the left, i.e. greater effects are observed than would be predicted using CA, however, at higher concentrations the agreement between the observed and predicted data improved considerably, indicating additivity.

3.7 Discussion

As we demonstrate, the method of effect summation may be unsatisfactory for the analysis of mixture effects when using data from endocrine disrupters. The dose-response curves generated by our agents in the MCF-7 cell proliferation assay are neither linear nor do they pass through the zero origin. This prompted us to investigate alternative methods of examining binary mixture effects.

Initially we constructed concentration-response relationships for the two estrogen-like agents *o,p'*-DDT and *p,p'*-DDE. From these graphs it was then possible to construct additivity isoboles for a number of effect levels, predicting the behaviour of mixtures of the two chemicals. Using experimentally generated mixture data *for o,p'*-DDT and *p,p'*-DDE, we were able to determine the concentrations of each in the mixture which produced the given effect levels. Here we observed that at high effect levels, the response is additive and in one case antagonistic, yet at low effect levels there is apparently a weak synergy. This observed deviation from additivity is certainly of

interest and merits further investigation. However, it should be noted that the binary data were generated from only a single experiment, so the observations may simply be attributed to inherent variability. Alternatively the deviations from additivity may come from a systematic error in mixture production, or the presence of impurities.

In addition, we observed that the largest deviations from additivity are found with the equimolar mixtures, while those with more DDE than DDT elicit the least deviation. We note that both the mixture ratio and the effect levels chosen strongly influence the type of response obtained. The observation of additivity at one mixture ratio does not necessarily hold true for other mixtures of the same chemicals. This again highlights the need to test several mixture ratios at a number of effect levels, making this form of analysis fairly labourious.

Although the method of isoboles is a useful tool for analysing deviations from additivity in binary mixtures, we became interested in the possibility of predicting entire dose-response relationships for the mixtures. This would allow us to directly compare the additivity predictions with observed data on the same graph. To achieve this, we rearranged the formula for CA, allowing us to plot predictions on a traditional log concentration-response curve. Here, we have used this modification to carry out comparisons of our observed mixture data with predictions using the data from the binary mixture experiments. As we show, at low concentrations the experimental mixture data show significant deviation from the CA prediction curve with all three mixtures. This observation, that experimental responses are greater than predicted, is consistent with synergy and requires further study.

Analysis of mixture effects with environmental estrogens has powerful

ramifications for risk assessment and as such it requires a solid methodological basis. This clearly requires further investigation to determine the validity of the models using other agents, more complex mixtures and different test systems.

3.8 Conclusions

Consequently, we propose that the method of isoboles is a more accurate tool than effect summation for analysing binary mixture effects with xenoestrogens. However, owing to the large numbers of potentially estrogenic agents to which we are exposed, we require mathematical concepts capable of dealing with mixtures containing large numbers of components. CA is an example of such a concept, and it will be interesting to see how it performs with mixtures containing more than two components. In addition, it would be helpful to compare CA with the model of independent action (IA), These points will be addressed in Chapter 4.

Chapter 4

Assessment of the effects of a four-component mixture in the MCF-7 ceil proliferation assay

4.1 Abstract

In order to address one of the issues of combination effects we demonstrate, for the first time, the application of the well documented models of concentration addition and independent action to the analysis of xenoestrogen mixtures. Four recognised endocrine disrupters (EDs), *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDE and β-HCH were selected and concentration-response relationships recorded using the MCF-7 cell proliferation assay. From these observations we were able to construct predictions of four-component mixture response for the two models. The two mixtures were then tested experimentally and compared with predictions. The observed data fitted the concentration addition prediction excellently, demonstrating an additive interaction between the four agents regardless of mixture ratio. However, independent action overestimated effects at high concentrations. In addition we highlight the ability of EDs to produce mixture effects when present at individually ineffective concentrations. These findings are of clear significance for epidemiological study design, and the information obtained should allow more confident assessment of the risk posed by xenoestrogens.

4.2 Introduction

The determination of mixture effects relies completely on the generation of a reliable reference point for mixture behaviour. This is usually a prediction of additivity. Observed deviations from this reference point can then be classified either as synergy (greater effect than predicted) or antagonism (lower effects). Over the last century, two suitable concepts have been developed, concentration addition (CA) and independent action (IA). The former is considered the most appropriate for the study of agents with a similar mode of action, while the latter is more appropriate for agents with differing activities. The concepts of CA and IA were developed independently to suit different experimental contexts and there are no rational criteria for choosing between them. Nevertheless, both models are currently regarded as equally valid reference points for predicting the effects of mixtures of chemicals (Greco et al. 1995). In addition, since they function independently of the profile of the individual curves, these models do not suffer the constraints of effect summation. One major benefit of these concepts is that they can be adapted to plot mixture effect predictions based upon additivity as a traditional log concentration-response curve, allowing a direct comparison with observed mixture data (Faust et al. in press).

Here we will examine the ability of the two concepts to model the behaviour of a four component mixture of estrogen-like agents in the MCF-7 cell proliferation assay. The four compounds, *o,p'*-DDT, *p,p'*-DDE, *p,p'*-DDT and β-HCH were selected on the basis of their estrogen-like activities and high prevalence in human adipose tissue. Also of interest is their differing mechanisms of action, the two DDT isomers act via ER (Chen et al. 1997), while *p,p'*-DDE is primarily an androgen receptor antagonist (Kelce

et al. 1995), and β-HCH acts through unknown mechanism distinct from that of E2 (Steinmetz et al. 1996). This has considerable implications for the understanding of similarity, where there is still no consensus in the field. Berenbaum (1985) proposes CA to be the "general solution" to mixture effects, while more recently, Pöch (1993) has claimed it is only applicable to agents acting at an identical molecular acceptor site. Despite the similarity of endpoint in the MCF-7 assay (proliferation), the mechanisms of action may be considered to be dissimilar. This may therefore highlight differences between the two model concepts.

4.2.1 Independent action

The model of independent action (also called response addition) can be traced back to the work of Bliss (1939) and was developed on the basis of stochastic considerations. It is thought to be more applicable to chemicals that have "different" sites or modes of action within an organism or test system. Although as with CA, the understanding of "different" is not always clear, especially with reference to endpoints such as cell death.

The general formula for any number of components is given by:

$$E(C_{1,2,\ldots n}) = 1 - \prod_{i=1}^{n} [1 - E(C_i)]$$

Where C represents the concentrations of a number of chemicals (i) applied, and E, the corresponding effect. When used for four components, the formula simplifies to:

$$E(C_1 + C_2 + C_3 + C_4) = 1 - ((1 - EC_1)(1 - EC_2)(1 - EC_3)(1 - EC_4))$$

Effect concentrations (EC) are in the form of the fractional effect of that agent in the mixture relative to the maximal achievable effect giving a range of 0 - 1.0. In our case fractional effects were calculated relative to the maximal effect of estradiol, so as the effect for a given concentration of X approached the E2 maximum, EC_x approaches 1.0. Consequently the multiplication term tends towards zero in the above equation. In that case the product of the four multiplication terms will also tend towards zero, yielding a final sum of almost 1.0. Conversely low ECs will yield a final product tending towards zero.

Recently, this model has been used to determine the predictability of multiple chemical mixture toxicity in the marine bacterium *vibrio fischeri* (Backhaus et al. 2000). Here, IA was found to be an excellent predictor of effect with a mixture of 14 dissimilarly acting chemicals.

4.3 Objectives

Here, for the first time, we report effects of four-component mixtures of endocrine disrupters and predict effects for the entire concentration-response curve. This will allow us to evaluate the performance of each model in our test system. In addition we explore the relevance of mixture effects for mixtures where each compound is present at low effect concentrations.

We have used the MCF-7 cell proliferation assay to record comprehensive concentration-response relationships for the endocrine disrupters *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDE and β-HCH, both as single agents and in combination, using the fixed mixture ratio design. i.e. a mixture if the agents is prepared at a predetermined ratio, and

subsequently diluted to yield a range of concentrations. In this way, the relative proportion of each agent remains constant regardless of dilution. Experimental observations of mixture effect were then compared with the additivity predictions using the two aforementioned models.

4.4 Methods

4.4.1 Measurement of cell proliferation in response to test compounds

Cells were maintained as previously reported, and assays were carried out according to our improved protocol (Chapter 2).

o,p'-DDT (Lancaster), *p,p*'-DDT and *p,p*'-DDE (Sigma), and β-HCH (J.T.Baker) were made up to 1 mM in absolute ethanol, diluted and administered so that the final ethanol concentration was 1% in the media. Concentrations greater than 1 mM were not made, owing to concerns regarding solubility. Mixtures were prepared from 1mM stocks of each agent such that dilutions took no more than three steps.

4.5 Results

4.5.1 Single agent concentration-response relationships

In order to study the predictability of more complex, four-component mixtures, we recorded concentration-response relationships for two additional EDs, *p,p*'-DDT and β-HCH (Figure 4.1). Because of concerns regarding limited compound solubility, we could not generate maximal responses with these two agents. However, assuming that their respective maxima were no greater than that of E2, their EC_{50} values were estimated to be 12.3 μM and 4.6 μM respectively, from their respective Hill function

parameters. Unfortunately, a lack of thorough concentration-response analysis in the literature precluded us from drawing direct comparisons between our data and previous reports. However, it is apparent that our observations agree well with data reported by Andersen et al. (1999) with regard to EC_{50} values.

These data, together with those of *o,p'*-DDT and *p,p'*-DDE allowed us to calculate predictions for four-component mixtures.

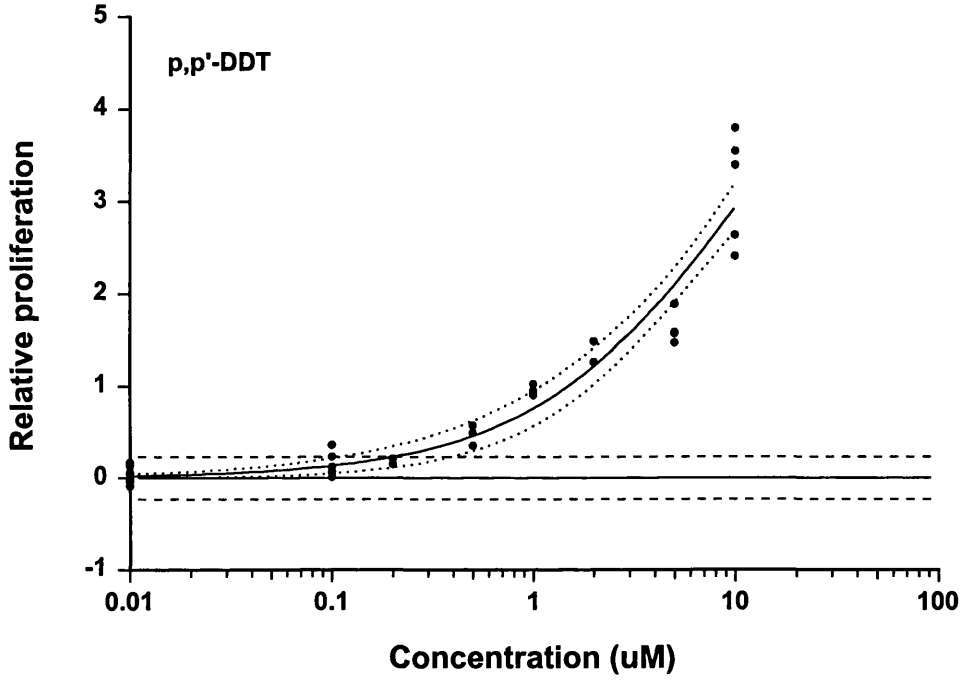
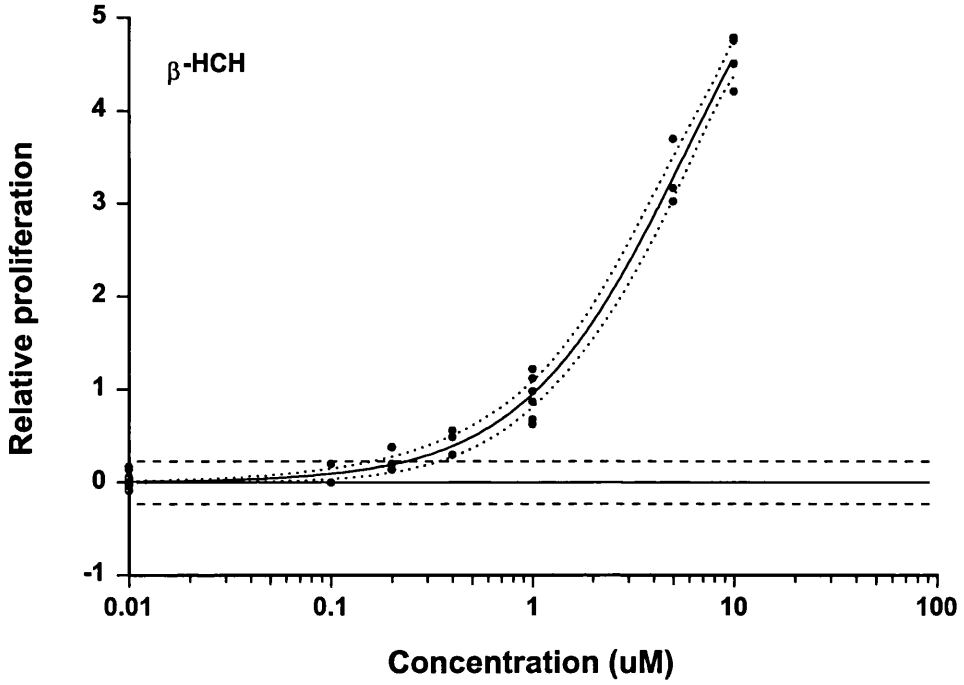


Figure 4.1 Concentration-response relationships for *p,p'*-DDT and β-HCH. Concentration-response relationships for *p,p'*-DDT and β-HCH generated using our improved MCF-7 protocol. For details see Figure 3.3, β-HCH graph is as shown on p. 62. Dotted lines represent 95% CI for curve fit, dashed lines show 3 x SD of controls.



4.5.2 Curve fitting and generation of predictions

Concentration-response relationships for single agents and mixtures were recorded and fitted to the asymmetric Hill function using the graphical package Fig. P. This model was selected due to its pharmacological usage for ligand-receptor interactions, and was found to yield better fits than with logistic sigmoid or statistical sigmoid dosimetric models.

In order to facilitate rapid calculation of IA and CA curve predictions, appropriate spreadsheets were constructed. For CA, the spreadsheet shown in Chapter 3 was modified to deal with four components, while for IA, a new layout was required (see Figure 4.2 for explanation). For each model, predicted relationships could then be plotted as log concentration-response curves for direct comparison with the observed

mixture data.

For IA, expected effects were generated for twenty evenly-spaced mixture concentrations. With a knowledge of the mixture ratio, we calculated the concentration of the individual components at the given mixture concentration. From this we determined the corresponding effects of those agents from their respective best fits to the Hill function. These were then expressed as fractions of the theoretical maximal response. These fractional effects were then used to generate the fractional expected combined effect using the formula given (F_x is the fractional effect of that agent in the mixture):

$$1-((1-F_{opDDT})*(1-F_{ppDDT})*(1-F_{ppDDE})*(1-F_{\beta HCH}))$$

Values were then re-scaled relative to E2 such that a final sum of 1.0 would correspond to an effect level of 6.4, the maximal response to E2 in this test system, or a sum of 0.5 would correspond to effect level 3.2.

Figure 4.2 Spreadsheet layout for independent action predictions, shown overleaf.

IA-Model

E = M i n + (M a x -

Min)/(1+((X/X50)^-P))

Parameters	Agent 1	Agent 2	Agent 3	Agent 4
Substance	**op'-DDT**	**pp'-DDE**	**pp'DDT**	**HCH**
X50	0.66	3.28	12.27	4.63
P	3.31	2.08	0.8	1.15
Max	2.69	4.36	6.4	6.4
Min	0	0	0	0
Fraction in mix	0.25	0.25	0.25	0.25

dilution factor: 1.3

Total conc of mix:

	expected effect	**concentration of mix agents**				**expected combined effect**	
						fractional	rescaled
10	4.92	2.50	2.50	2.50	2.50	0.7695	4.9246
7.69	4.52	1.92	1.92	1.92	1.92	0.7062	4.5199
5.92	4.10	1.48	1.48	1.48	1.48	0.6403	4.0978
4.55	3.63	1.14	1.14	1.14	1.14	0.5676	3.6329
3.50	3.07	0.88	0.88	0.88	0.88	0.4794	3.0678
2.69	2.39	0.67	0.67	0.67	0.67	0.3727	2.3854
2.07	1.70	0.52	0.52	0.52	0.52	0.2649	1.6955
1.59	1.15	0.40	0.40	0.40	0.40	0.1793	1.1475
1.23	0.78	0.31	0.31	0.31	0.31	0.1220	0.7811
0.94	0.55	0.24	0.24	0.24	0.24	0.0860	0.5505
0.73	0.40	0.18	0.18	0.18	0.18	0.0629	0.4027
0.56	0.30	0.14	0.14	0.14	0.14	0.0474	0.3031
0.43	0.23	0.11	0.11	0.11	0.11	0.0363	0.2324
0.33	0.18	0.08	0.08	0.08	0.08	0.0282	0.1803
0.25	0.14	0.06	0.06	0.06	0.06	0.0220	0.1409
0.20	0.11	0.05	0.05	0.05	0.05	0.0173	0.1107
0.15	0.09	0.04	0.04	0.04	0.04	0.0136	0.0872
0.12	0.07	0.03	0.03	0.03	0.03	0.0108	0.0689
0.09	0.05	0.02	0.02	0.02	0.02	0.0085	0.0546
0.07	0.04	0.02	0.02	0.02	0.02	0.0068	0.0433
0.05	0.03	0.01	0.01	0.01	0.01	0.0054	0.0344

maximal effect
reference: estradiol
max. effect: 6.40

op'-DDT	pp'-DDE	pp'DDT	HCH	op'-DDT	pp'-DDE	pp'DDT	HCH
effect of single agents at conc in mix				fractional effects of mix agent			
2.6576	1.5802	1.4003	2.1113	0.4153	0.2469	0.2188	0.3299
2.6141	1.0803	1.1842	1.7081	0.4085	0.1688	0.1850	0.2669
2.5160	0.6988	0.9949	1.3576	0.3931	0.1092	0.1555	0.2121
2.3094	0.4341	0.8310	1.0627	0.3608	0.0678	0.1298	0.1661
1.9314	0.2625	0.6906	0.8215	0.3018	0.0410	0.1079	0.1284
1.3895	0.1561	0.5716	0.6285	0.2171	0.0244	0.0893	0.0982
0.8327	0.0918	0.4713	0.4770	0.1301	0.0143	0.0736	0.0745
0.4259	0.0537	0.3875	0.3598	0.0665	0.0084	0.0605	0.0562
0.1968	0.0313	0.3178	0.2700	0.0308	0.0049	0.0497	0.0422
0.0862	0.0182	0.2600	0.2019	0.0135	0.0028	0.0406	0.0315
0.0369	0.0105	0.2124	0.1506	0.0058	0.0016	0.0332	0.0235
0.0156	0.0061	0.1733	0.1120	0.0024	0.0010	0.0271	0.0175
0.0066	0.0035	0.1412	0.0832	0.0010	0.0006	0.0221	0.0130
0.0028	0.0021	0.1150	0.0618	0.0004	0.0003	0.0180	0.0097
0.0012	0.0012	0.0935	0.0458	0.0002	0.0002	0.0146	0.0072
0.0005	0.0007	0.0760	0.0339	0.0001	0.0001	0.0119	0.0053
0.0002	0.0004	0.0618	0.0251	0.0000	0.0001	0.0097	0.0039
0.0001	0.0002	0.0502	0.0186	0.0000	0.0000	0.0078	0.0029
0.0000	0.0001	0.0407	0.0138	0.0000	0.0000	0.0064	0.0022
0.0000	0.0001	0.0331	0.0102	0.0000	0.0000	0.0052	0.0016
0.0000	0.0000	0.0268	0.0075	0.0000	0.0000	0.0042	0.0012

For example, in the spreadsheet illustrated we have an equimolar mixture of the four agents, with their respective Hill function parameters and mixture prevalence marked in the boxed area. In order to predict response, we first set the highest concentration with which to calculate, in this case 10 μM. With the equimolar mixture, the concentrations of each component (2.5 μM) can be viewed in the subsequent columns. For each agent we then calculate the effect it would individually elicit at that concentration, e.g. with 2.5 μM *o,p'*-DDT we would obtain a proliferation of 2.6576. By expressing this as a fraction of the maximum achievable response (E2 = 6.4, see 4.2.1 for explanation) we obtain a fractional effect for *o,p'*-DDT of 0.4153. By repeating this procedure for each component we can obtain the sum of fractional effects when the total concentration of the mixture is 10 μM. This sum is found to be 0.7695, which when multiplied by 6.4 yields a proliferation prediction of 4.92. This process is then repeated for a number of different mixture concentrations, to yield a predicted concentration-response curve.

4.5.3 Mixture responses and comparison with predictions

To test the applicability of the models CA and IA, predictions were tested experimentally. Two different combinations were investigated, one equimolar and another reflecting the prevalence of the agents in human blood (Toppari et al. 1996), 1:4:5:10 of *o,p'*-DDT, *p,p'*-DDT, β-HCH and *p,p'*-DDE, respectively (Figure 4.3, see chapter 6 for quantification of agents in man). The observed data were then compared with the two additivity models, and the quality of the prediction assessed.

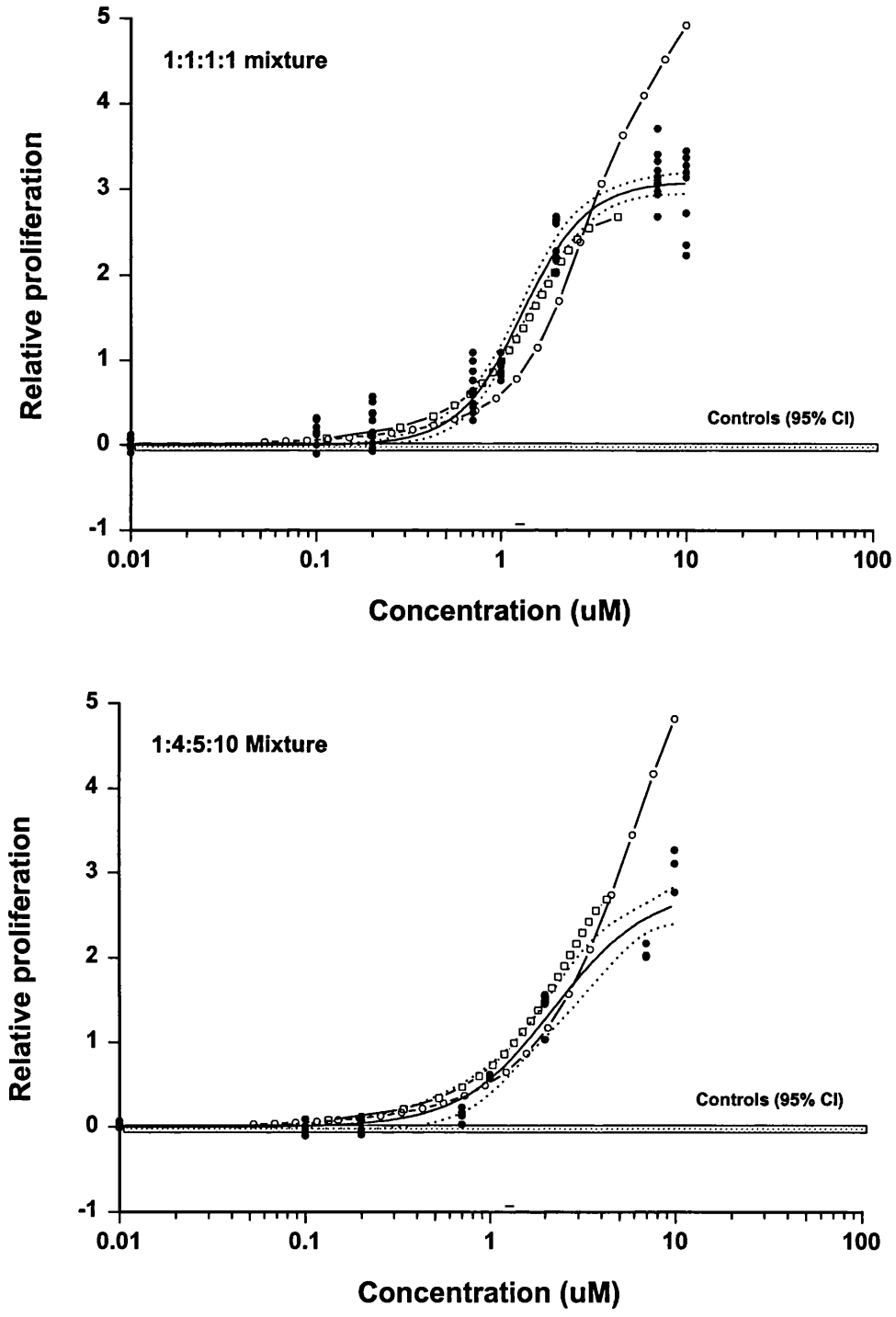


Figure 4.3 Experimental mixture data can be directly compared with predictions using IA and CA. Experimental concentration-response relationships (●) were generated in the MCF-7 cell proliferation assay for the mixture ratios 1:1:1:1 and 1:4:5:10, and fitted to the asymmetric Hill function (solid line) with 95% CI (dashed line). Using the single agent curve parameters, IA (○) and CA (□) predictions were calculated.

As an assessment criterion, we examined the degree to which the observed data overlapped with the 95% CI of the predicted fit. Assessments in relation to the model predictions showed that the combination effects of the mixtures did not deviate significantly from the additivity expectations of the models CA and IA (Fig 4.3). Applying the overlap criterion between the prediction curves and the confidence belts of the best fit to observed effects, no marked deviations could be identified. However, both models slightly overestimated the effects of the 1:4:5:10 mixture in the low effect range. At maximum effect concentrations the observed mixture responses began to plateau off, a feature modelled well by CA for the equimolar mixture. With both mixtures the performance of IA was poor in this effect range.

Of the two models, CA predicted effects which more closely matched the experimental data and indicated that the agents selected act additively in our test system. However, we were unable to generate a complete concentration-response curve for CA, because the highest predictable effect level cannot be greater than the maximal response of the weakest mixture component. Generally, both models agreed well with observed data.

4.5.4 Mixture effects at low effect concentrations

Figure 4.4 shows responses elicited by low concentrations of the test agents individually and compares these to effects seen with mixtures. A concentration of 1 μM was selected as this is the lowest mixture concentration at which individual agents were reasonably expected to elicit an effect if administered singly at their concentration in the mixture. At 1 μM the equimolar mixture produces a relative proliferation of 1.04. The

concentration of each agent in that mixture is 250 nM and it is therefore possible to determine, from the single agent curves, the effect produced by each chemical alone.

Under these conditions, the effects produced by such low concentrations of the test compounds are in some cases within the control variability, and so probably could not be detected in the assay. Yet, as we show, the failure to observe an effect with single agents does not preclude one existing in the mixture.

For comparison we also show the result that would be predicted by the model of effect summation (ES), where the prediction of the mixture effect equals the sum of the effects of the individual components. This predicts a mixture response of 0.54 at 1 μM, lower than the observed data, which would result in an incorrect assumption of synergy, however, CA and IA clearly predict additivity. For the purposes of these graphs, we were interested in determining whether the responses to low concentrations of agents would be distinguishable from background "noise". To achieve this, we set a threshold of 3xSD of control values, in line with the methods of Veritt and Parry (1984). They state that increases of 1.5-fold over the control value are rarely considered biologically significant, whereas increases of more than 3-fold will rarely be considered insignificant.

Similar observations can be made with the 1:4:5:10 mix, with test agents again present singly at concentrations within 3 x SD of control values. Here, we also observe that individually, test agents eliciting low effects can act additively to produce measurable responses. Again, ES underestimates the observed mixture effect, which could lead one to wrongly conclude synergy.

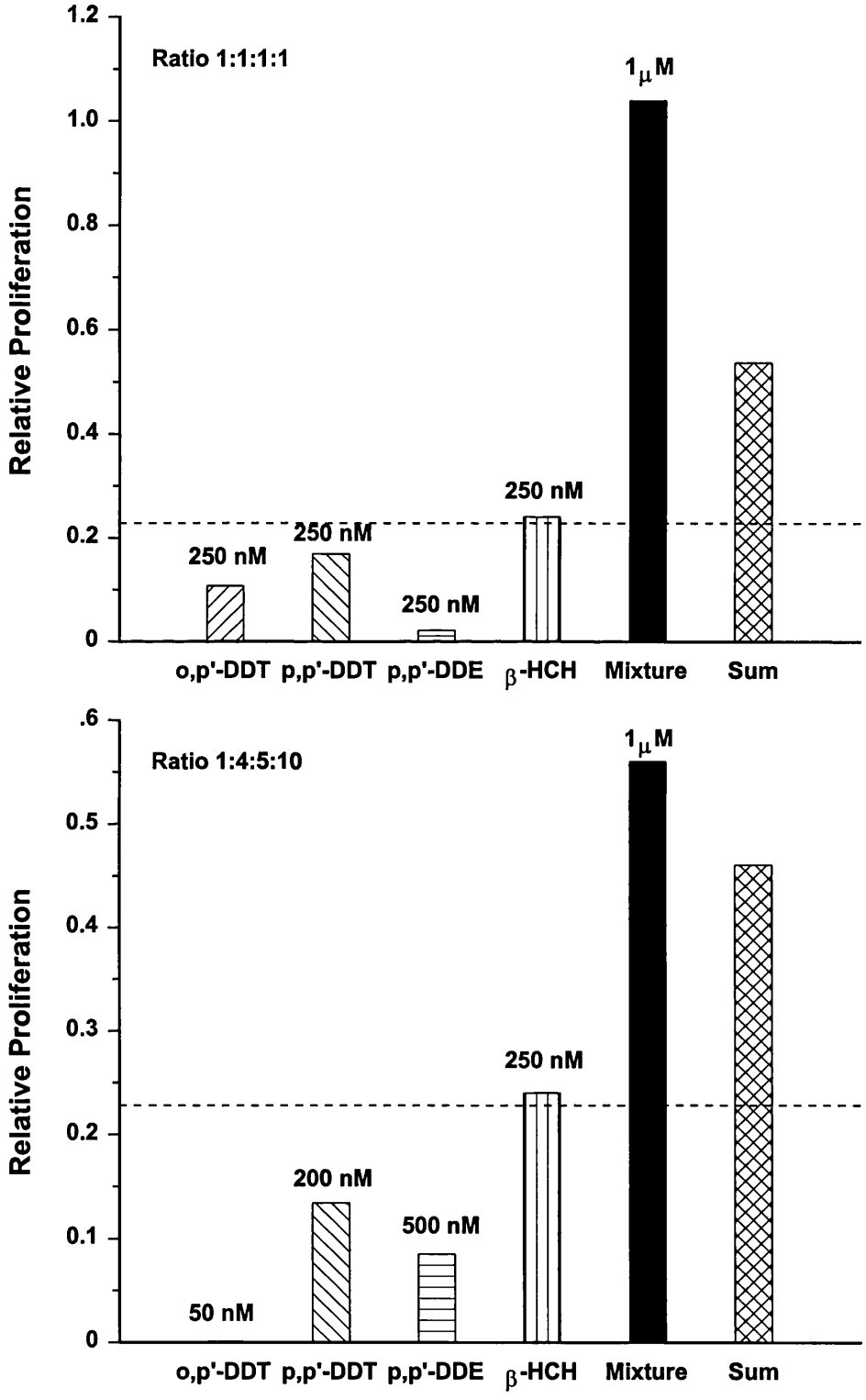


Figure 4.4 Mixture effects can be observed at low concentrations of agents. For each mixture, the response elicited at a concentration of 1 μM is shown and compared with the response for each agent singly at its concentration in the mix. In addition we show the response prediction generated by the model of effect summation. Dashed line indicates upper limit of 3 x SD of controls.

4.6 Discussion

Until now, nobody has been able to successfully predict the effects of four-component mixtures of xenoestrogens. To resolve this problem, we have thoroughly investigated the methods available for the analysis of combination effects which has led us to the long-standing and well validated models of concentration addition and independent action.

Employing the widely-used MCF-7 cell proliferation assay, we assessed the estrogenic effects of four well-documented MCF-7 endocrine disrupters. This was carried out for the single agents and for two- and four-component mixtures. Utilising the Hill function parameters for the single agents, we generated additivity predictions for the mixtures using two model concepts, CA and IA. By representing these predictions as log concentration-response curves it was possible to make direct comparisons with the observed mixture data.

It had previously been shown that the models of IA and CA often produce similar predictions when based upon exponential response curves (Berenbaum 1985). This is certainly the case with our observations, however, in other test systems and with other compounds, the two predictions may well be more distinct.

Having generated predictions for the responses to two four-component mixtures, we examined the quality of the predictions against experimentally generated data. The agreement between observed and predicted data was excellent for both mixtures and indicated that the agents selected acted additively in combination in this test system. This is interesting in the light of the weak synergy observed with binary mixtures of *o,p'*-DDT and *p,p'*-DDE in Chapter 3. It seems unlikely that such an interaction would simply

disappear when the mixture was increased to four agents, and so draws into question the validity of the observed mixture data in Chapter 3. This highlights the need to rigorously repeat experiments in order to be confident of the outcomes.

These observations of additivity are of particular interest in the light of the findings of Shekhar et al. (1997). They report a synergistic interaction of binary mixtures *o,p'*-DDT and *p,p'*-DDT in the MCF-7 cell proliferation assay. In their study, 1 μM *o,p'*-DDT elicited a proliferative increase of 0.75 over controls, while an identical amount of *p,p'*-DDT yielded a response of 1.63. However, when administered as an equimolar mixture, only 200 nM was required to produce an effect of 4.42. Any scenario where an increased response can be obtained by reducing the concentration of agents can only be interpreted as synergy. Although this is in line with our findings in chapter 3, it is not the case with our four-component mixtures and merits further investigation. With the well-documented inter-laboratory variability of the MCF-7 cell assay, it would be of great interest to see what outcomes are obtained by other research groups.

The situation with our test agents is not ideal, the two models are usually applied to mixtures where the individual agents all elicit the same maximal response, however, as is commonly found with xenoestrogens, many are unable to match the proliferative effect of E2 regardless of concentration. It would be of interest to compare predictability when using a mixture of agents with full agonist abilities.

The absence of synergy should not be considered to represent an absence of risk. With multiple chemical exposure it is quite possible that additivity alone between chemicals could produce adverse effects. We have demonstrated that a mixture of four

agents which individually would be considered to have no effect, can in combination elicit a measurable response. This has strong implications for the design of epidemiological studies. Consideration of agents in isolation may lead to gross underestimation of effects. In fact, many studies have so far failed to find associations between breast cancer and xenoestrogens, probably because they focus solely on the impact of single agents (Wolff and Toniolo 1995). Future studies examining total body burden of xenoestrogens may yet reveal strong associations. In addition, assuming the observation of additivity holds true for mixtures of any number of xenoestrogens, it is possible to calculate the required concentration of a mixture that would modulate the response to physiological levels of E2 in our assay. This would in turn shed some light on the health risks posed by these compounds.

Thus, of the two models, CA produced predictions of effects which more closely resembled the experimental data. This is of interest since CA is thought to be most relevant to mixtures of similarly acting chemicals, yet we selected agents possessing different mechanisms of action. One potential explanation for the discrepancy between our observations and the theory behind the two models can be traced back to previous studies on "similarity" of action. In each case the research has focussed on the ability of chemical mixtures to induce cell death. However, we are interested in cell growth, which operates via completely different pathways.

Cell death can result from numerous diverse insults. DNA and protein damage, lipid peroxidation, uncoupling of the electron transport chain and interference with enzyme function can all lead to cell killing. This in turn can occur via different pathways, either by organised cell suicide (apoptosis) or gross pathology leading to necrosis.

Hence, cell death can be the result of a number of divergent pathways.

On the other hand, although cell proliferation can be stimulated through several routes (growth factors, survival factors and steroid hormones), there is only one final common pathway to cell division. This can therefore be seem as a more convergent pathway, and one where similarity of endpoint (cell division) may be more relevant than similarity of chemical mechanisms. This would certainly provide an explanation of why in the MCF-7 assay, agents with differing mechanisms of action are better modelled by CA. These discrepancies clearly require further examination, but it is apparent that the effective application of each model is dependent on not only the test system employed but also the endpoint measured.

4.7 Conclusions

We have shown the model of CA to be an excellent predictor of mixture effects in the MCF-7 cell proliferation assay, and propose its generalised usage within the field of xenoestrogens. We also highlight the problems associated with the model of effect summation and draw attention to the ability of xenoestrogens to contribute to mixture effects at concentrations that singly would produce no effect. Although we have not observed synergy between EDs, we would like to draw attention to the relevance of additivity in risk assessment. It would be of great interest to see whether the additivity observed between four EDs can be reproduced using mixtures containing greater numbers of components.

Chapter 5

Mixtures of xenoestrogens exhibit additivity in the yeast estrogen screen

5.1 Abstract

As we have shown, the accurate prediction of responses to mixtures of xenoestrogens relies heavily upon the appropriate application of mathematical models. Here we investigate the application of the models of concentration addition and independent action to the analysis of mixture behaviour in the yeast estrogen screen. This assay is rapid and highly reproducible, allowing us to screen a large number of potentially estrogenic agents. The greatest responses were obtained with the xenoestrogens *o,p*'-DDT, octylphenol, nonylphenol and genistein. Hence, these were selected for mixture experiments. From the single agent curves it was possible to generate predictions of the behaviour of mixtures of these agents. Experimentally, we tested two-, three- and four-component equimolar mixtures of these agents and compared the observed responses with those predicted. There was excellent agreement between both models and the observed data, however, it was not possible to determine which provided the better fit, since the low maximal response of *o,p*'-DDT makes it impossible to construct a complete concentration-response curve for the model of concentration addition. However, it is interesting to note the quality of the agreement between independent action and the observed data. All four test compounds possess the same mode of action, but the model is more applicable to agents with different mechanisms, questioning the interpretation of "similarity". As far as we are aware, this is the first time these models have been applied in the field of xenoestrogens. Additionally, the observation of additivity is of critical importance for the confident assessment of the risk posed by xenoestrogens.

5.2 Introduction

As we have demonstrated in Chapters 2 and 3, the analysis of mixture effects relies strongly on the sound prediction of mixture action based upon the activity of its individual components. To achieve this we have employed the long-standing and well validated models of concentration addition and independent action to generate additivity predictions for several different mixtures (Berenbaum 1985, Drescher and Boedeker 1995). One feature of CA is its inability to predict effect levels greater than the maximal effect of the mixture constituent with the lowest maximum. Consequently we will screen a number of EDs for those able to elicit the greatest effects.

Previously, the effects of EDs have been assessed in the MCF-7 cell proliferation assay. In order to probe the activity of ED mixtures at a different organisational level, we selected the yeast estrogen screen (YES) for further studies.

YES is a rapid yet sensitive test system (Routledge and Sumpter 1996, Andersen et al. 1999), utilising yeast cells with human estrogen receptor (hER) DNA stably integrated into the main chromosome. Receptor activation is measured via expression plasmids bearing estrogen response elements (ERE) and the reporter gene *lac-Z*. This represents a far simpler pathway between receptor activation and endpoint than in MCF-7 cells. However, this is not without a price, as YES is not always capable of discriminating between agonists and antagonists. For example, the pure antiestrogen ICI 182.780 and the partial antiestrogen tamoxifen both induce increased β-galactosidase activity. In addition, it is unable to detect agents which induce an estrogen-like effect without binding to ER (Andersen et al. 1999).

Endocrine disrupters are structurally diverse and to reflect this we selected

agents from different classes. These were all previously characterised agents (Routledge and Sumpter 1996): the ubiquitous organochloride pesticide *o,p'*-DDT, the phytoestrogen genistein, and two alkylphenols, n-4-octylphenol (OP) and 4-nonylphenol (NP). These were tested both singly and in combination using the yeast estrogen screen (YES). In designing experiments we have opted for the so-called fixed ratio design: Additive mixture effects were computed for equimolar mixtures over the complete effect range and predictions tested experimentally.

5.3 Objectives

In order to examine mixture effects at a different organisational level, we have selected YES for this study. Here we report, for the first time, the activity of four environmental estrogens both singly and in combination, and compared mixture results with the two model predictions. These data can then be compared with that observed in the MCF-7 cell proliferation assay.

5.4 Methods

5.4.1 The recombinant yeast estrogen screen

This assay was carried out as previously reported (Routledge and Sumpter 1996). Briefly, growth medium was inoculated with 125 µl of 10x concentrated stock yeast and incubated at 28°C in an orbital shaker (150 rpm), until an absorbance of 1.0 at 640 nm was obtained. Assay medium was then prepared by adding 0.5 ml of the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG, Boehringer Mannheim) and 2 ml of the yeast culture to 50 ml of fresh growth medium. See

Appendix 1 for full details of media preparation.

Test compounds, *o,p'*-DDT (purity 99+%, Lancaster), *p,p'*-DDT (98%, Sigma), *p,p'*-DDE(99%, Sigma), β-HCH (98%, JT Baker), OP (99%, Aldrich), NP (Technical grade, Aldrich), DEHP (99%, Sigma), BBP (98%, Aldrich) and genistein (98%, Sigma) were prepared as stock solutions in ethanol at 1 mM and stored at -20°C prior to use in the assay. Test agents then diluted in ethanol over a range of concentrations, and aliquots of 10 µl of each concentration were then transferred to 96-well optically flat bottom microtiter plates and allowed to evaporate to dryness. Equimolar mixtures of the four agents were also prepared by combining equal volumes of their respective 1 mM stocks, diluted and assayed as outlined.

Aliquots (200 µl) of the seeded assay medium were then added to the wells. Each plate also contained one row of ethanol controls (assay medium without test compounds), and in each experiment E2 positive controls and blanks (unseeded assay medium) were also run. Owing to the ability of OP and NP to permeate the plastic of the plate, empty wells were left between differing concentrations of these agents.

Prepared plates were sealed with autoclave tape and shaken vigorously for 2 min on a titer plate shaker. They were then incubated at 32°C in a humidified box for 72 h. Plates were again shaken at 24h and at the end of the experiment. After the final shake, plates were left to stand for 60 min before spectrophotometric analysis at 540 nm (colour change) and 620 nm (turbidity) using a Labsystems Multiskan Multisoft. Experiments with each compound and mixture were carried out in duplicate on not less than two separate occasions.

Responses to test agents were calculated by correcting results for colour change against turbidity and untreated controls:

$$\text{Corrected value} = \text{Test}_{540} - [\text{Test}_{620} - \text{Control}_{620}] - \text{Control}_{540}$$

In order to correct not only for turbidity, but also for variations in seeding density, we subtracted the readings for the colour change at 540 nm in ethanol treated controls.

5.4.2 Curve fitting and generation of predictions

Plots of log concentration vs corrected responses were then constructed for each compound, and curves generated by non-linear fitting to the asymmetric Hill function using the graphical package Fig P. This model was selected due to its pharmacological usage for ligand-receptor interactions, and was found to yield better fits than with logistic sigmoid or statistical sigmoid dosimetric models.

In order to facilitate rapid calculation of IA and CA curve predictions, appropriate spreadsheets were constructed. For CA, the spreadsheet shown in Chapter 3 was modified to deal with four components, while for IA, a different layout was required (Chapter 4). For each model, predicted relationships could then be plotted as log concentration-response curves for direct comparison with the observed mixture data.

5.5 Results

5.5.1 Concentration-response relationships for single agents

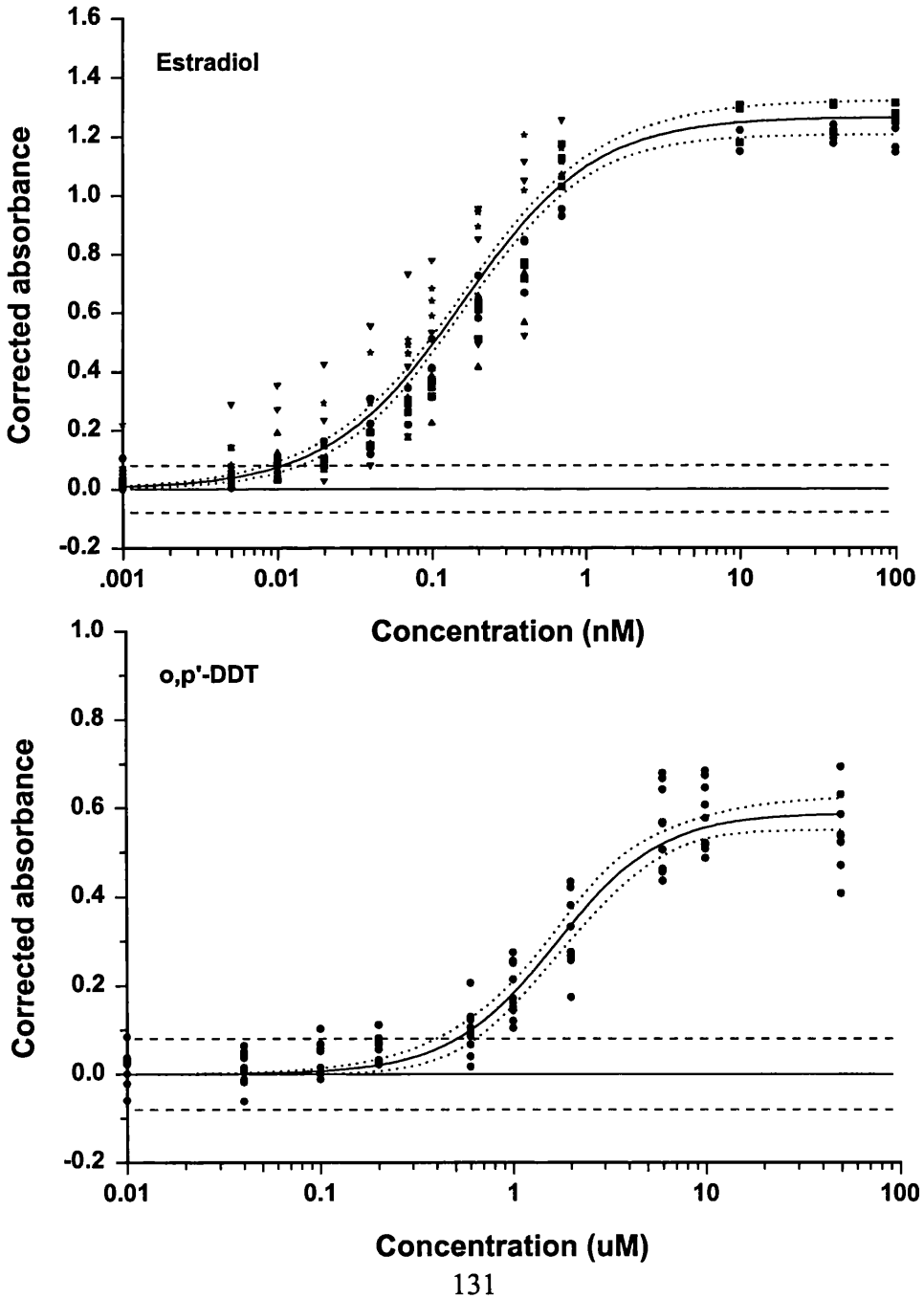
E2 was used as a positive control, it yielded a maximal corrected absorbance of

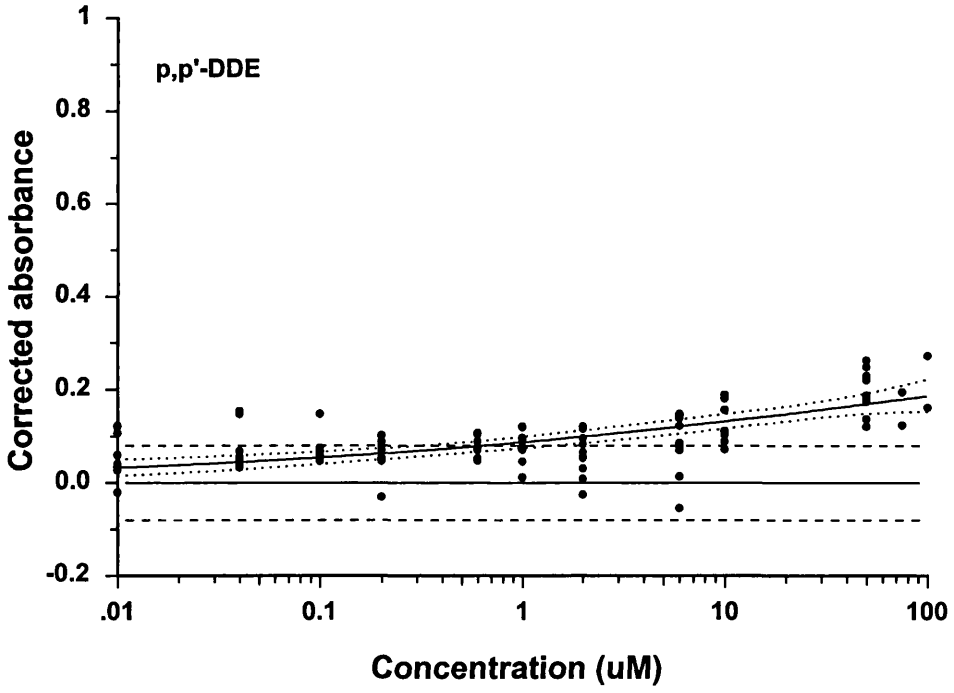
1.4. This corresponded to an EC_{50} of 0.16 nM, in good agreement with other groups (Routledge and Sumpter 1996, Arnold et al. 1996b). Also, using the estradiol curve as a standard, we explored the inter- and intra-experimental variability of the assay. In the majority of cases, the intra-experimental variability was not more than 20 %, while, the inter-experimental variability was somewhat poorer, nearer 40 %. However, much of this variability could be attributed to one dataset (designated ▼). By removing these points, the inter-experimental variability could be markedly reduced, however, as with the MCF-7 assay, these outliers were included for completeness.

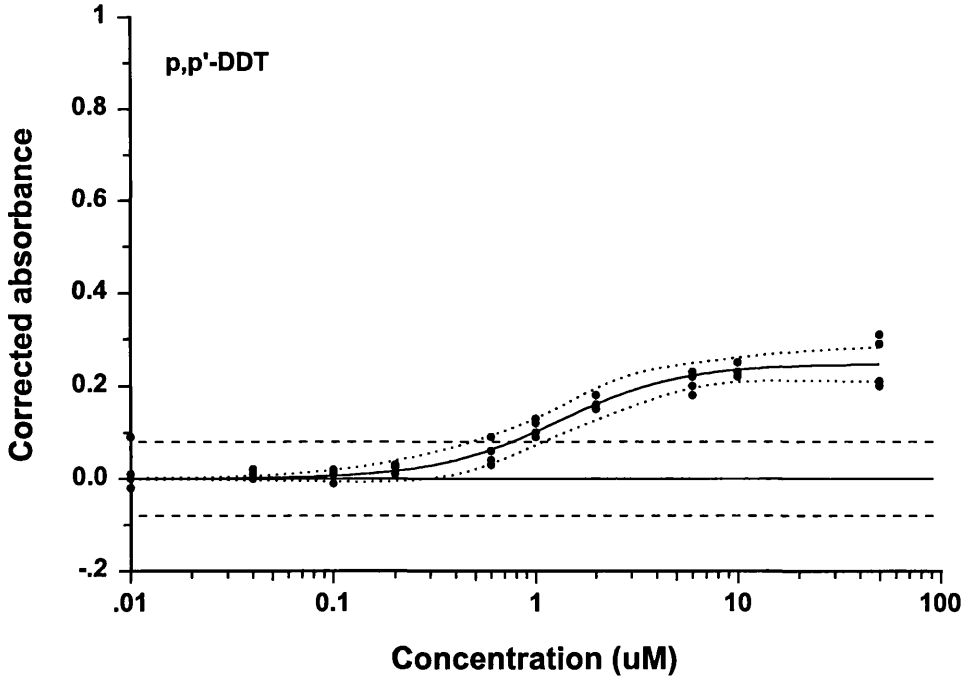
Each of the four single agents previously used for mixture experiments (Chapter 4) was also tested (Fig 5.1), however the maximal responses achieved in each case were considerably lower than that of E2, complicating the application of CA as a model, since it cannot predict effect levels greater than the maximal effect of the agent with the lowest maximum. Ideally, all agents in a mixture would possess full agonist qualities, allowing the generation of predictions over the full range of the concentration-response curve. Consequently, we also tested a number of other agents, two alkylphenols, OP and NP, two phthalates, DEHP and BBP, as well as the phytoestrogen genistein to determine which four yielded the greatest maximal response.

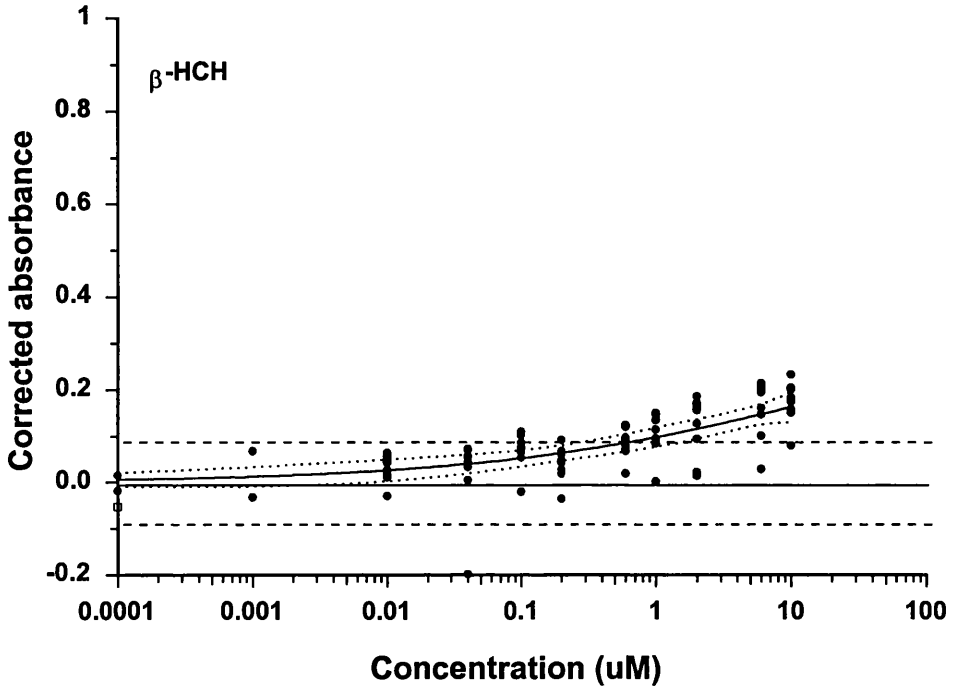
Two of the test agents, genistein and NP, achieved similar maximal reponses to E2 with EC_{50} values of 0.3μM and 1.9μM respectively. This too agreed well with previous reports (Routledge and Sumpter 1996). *o,p'*-DDT was similar in potency to NP (EC_{50} 1.7 μM) but elicited a maximal response of only 0.6, again, as previously reported (Routledge and Sumpter 1996). However, OP (Max. 0.75, EC_{50} 12.8μM) was less potent both in terms of maximal effect and half-maximal concentration than had been

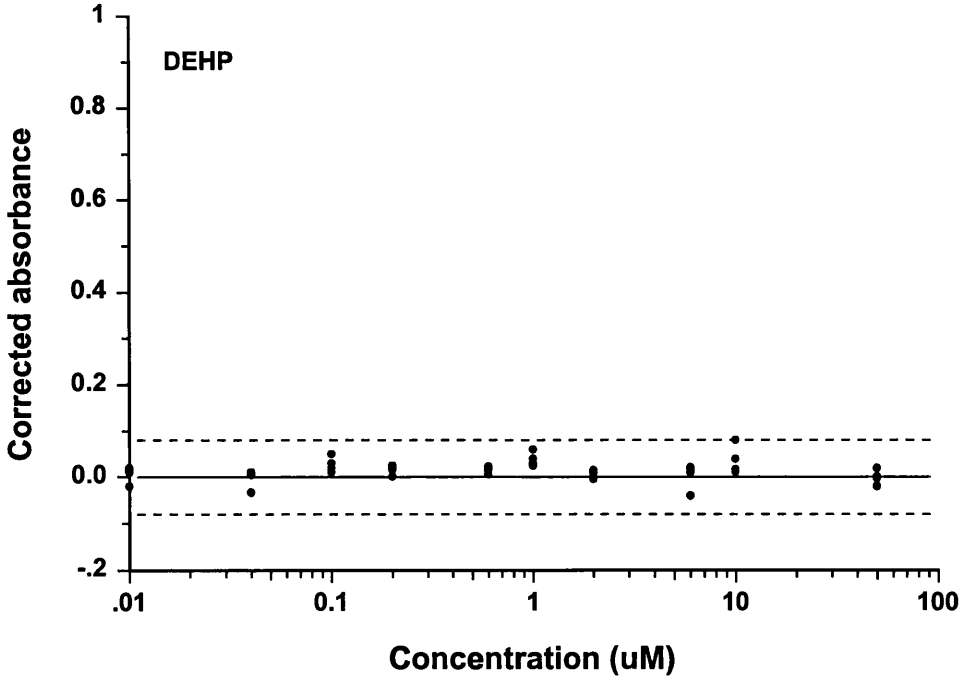
seen by Routledge and Sumpter (1996). They observed OP to produce the same maximum as E2 with an EC_{50} at approximately 1μM, although there is disagreement in the literature. Coldham et al. (1997) observed responses to OP similar to ours in terms of EC_{50} and maximal effect. All other agents yielded lower maxima, so OP, NP, *o,p'*-DDT and genistein were selected for mixture analysis.

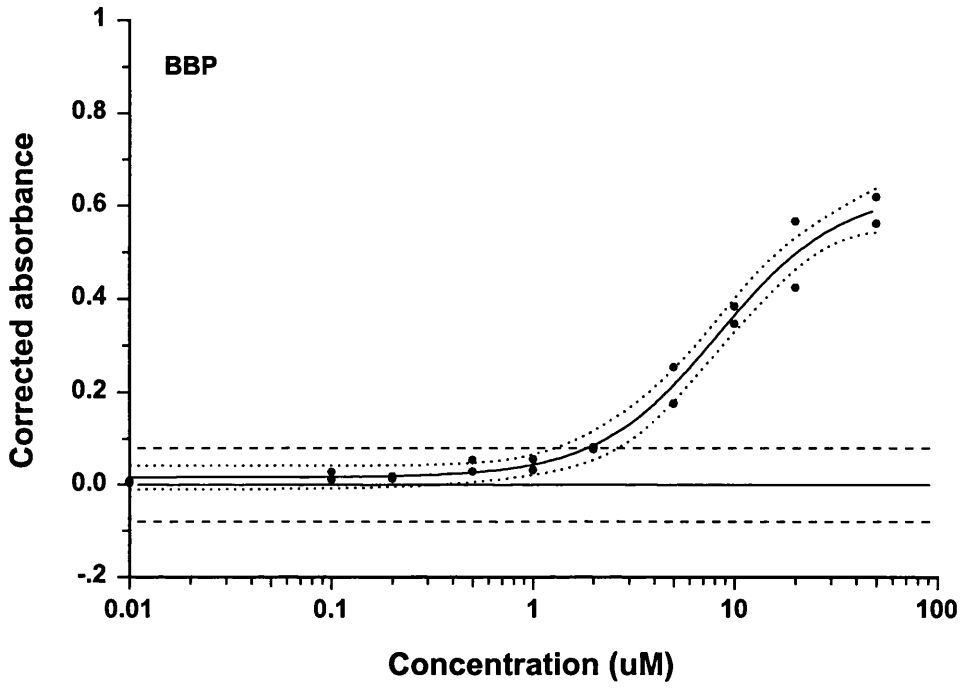


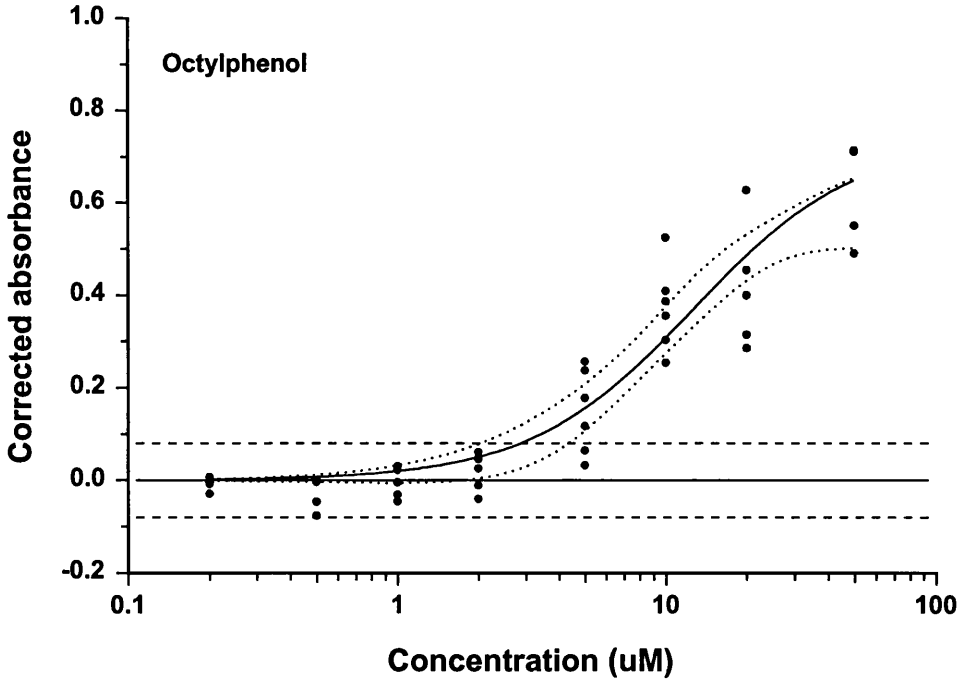

p,p'-DDE
Corrected absorbance
1
0.8
0.6
0.4
0.2
0.0
-0.2
.01
0.1
1
10
100
Concentration (uM)


p,p'-DDT
Corrected absorbance
1
0.8
0.6
0.4
0.2
0.0
-.2
.01
0.1
1
10
100
Concentration (uM)


β-HCH
Corrected absorbance
1
0.8
0.6
0.4
0.2
0.0
-0.2
0.0001
0.001
0.01
0.1
1
10
100
Concentration (uM)


DEHP
Corrected absorbance
1
0.8
0.6
0.4
0.2
0.0
-.2
.01
0.1
1
10
100
Concentration (uM)


BBP
Corrected absorbance
1
0.8
0.6
0.4
0.2
0.0
-.2
0.01
0.1
1
10
100
Concentration (uM)


Octylphenol
Corrected absorbance
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0.1
1
10
100
Concentration (uM)

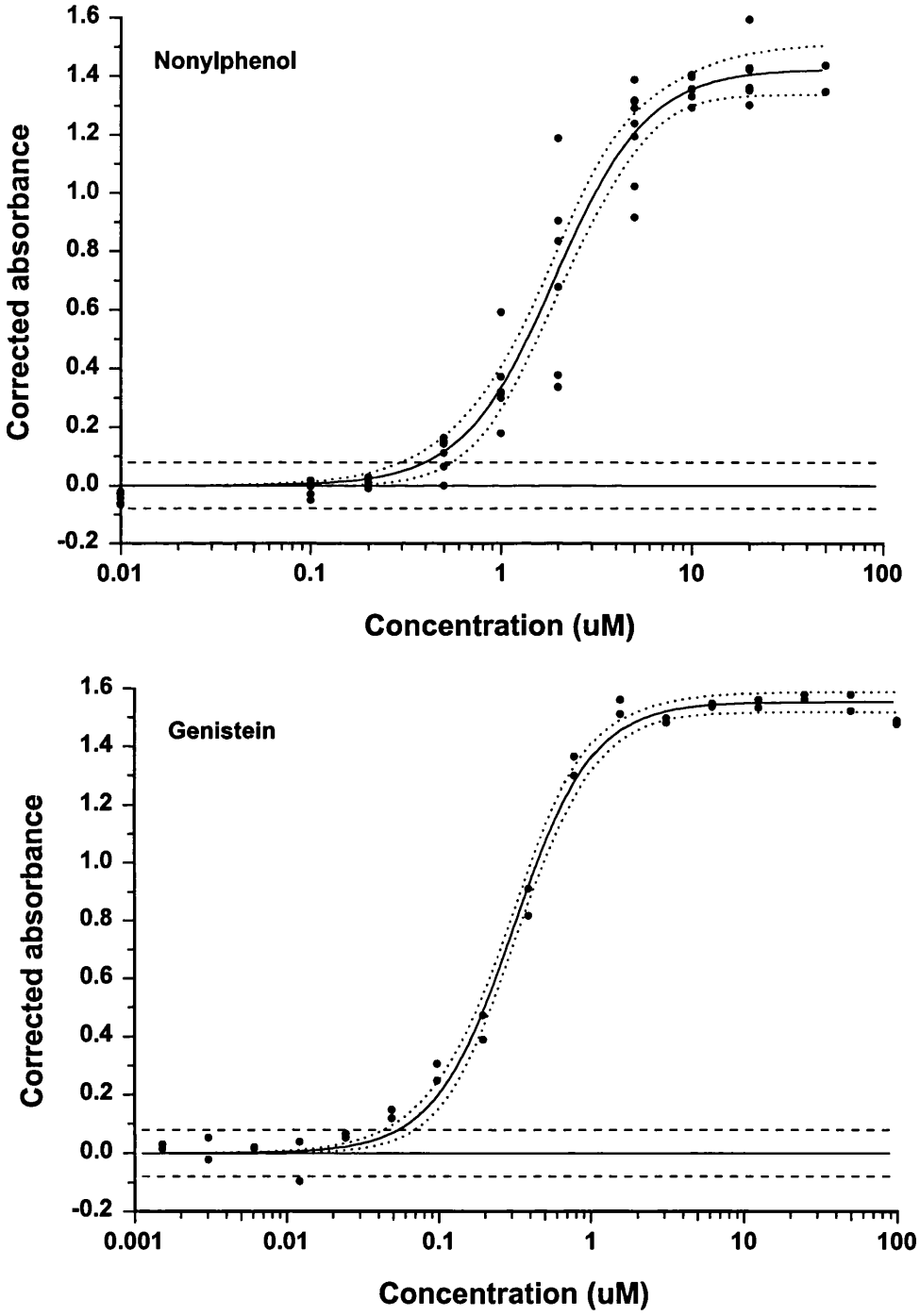


Figure 5.1 Concentration response analyses for test agents in the yeast estrogen screen. Compounds tested are E2, *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDE, β-HCH, DEHP, BBP, OP, NP and genistein Data represents individual wells from 96-well plates, experiments carried out in duplicate on at least two occasions (E2 = 5 occasions). Curves generated by non-linear fitting to the asymetric Hill function with 95% confidence interval (dotted lines). Control ± 3x SD are also shown (dashed lines). Number of experiments $n \geq 2$.

5.5.2 Mixture analysis and generation of predictions

From the parameters of the single agent curves we constructed additivity predictions for equimolar mixtures of OP, NP, *o,p'*-DDT and genistein using CA and IA, as outlined in Chapter 4. The only modification to the spreadsheets was the fixed maximum used for IA. This was altered to 1.4, since this was the maximal response achieved with E2. Curve parameters used for the mixture components are shown in Table 5.1. Construction of full response predictions allowed us to then make direct comparisons with the observed mixture data. Predicted and experimental concentration-responses were generated for three equimolar mixtures. These mixtures were: *o,p'*-DDT and genistein; *o,p'*-DDT, genistein and NP; *o,p'*-DDT, genistein, NP and OP (Figure 5.2).

As in Chapter 4, we employed an assessment criterion, whereby the degree of overlap between observed data and the 95% CI of the prediction was compared. In each case there was excellent agreement between the observed data and the prediction based upon the model of independent action. However, it was impossible to determine the quality of the agreement with concentration addition, because the model is unable to yield predictions above the maximal effect of the weakest agent. In this case the cut-off at response level 0.6 was due to the presence of *o,p'*-DDT in the mixture.

It is of interest to note that at a concentration of 50 μM, each mixture began to elicit a reduction in the absorbances measured. This is most likely due to toxicity of the mixtures at this concentration, resulting in a decrease in viable yeast cell numbers.

Hili Parameter	*o,p'*-DDT	Genistein	Octylphenoi	Nonylphenol
X50	1.67	0.31	12.8	1.94
P	1.57	1.77	1.42	1.79
Max	0.59	1.55	0.74	1.43
Min	0	0	0	0

Table 5.1 Hill function parameters for the four mixture components used in YES.

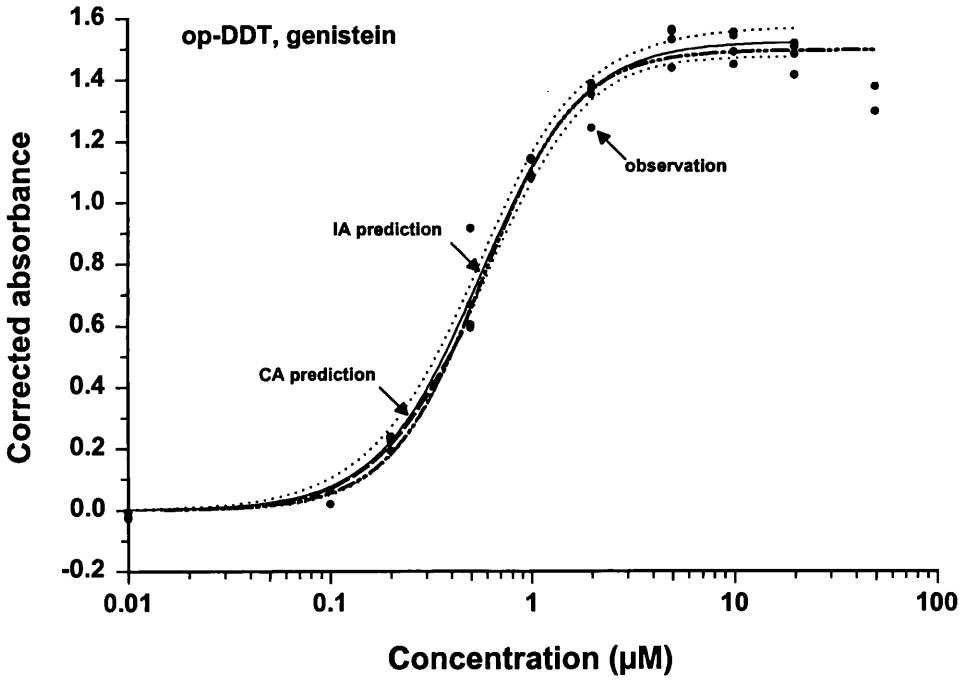
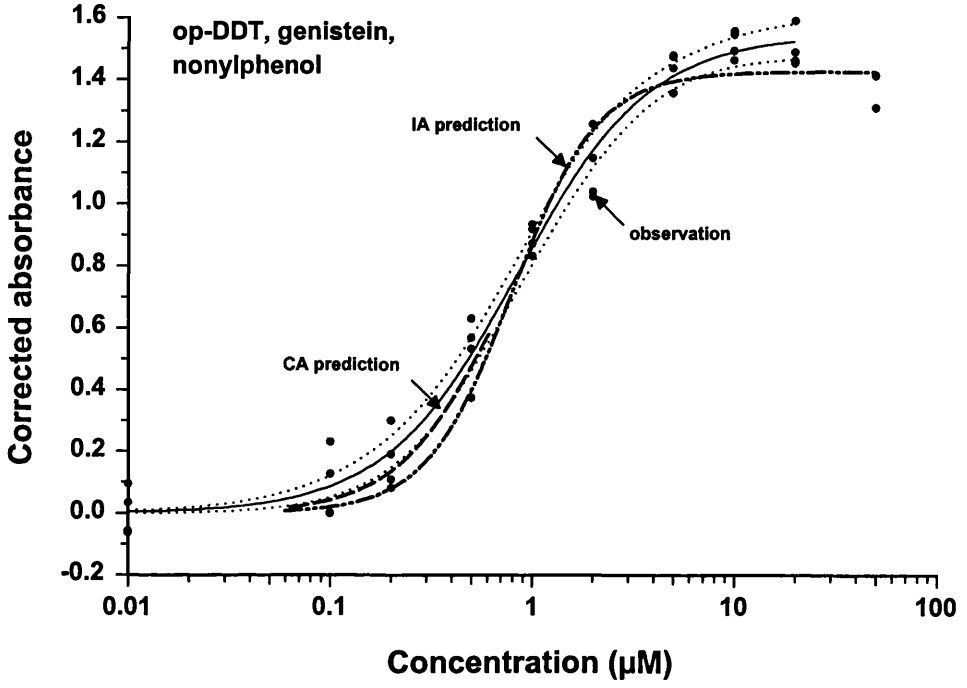
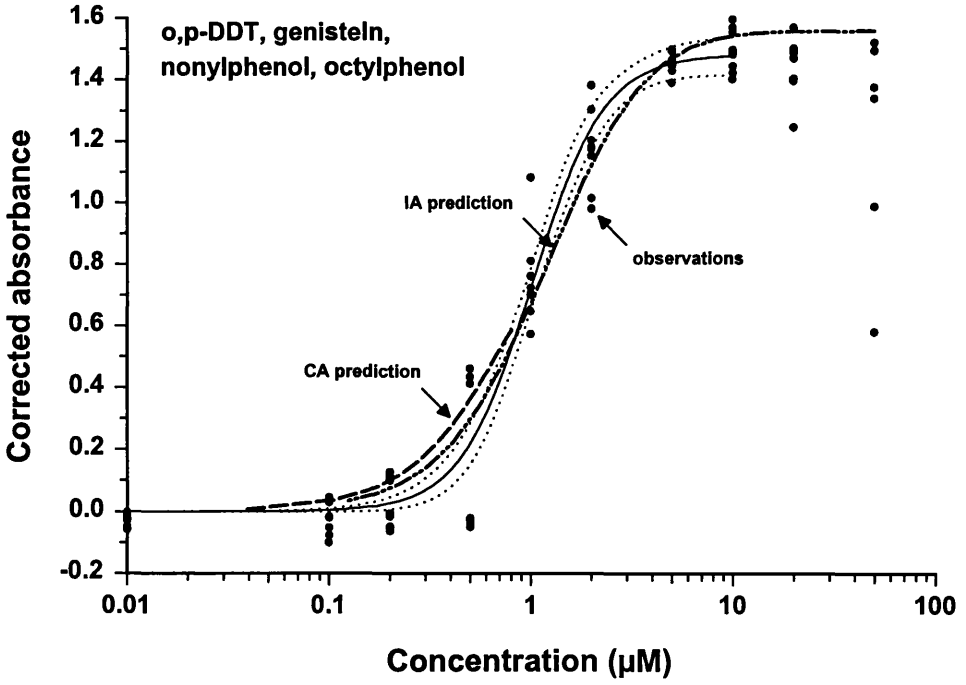


Figure 5.2 Concentration-response relationships for mixtures of EDs in the yeast estrogen screen. Effects of equimolar mixtures of *o,p'*-DDT and genistein; *o,p'*-DDT, genistein and NP; and *o,p'*-DDT, genistein, NP and OP were analysed using YES (●). Datapoints represent individual wells from 96-well plates, experiments carried out in duplicate on at least three occasions. Predictions based on IA (dot-dash line) and CA (dashed line) are shown for comparison with observed data (solid line: best regression and dotted line: 95% CI of fit).


op-DDT, genistein,
nonylphenol
IA prediction
observation
CA prediction
Corrected absorbance
Concentration (μM)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0.01
0.1
1
10
100


o,p-DDT, genisteln,
nonylphenol, octylphenol
IA prediction
observations
CA prediction
Corrected absorbance
Concentration (μM)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
0.01
0.1
1
10
100

5.6 Discussion

We have found the yeast estrogen screen to be a simple and highly reproducible assay for the identification of weak estrogens. Experimental data were generated on several occasions, by different researchers using separate dilutions of test agents and mixtures. Despite this, the data generated were in excellent agreement in each case. Data were also in good agreement with literature values, except in the case of OP, where some discrepancy already exists between other laboratories. This is probably due to the use of a number of different isomers of the compound.

When used to model mixture effects, the model of independent action provided an excellent prediction of mixture behaviour regardless of the number of components in that mixture. This indicates that the interaction between these agents is additive, and we are potentially dealing with agents possessing different modes of action. By comparison, concentration addition is thought to be an appropriate model for similarly acting agents, however, there is still no consensus of opinion on the exact meaning and interpretation of similar vs dissimilar action (Faust et al. in press). On the one hand, CA has been proposed to be the "general solution" to combination effects (Berenbaum 1985), but more recently Pöch (1993) has claimed it is only applicable to agents acting at an identical molecular acceptor site. This again suggests that there is the need for a re-examination of the interpretation of "similarity" when applied to EDs in our assay systems.

Despite the apparent limitations of the model of concentration addition, it would almost certainly yield a similar prediction to IA in our experiments owing to the similarity in slope parameters for all four single agents (see Table 5.1). Full concentration-

response predictions could have been generated if all the mixture components possessed full agonist action, and it would be interesting to see how the predictions differ with a mixture of such agents.

Both models are adaptable to any number of compounds and the next step will be to determine whether mixtures containing a greater number of agents also behave additively. If this proves to be the case, then a large step forward will have been made in the assessment of the risk posed by such chemicals.

It is important to note that the absence of synergy between these agents does not imply absence of risk. With multiple chemical exposure, additivity between agents may still be of importance, and this remains a crucial question in the field of xenoestrogens. With the speed and reproducibility of the YES assay coupled to the use of accurate model predictions means, such risk assessments are within our grasp.

5.7 Conclusions

The yeast estrogen screen is a simple and highly reproducible assay for the characterisation of xenoetrogens. In addition, the rapid time-frame of this technique (72 h) is ideally suited to generating the large quantities of data required for the analysis of mixture effects. However, care must be taken not to overlook agents which elicit estrogen-like effects via non-ER mechanisms.

From the experiments performed, it appears that the agents selected act in an additive fashion, which is well-modelled by IA. This is unusual since IA is thought to be most applicable to differently acting chemicals, yet all four tested are ER agonists. This clearly merits further investigation, but is not completely surprising. Greco et al. (1995)

state that most examples of theoretical systems that follow the IA model are relatively simple in terms of biochemical pathways. So, it may be that the complexity of the assay system is a more relative determinant of model applicability than the agents selected.

Chapter 6

What is the likely risk posed by environmental estrogens ?

6.1 Abstract

In the field of xenoestrogens, the ultimate question remains, as to what extent these agents account for recent increases in the incidences of breast and testicular cancers. With a knowledge of the potencies and prevalence of both xenoestrogens and E2, and an appreciation of the risks associated with their exposure, it should be possible to begin addressing this issue. Here we explore the impact of weak estrogens on endogenous E2 and discuss the implications for breast cancer risk assessment. Assuming that the carcinogenic mechanism of action is linked to cell proliferation, then other agents that produce this effect may act in combination with E2 to increase proliferation, thereby increasing the risk. Therefore, we carried out model calculations to examine the ability of increasing concentrations of *o,p*'-DDT to increase the proliferative response of MCF-7 cells to physiological amounts of E2 (1pM). From the calculations it was estimated that at least 75 times the present mean serum level of *o,p*'-DDT was required to significantly increase cell proliferation. Although it is unlikely that many women are exposed to 375 nM *o,p*'-DDT, it is not unreasonable that some are exposed to 75 agents with the potency and prevalence of this ubiquitous pollutant. Since some individuals are highly exposed to these chemicals either occupationally or through their diet, we investigated the effect of xenoestrogens being present at levels corresponding to the upper limit of the 95% confidence interval of serum levels. With this group, the required exposure ran to only 21 chemicals, a figure indicating a potentially increased breast cancer risk in such high risk populations. Our assessment indicates that, given the large numbers of potentially estrogenic agents, xenoestrogens could pose a significant breast cancer risk in many women.

6.2 Introduction

Circulating levels of E2 fluctuate dramatically throughout a woman's life. Exposure begins *in utero* with maternal circulating E2, and after birth levels decrease until the burst at puberty. Following this, levels fluctuate monthly during the ovulatory cycle until menopause, when they fall again. Further alterations in this pattern can be induced by pregnancy, oral contraceptives or hormone replacement therapy. Considerable data indicate that a woman's lifetime exposure to estradiol is a major contributing factor in the etiology of breast cancer (Dorgan et al. 1997). However, exposure to endogenous hormones cannot explain every instance of the disease, nor can it account for the rising increasing incidence (Wolff and Weston 1997).

The rapid time frame of increases in breast cancer suggests that the underlying causes are environmental rather than genetic, and the existence of environmental contaminants with estrogen-like activities offers a tempting explanation. Many such agents have been found at high levels in human tissues, which has prompted researchers to speculate about the role of these chemicals in the rising numbers of breast cancer cases. Despite the wealth of knowledge regarding xenoestrogens, there is still no conclusive epidemiological evidence of a link with breast cancer, probably due to xenoestrogen's low individual potency compared to E2. Yet, there exists the possibility that these agents, acting together, could produce an effect capable of influencing normal physiological responses to E2.

6.3 Objectives

Having evaluated IA and CA as tools for the prediction of mixture effects, we can now apply them to addressing the question as to what impact weak xenoestrogens can have on the already strong effects of endogenous steroidal estrogens. This could be of use in approaching the main problem, i.e. to what extent do weak estrogens pose a risk of breast cancer ? Here, we hope to estimate the concentration of weakly estrogenic agents required to significantly modulate physiological levels of E2 in the MCF-7 assay. From this we should be able to begin examining the risks posed by realistic levels of xenoestrogens.

In addition, this framework would also provide a useful tool to critically assess the design of epidemiological studies, which have so far failed to demonstrate a conclusive link between exposure to organochlorides and breast cancer.

6.4 Can we assess human risks from *in vitro* data ?

With a knowledge of the relative potencies of E2 and some EDs from *in vitro* studies, combined with information about their concentrations in human serum, it should be possible to begin to estimate the potential human health risks associated with EDs.

In order to achieve this we require information on serum levels of E2 and also selected xenoestrogens. E2 levels vary in women, and it is also unclear how much of the total serum E2 is available and able to act upon estrogen receptors. Only 1-2% is freely available, with a further 60% being loosely bound to albumin and 40 % tightly bound to SHBG (Nagel et al. 1998). A summary of E2 levels in women is given in Table 6.1. Typical levels of total endogenous E2 are 50-100 pM in postmenopausal women

and 150-200 pM in premenopausal women. Assuming that the unbound, "free" fraction is 1-1.5% in each case (Toniolo et al. 1995), then the concentration of this bioavailable fraction will be approximately 1 pM in postmenopausal women and 2.5 pM in premenopausal ones.

Because of their proliferative effects on mammary glands, estrogens, especially estradiol and estrone, have long been linked to the promotion and growth of breast cancer (Saceda et al. 1988). However, it is only in the last few years that this could be confirmed epidemiologically (Hunter et al. 1997, Helzlsouer et al. 1994). The strongest epidemiological associations between estrogens and breast cancer have been observed when relating the free fraction of E2 in postmenopausal women with cancer incidence (Toniolo 1997). Thus, for the purposes of calculations, mean free E2 levels from post-menopausal women will be used as our benchmark (1 pM).

Secondly, we require data on the levels of xenoestrogens in human serum. Data on serum xenoestrogen levels from a number of studies are summarised in Table 6.2. Although there is considerable individual variation, we will use mean concentrations. Some of these agents, like E2, may also be bound to albumin, however, there is no evidence of their binding to SHBG (Nagel et al. 1998). Therefore, reported figures for xenoestrogens will be taken as being the bioavailable quantity. With a knowledge of the potency of these agents from assays using MCF-7 cells, it is possible to create predictions of the effects of mixtures containing E2 and xenoestrogens. This will be carried out using the model of concentration addition, as CA is regarded as producing the more conservative result, or "worst case scenario" (Backhaus et al. 2000).

Total serum levels (pM)		**Case**	**Control**	**Reference**
Postmenopausal:		54 ± 4	49 ± 4	Dorgan 1997
		123	102	Toniolo 1995
		88 ± 4	85 ± 36	Berrino 1996
		63	59	Helzlsouer 1994
Premenopausal:	*Follicular*	167	143	Helzlsouer 1995
	Luteal	162	226	Helzlsouer 1994
Distribution (%)				
Free		1.42	1.34	Toniolo 1995
		1.47	1.46	Helzlsouer 1994
Albumin-bound		58.9	54.1	Toniolo 1995
SHBG-bound		39.7	44.6	Toniolo 1995
Bioavailable (pM)				
Free				
Postmenopausal		0.65	0.53	Dorgan 1997
		1.85	1.35	Toniolo 1995
		1.33	1.32	Berrino 1996
		0.95	0.88	Helzlsouer 1994
Premenopausal:	*Follicular*	2.5	2.2	Helzlsouer 1994
	Luteal	2.4	3.4	Helzlsouer 1994
Non-SHBG				
Postmenopausal		26 ± 2	21 ± 2	Dorgan 1997
		74	54 (Max 400)	Toniolo 1995
		53 ± 2	51 ± 22	Berrino 1996
		38	35	Helzlsouer 1994
Premenopausal:	*Follicular*	100	86	Helzlsouer 1994
	Luteal	97	136	Helzlsouer 1994

Table 6.1 Summary of levels of E2 in women with and without breast cancer.

	Serum levels (μM)		
Study	**o,p'-DDT**	**p,p'-DDE**	**β-HCH**
Yugoslavia '85-'86	0.009 (14)	0.029 (14)	0.006 (14)
	---	0.024 (14)	0.005 (14)
Norway '81-'82	---	0.060±0.066 (15)	≤ 0.004 (15)
	---	0.035±0.030 (20)	≤ 0.004 (20)
Mexico '94-'96	0.0003 (141)	---	---
Spain '97	---	---	0.030±0.050 (625)

Table 6.2 Mean serum levels of selected xenoestrogens from female volunteers. Number of subjects shown (n). Data reproduced from published results (Toppari et al. 1996, Dorgan et al. 1997, Lopez-Carillo et al. 1997).

6.4.1 E2 levels and their impact on cancer risks.

Before addressing the impact of xenoestrogens on the activity of E2, it is necessary to designate an effect level which constitutes a measurable deviation from the response to E2 alone. Using the E2 concentration-response relationship data from the MCF-7 cell assay, it is possible to construct a 95% confidence belt about the response curve. Datapoints lying outside this belt can then be designated as deviations from that curve.

In the MCF-7 cell proliferation assay, 1pM E2 elicits a proliferative response of 1.05, with an upper 95% confidence value of 1.5. So, a mixture of EDs, which in conjunction with 1 pM E2 yielded a relative proliferation of 1.5 or greater would be considered to have modulated the normal E2 response (Figure 6.1). From this it should be possible to address the risks posed by EDs, when present at physiological levels.

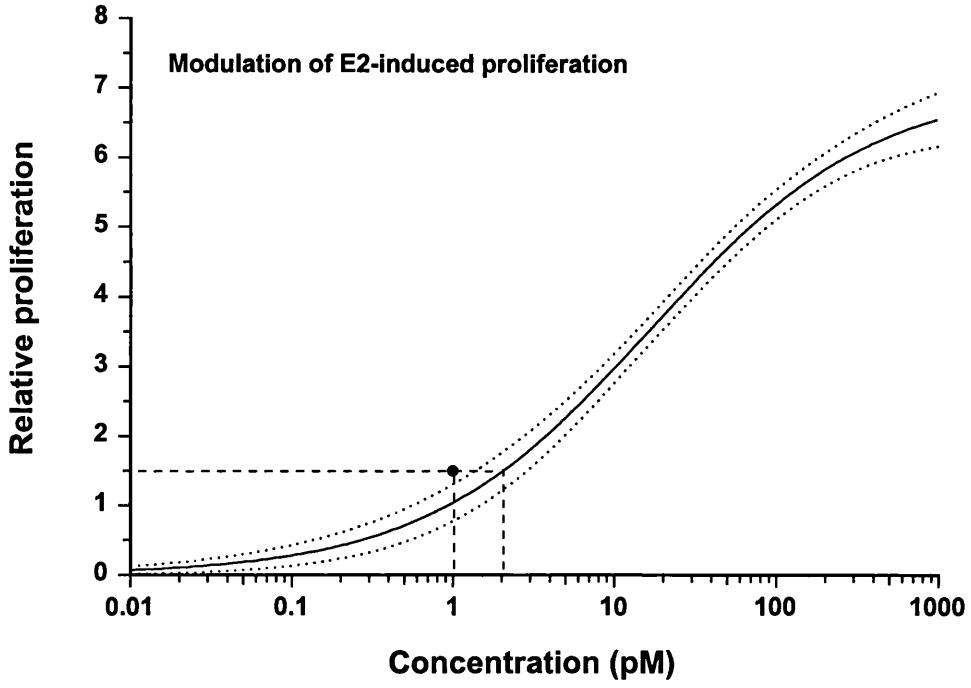


Figure 6.1 Concentration-response relationship and 95% CI for E2 in the MCF-7 cell proliferation assay. Datapoint (●) indicates the lowest effect level (1.5) lying outside the 95%CI for 1 pM E2. Also shown is the best estimate of a concentration of E2 corresponding to effect level 1.5 (2.03 pM).

With a knowledge of the risks associated with different levels of E2, it would then be possible to extrapolate from *in vitro* observations to the "real world" situation, by comparing effect levels *in vitro* with relative risks for different E2 exposures. 1 pM E2 (0.27 pg/ml) is the typical free fraction of the hormone in postmenopausal women. From the regression analysis, we estimate that 1 pM E2 elicits a response of 1.05 in the MCF-7 assay, similarly, the concentration estimated to elicit an effect level of 1.5 is 2.03 pM E2 (0.55 pg/ml). We can compare these two concentrations with published data on E2 and breast cancer risk. Berrino et al. (1996) quoted a relative risk (RR) of 1.0 for

women with 0.27 pg/ml E2, but a RR of 2.4 for those with 0.55 pg/ml E2. This observation is also supported by Toniolo et al. (1995), who reported an odds ratio (OR) of 1.0-1.5 with 0.27 pg/ml E2 and OR of 3.8 with 0.55 pg/ml E2. This clearly represents an increased risk of breast cancer from the additional hormonal burden. Assuming that xenoestrogens act via increasing breast cell proliferation to exert their influence in breast cancer, then we can begin to estimate concentrations required to significantly modulate E2 activity, i.e. mixture concentrations corresponding to effect level 1.5 in the MCF-7 assay

6.4.2 How much *o,p*'-DDT is required to modulate responses to E2 ?

In calculating the concentrations of EDs required to modulate the effects of E2, the crucial parameters required are the potencies of the respective agents, and their relative prevalence within the mixture.

Taking *o,p*'-DDT as a representative xenoestrogen, and assuming that the combined effect of *o,p*'-DDT and E2 is additive, we used the model of concentration addition to generate predictions of effects for various mixtures of *o,p*'-DDT and E2, based on their individual potencies in the MCF-7 cell proliferation assay. Data shown in Chapter 2 were used for the calculations. Figure 6.2 shows the influence of increasing concentrations of *o,p*'-DDT on the activity of 1 pM E2, and as the figure shows, a 375 000 : 1 mixture would be expected to yield a response significantly different to 1 pM E2 acting individually. That is, 375 nM *o,p*'-DDT would be required to increase the response to 1 pM E2 from 1.05 to 1.5. Since serum *o,p*'-DDT levels are approximately 5 nM, MCF-7 cells must therefore be exposed to 75 times more *o,p*'-DDT

than is typically present in serum to produce a significant increase in proliferation. Or, looking at this in another way, 75 distinct agents with the same prevalence and potency as *o,p'*-DDT would also significantly increase E2-mediated cell proliferation.

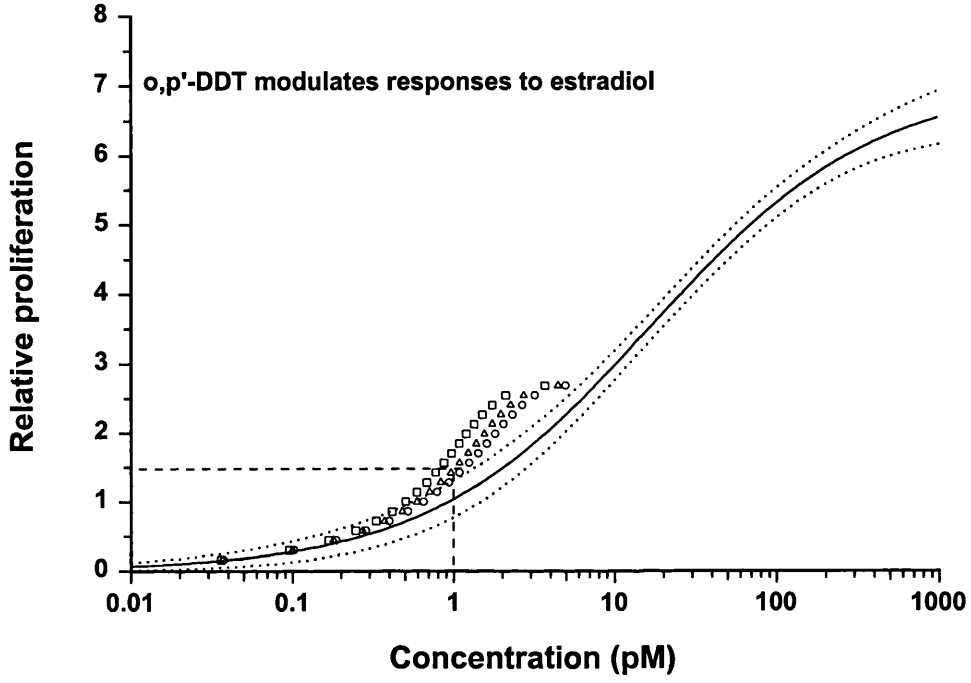


Figure 6.2 *o,p'*-DDT is able to increase the response to 1 pM E2 in the MCF-7 cell assay. Given that serum levels of *o,p'*-DDT and E2 are 5 nM and 1 pM, respectively (ratio 5000:1), concentration-response predictions were calculated for a number of ratios of the two. By increasing the proportion of *o,p'*-DDT in the mixture, we present additivity predictions for the effects of 500 000:1(□), 375 000:1 (△) and 250 000:1(○) mixture ratios, plotted relative to the concentration of E2 in the mixture. From this we can observe that at 1 pM, the 375 000:1 and 500 000:1 mixtures, but not the 250 000:1 mixture elicit a response significantly greater than that obtained with E2 alone (solid line, shown for comparison), since the predicted effects are larger than the upper 95% CI of the best fit (dotted line). That is 375 nM *o,p'*-DDT is required to modulate the response to 1 pM E2.

Given the numbers of estrogenic agents that we are already aware of, as well as the vast numbers of untested compounds, exposure to 75 environmental estrogens seems to be well within the realms of possibility. However, many of these agents are much less potent and less prevalent than *o,p'*-DDT. It is therefore necessary to consider concentrations of a mixture of agents which could influence responses to E2.

6.4.3 *o,p'*-DDT is not the only xenoestrogen of interest

To reflect the differing potencies and prevalences of xenoestrogens, we considered the ability of a mixture of agents to influence the action of E2. In addition to *o,p'*-DDT (5 nM) we considered *p,p'*-DDE. which is less potent but more prevalent (serum concentration taken as 30 nM) and β-HCH, which is less potent but similarly prevalent (5nM). Taking a mixture of these three agents, we calculated the concentration required to shift the response to 1 pM E2 from 1.05 to 1.5 (Fig 6.3). Again, the predictions were based on data for the individual potencies of the agents in the MCF-7 cell assay, as shown in Chapter 2. From this data we calculated that individual xenoestrogens would have to be present at 20 times higher than mean serum concentrations, in other words, a person would have to be exposed to approximately 60 such agents to increase their risk of breast cancer. As before, this figure is not considered unreasonable given the structural pervasiveness of estrogenic activity.

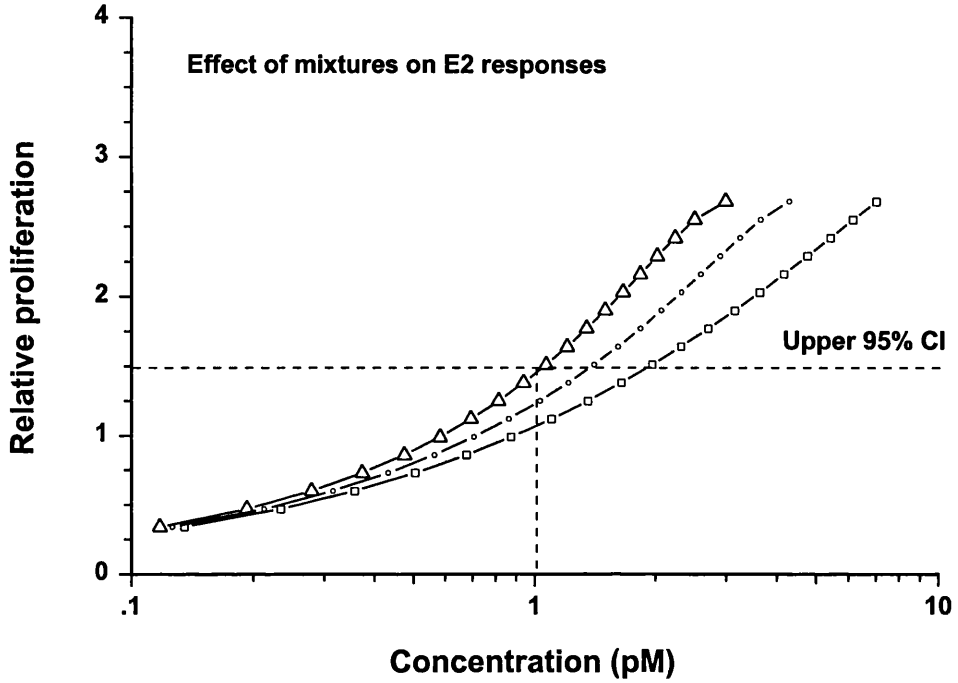


Figure 6.3 Mixtures of xenoestrogens are able to modulate the effects of E2. *o,p'*-DDT, *p,p'*-DDE and β-HCH are present in serum at 5, 30 and 5nM, respectively. To examine the ability of this mixture to increase responses to 1 pM E2, CA predictions were calculated for a number of mixtures of the four, at ratios proportional to their relative abundance in human serum. Ratios 5000:30000:5000:1 (□), 50000:300000:50000:1 (○) and 100000:600000:100000:1 (△) are represented. From this we can observe that at 1 pM, the 100000:600000:100000:1 mixture elicits a response significantly greater than that obtained with E2 alone, since the effects are greater than the upper 95% CI of the best fit for E2 alone (dashed horizontal line). That is, 100 nM *o,p'*-DDT, 600 nM *p,p'*-DDE and 100 nM β-HCH together are required to modulate the response to E2. E2 response curve omitted for clarity.

6.4.4 Some individuals may have increased exposure to xenoestrogens

To pursue this further, we examined what levels of xenoestrogens might constitute a heavy exposure. From data on the variation of *p,p'*-DDE levels in serum, we obtained standard deviation values for the mean serum levels, from which we calculated the likely upper 95% CI for serum *p,p'*-DDE, i.e. the level of the most highly exposed 5% of the population.

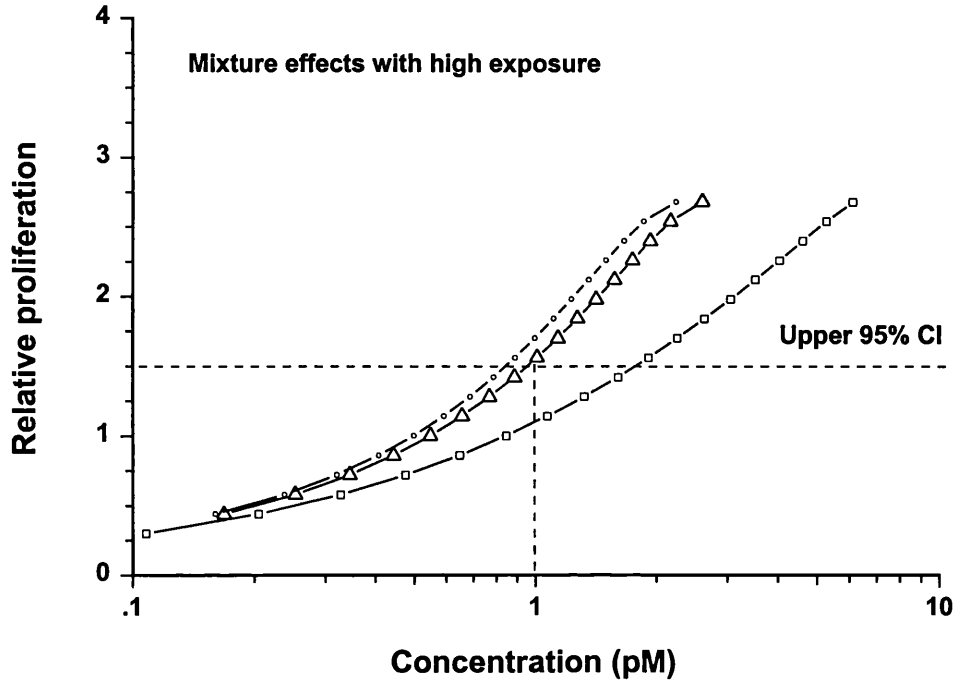


Figure 6.4 Mixtures of xenoestrogens in highly exposed individuals modulate the effects of E2. In the most highly exposed 5% of the population, *o,p'*-DDT, *p,p'*-DDE and β-HCH are present in serum at approximately 15, 100 and 15nM, respectively. To examine the ability of this mixture to increase responses to 1 pM E2, CA predictions were calculated for a number of mixtures of the four. Ratios 15000:100000:15000:1 (□), 120000:800000:120000:1 (△) and 1500000:1000000:1500000:1 (○) are represented. From this we can observe that at 1 pM, the 120000:800000:120000:1 mixture elicits a response significantly greater than that obtained with E2 alone, since the effects are greater than the upper 95% Ci of the best fit for E2 alone (broken horizontal line). That is, 8 times the mean serum levels of each are required to modulate E2 activity. E2 response curve omitted for clarity.

In the case of *p,p'*-DDE this was a concentration of approximately 100 nM (Toppari et al. 1996), roughly three times above mean levels. In the absence of equivalent data for *o,p'*-DDT and β-HCH, we assumed that this held true, and assigned these agents serum concentrations of 15 nM. We then examined the ability of this new mixture to modulate E2 responses. As can be seen in figure 6.4, we estimate that 7 times as much of each agent is required; that is, individuals highly exposed to 21 such

agents would be at a significantly greater risk of developing breast cancer.

This may well be realistic, since exposure to different xenoestrogens often comes from the same sources. Due to the ability of many of these chemicals to bioaccumulate in lipids, people consuming large quantities of meat and dairy products may well receive large quantities of many xenoestrogens via their diet. In addition, there are potentially many unknown environmental estrogens which may well be more potent than the agents examined here.

6.5 Discussion

The primary objective of this chapter was to estimate the numbers of EDs which at environmentally relevant levels could impact upon the activity of E2. Assuming the carcinogenic effect of EDs is related to their proliferative effects, then from the model calculations it is apparent that those with average serum levels are potentially exposed to sufficiently high concentrations of xenoestrogens to be at an increased risk. This leads us to believe that in a number of cases, these chemicals could contribute to the genesis of breast cancer and may partly account for the increasing incidence of the disease. Secondly, individuals with raised exposure to such agents, either occupationally or in their diet, would experience a greater than average risk.

However, it must be mentioned that these calculations assume that xenoestrogens exert their role in breast cancer by increasing cell proliferation. This may not be strictly the case, and some of these agents may exert their carcinogenic effects by means other than cell proliferation. This is therefore certainly one limitation of the model I have proposed, and will not be resolved until conclusive evidence on the

carcinogenic mechanism of EDs has been determined.

6.6 Conclusions

It is quite possible that there is an increased risk of breast cancer associated with serum levels of xenoestrogens in the majority of women. Those with excessive exposure may well be at greater risk.

Chapter 7

General discussion and future work

7.1 Introduction

Over the last few decades there has been mounting concern regarding the increasing incidence of endocrine related disorders in humans and wildlife. The speed with which these changes have occurred has prompted researchers to speculate about a common underlying environmental cause.

It is well recognised that E2 exposure is a major risk factor for the genesis of breast cancer, and since the 1960s it has become apparent that a number of persistent environmental pollutants are able to exert effects similar to those of the hormone both *in vitro* and *in vivo*. Thus it was hypothesised that these xenoestrogens may represent a preventable cause of breast cancer. However, a multitude of epidemiological studies have failed to confirm an association between environmental pollution and breast cancer, since no single ED would be sufficient to influence breast cancer risk at observed concentrations.

Despite this, research into the action of these chemicals has continued unabated, although this too has not been without problems. The MCF-7 cell proliferation assay, which is widely used for the study of xenoestrogens, has been dogged by inter-laboratory variability. The extensive variability between supposedly identical MCF-7 stocks, coupled with the lack of a standardised protocol, has made it difficult to draw meaningful comparisons between the findings of different research groups. Additionally, even when researchers have utilised the same cell line and protocol, there have been major differences in the way data is represented, with few groups carrying out thorough concentration-response analyses. The one issue everyone seems to have agreed upon is that singly, these weak estrogens are so low

in potency relative to E2 that it is difficult to explain their role in disease.

So, in an attempt to explain the action of these weak agents, a number of groups began experimentally investigating the action of these agents in combination. Again, with mixed results. Groups began reporting synergistic interactions between mixtures of weak estrogens (Soto et al. 1994, Arnold et al. 1996a), which at first appeared to bridge the potency gap regarding estradiol. However, following the retraction of one key paper (McLachlan 1997), it became clear that the tools for analysing combination effects were not in place.

These shortcomings, both in experimental procedure and data analysis, prompted us to examine thoroughly the methods available for testing and predicting mixture effects.

7.2 The MCF-7 assay and YES are useful systems for the study of xenoestrogens

Despite some criticism of the MCF-7 cell proliferation assay in recent years, we have found it to be a sensitive and highly reproducible tool for the investigation of xenoestrogen mixture effects. Although perhaps not as complete as the rodent uterotrophic assay, working with MCF-7 cells is far more rapid and cost-effective. *In vivo* analysis of combination effects is prohibitively expensive due to the requirement for comprehensive concentration-response analysis both with the single agents and mixtures of interest. Despite their inability to model absorption, distribution and excretion of test compounds, the cells are of human origin and metabolically active, and so can give a strong indication of likely effects *in vivo*.

The YES is a somewhat different assay. The yeast cells probably do not

possess the same metabolic capabilities as human cells, and their cell walls represent an additional barrier to chemical permeability. In addition, the estrogen response machinery is artificially constructed and cannot respond exactly as it would in human cells. That said, the assay is a simple, rapid and highly reproducible way of characterising the estrogenic activity of test agents. Its high throughput is ideally suited to the analysis of combination effects, although one should always remember that not all breast cell proliferaters act via the ER. Consequently, some endocrine disrupters would be overlooked while some ER antagonists may show as positive.

On balance, neither assay is perfect, but their reproducibility, speed and cost-effectiveness make them ideally suited to generating the large quantities of data required for analysing combination effects.

7.3 CA and IA are appropriate models for analysing combination effects

With respect to the analysis of xenoestrogen combination effects, the biggest problem in recent years has been the inappropriate application of predictors of mixture behaviour. The majority of groups have implicitly adopted a model others would refer to as effect summation, which has led to some incorrect assumptions of synergy. To avoid this problem we selected two well-founded pharmacological models of mixture behaviour which function irrespective of the shape of the underlying concentration-response curves.

CA is an appropriate and well-validated model for analysing data on mixture effects. Here, for the first time, we demonstrate its application for generating complete concentration-response relationships with mixtures of xenoestrogens. Experimentally,

this model yields excellent predictions of the effects of xenoestrogen four-component mixtures in the MCF-7 cell assay, and indicates the combination effect to be one of additivity. Interestingly, this model is thought to be applicable to mixtures of similarly acting agents, yet the chemicals we selected act at different molecular target sites. Consequently, there is need for a re-evaluation of the definitions of similar and dissimilar action.

IA is also a suitable model of mixture behaviour with a long pedigree, and we are unaware of any previous application of it with xenoestrogens. In YES, this model was observed to produce an accurate prediction of 2-, 3- and 4-component mixture effects. However, no comparison can be drawn with CA as the latter model was unable to model entire curves for the chemicals selected. Despite this, we observed another interesting discrepancy. IA usually applies to mixtures of agents with distinct modes of action, yet our agents all acted via the ER, again requiring a closer look at what constitutes similarity.

7.4 Risk assessment and the lack of conclusive evidence from epidemiology

One explanation for the lack of conclusive results using epidemiological studies comes from the results of *in vitro* studies with these compounds. Researchers have consistently found that the vast majority of xenoestrogens are far less potent than estradiol, requiring concentrations about 100 000 times greater than estradiol to elicit a comparable response in most *in vitro* assays. This is supported by our calculation that 375 nM *o,p'*-DDT (or 75 agents with the potency and prevalence of *o,p'*-DDT) would be required to significantly modulate the response to physiological levels of E2.

Such concentrations of single agents are unlikely to occur even in the most heavily exposed individuals, making it virtually impossible to correlate breast cancer risk with body levels of individual agents. This is borne out by the majority of epidemiological studies where there is little evidence of xenoestrogen related risk. However, our calculations assume that increased cell proliferation is the key step in the etiology of breast cancer, a situation that may well not be the case.

7.5 The animal link discredited

Further questions regarding the impact of EDs in health disorders have recently been postulated, following a re-examination of reports on EDs in animals. Despite a lack of concrete epidemiological evidence in support of the xenoestrogen hypothesis, there have been numerous reports of the effects of endocrine disrupters on animal populations. In a recent book *Principles and processes for evaluating endocrine disruption in wildlife* (Kendall et al. 1998), the authors summarise 50 years of research into the effects of environmental pollutants on wildlife. However, as Risebrough (1999) has recently pointed out, a number of the book's arguments do not hold true.

In the book, the observation of skewed sex ratios and female-female pairing in the western gulls of southern California is attributed to a "chemical etiology" (Fry and Toone 1981). In addition, experimental work cited shows that agents such as DDT can produce reproductive feminisation in male gull embryos, a phenomenon observed in the wild. However, in reality, feminised adult male gulls have never been observed. Instead, the author attributes the skewed sex ratio to starvation which affects the larger, male chicks to a greater extent than the females (Risebrough 1999).

Also discredited by Risebrough is the often quoted story of the Lake Apopka alligators of Florida. Following observations of reduced reproductive function and genital malformation in this population, researchers linked these events to the discharge of organochlorides into the lake. However, this may only be a small part of an ongoing story, since the water had been contaminated even before this. Some years previously, dibromochloropropane (DBCP), which had been found to produce azoospermia in workers at the formulation plant, had already become a major contaminant of local ground waters. So it is conceivable that DBCP, not the organochlorides, was responsible for the alligator's fate.

Although we cannot totally discount the role of xenoestrogens in animal disorders, there is mounting evidence that the link is perhaps circumstantial. So, if the xenoestrogen explanation of increasing breast cancer does not hold true, then what other explanations may be offered ?

7.6 Other explanations of the rising trend in breast cancer

We are already aware that the most powerful risk factor for breast cancer is the lifetime exposure to E2 (Nandi et al. 1995). In post-menopausal women, the primary source of estrogens is the conversion of androstenedione to estrone in adipose tissue, hence obesity results in an overproduction of estrogens. In addition, over 95% of estrogens are carried in serum bound to proteins such as albumin and sex SHBG, but obesity is associated with a reduction in SHBG levels, leading to increased free serum estrogens (Kelsey and Bernstein 1996). One controversial area of study is the use of combined oral contraceptives (COC) and hormone replacement therapy (HRT). Results

so far have been mixed, but the emerging picture seems to be that COC use may increase the risk in young women (Feigelson and Henderson 1996), while prolonged HRT may confer a moderate increase in risk (Pike et al. 1993). On a more positive note, multiple pregnancies, prolonged lactation and regular exercise can all reduce the number of ovulatory cycles thereby providing some protection from the disease. Exercising for four hours per week can reduce breast cancer risk by up to 50% (Bernstein et al. 1994). However, due to smaller family sizes, bottle feeding and more sedentary lifestyles, many women may not derive these benefits. Other factors such as smoking, alcohol consumption, poor diet and exposure to electromagnetic fields have all been associated with this condition, although to a much lesser extent than estrogen burden.

7.7 Phytoestrogens

Despite the obvious concerns over man-made environmental pollutants with estrogen-modulating activity, the issue remains clouded by the existence of many natural estrogenic agents (phytoestrogens). These chemicals are produced in numerous plant species and are thought to act as fungicides, deter herbivores and regulate plant hormones (Barrett 1996). Although these agents have been used medicinally for thousands of years, only recently have they been studied in relation to human health and disease.

The two major classes of phytoestrogens are the lignans and isoflavonoids, and types of both have been found in many human food sources. Common dietary isoflavones include genistein, dadzein and equol, and are found abundantly in legumes.

Lignans such as secoisolariciresinol and matairesinol are found in whole grains and a number of vegetables and fruits, and are metabolised by gut flora to yield enterolactone and enterodiol.

These nonsteroidal plant estrogens were first identified in the 1930s, when soybeans were found to contain chemicals with a similar structure to human estrogens (Walz 1931). Later, fertility problems in sheep were attributed to some species of clover, rich in equol and coumestrol (Price 1985). When eqoul was found in human fluids at concentrations 5000 times that of estradiol, the question was raised regarding human health risks. However, epidemiological studies suggest that diets rich in plant estrogens, particularly soy and unrefined grain products, may be associated with lowered risks of breast and prostate cancer (Strauss 1988).

Asian populations consume a diet that is rich in plant estrogens such as genistein and daidzein, which are prevalent in soybean-based products (up to 300 mg / 100g). These populations suffer a 5 to 8-fold lower rate of hormone-dependent cancers (such as breast and prostate) compared to westerners (Muir 1987), as well as lower rates of osteoporosis and menopausal symptoms. A recent study in Singapore demonstrated that increased soy intake protects women from breast cancer (Barrett 1996). However, Asian immigrants to western countries tend to change their diets, consuming fewer soy products. This is accompanied by increases in the incidence of hormonally-related conditions (Muir 1987). This suggests that phytoestrogens may have a protective effect against these disorders. Asian women exhibit a menstrual cycle length 3 days longer than in westerners, therefore the follicular phase of the cycle is longer. During this phase breast cell division is at its lowest and this may repesent

a reason for reduced breast cancer (Henderson et al. 1985). In addition, epidemiological evidence suggests that vegetarians possess a reduced risk of hormone-dependent cancers (Rohan and Bain 1987), although it remains unclear whether this is due to an increased intake of phytoestrogens, or a reduced intake of animal fat and protein (Sathyamoorthy 1994).

7.7.1 *In vitro* studies with phytoestrogens

In vitro studies have demonstrated that plant estrogens can interact with ER as either agonists or antagonists, often in a concentration-dependent fashion. Genistein, daidzein and equol all interact with ER in MCF-7 cells (Willard 1998). At 0.1-10 μM (physiological concentrations), enterolactone, genistein and coumestrol increase DNA synthesis in MCF-7 cells by up to 200%, but at concentrations over 20 μM, they inhibit DNA synthesis (Wang 1997). At approximately physiological concentrations, daidzein, equol and enterolactone stimulate synthesis of the estrogen-responsive protein pS2 in MCF-7 cells, while enterolactone does not (Sathyamoorthy 1994).

Genistein-induced proliferation at low concentrations can be blocked by tamoxifen, but not at high concentrations. Lignans are also found to stimulate proliferation in a manner not inhibited by tamoxifen (Jordan 1985), perhaps indicating different mechanisms of action (Wang 1996). In addition, prolonged exposure of MCF-7 cells to genistein resulted in a downregulation of ER, accompanied by a decreased responsiveness to E2 (Wang 1996).

Genistein also elicits a number of non-ER effects, including inhibition of protein tyrosine kinases, DNA topoisomerases and angiogenesis. The inhibition of tyrosine

kinases may explain why genistein is able to inhibit cell proliferation in both estrogen-dependent (MCF-7) and estroge-independent (MDA-468) cell lines. Other effects of genistein include stimulation of SHBG synthesis and inhibition of aromatase, both of which indirectly reduce the amount of free estradiol in the body (Adlercreutz 1987).

However, there is also evidence that phytoestrogens are able to damage DNA. In cultured chinese hamster V79 cells coumestrol, genistein and daidzein induced DNA strand breaks and micronuclei containing acentric fragments, with coumestrol also inducing HPRT mutations (Kulling 1997).

7.7.2 In vivo phytoestrogen studies

In human studies, daily ingestion of 45 mg isoflavones for 1 month was found to increase the length of the follicular phase and delay menstruation in premenopausal women. Studies with postmenopausal subjects have been less conclusive, although soy supplemented diets did reduce the incidence of hot flushes over a 3 month period.

In addition, postmenopausal women given 60 g / d soya produced lower levels of LH, while 40 g / d linseed (rich in lignans) depressed both LH and FSH concentrations. This may be potentially beneficial with respect to risk factors for breast cancer (Cassidy 1998).

There remain concerns over neonatal and *in utero* exposure because during these developmental stages, sex steroids have a powerful effect on behaviour, reproductive physiology and central nervous system neurochemistry. Soy-based infant formulas have been available in the UK for over 20 years and account for approximately 7% of sales (20% in US) (Essex 1996)

In immature rats, coumestrol induces premature estrous cycles, and in mature rats, it disrupts the ovarian cycle, while, *in utero* exposure to genistein decreased birth weight and delayed the onset of puberty (Adlercreutz 1995).

However, powdered soybean decreases mammary tumour formation in rat breast cancer models (Barnes 1990), this is interesting since in addition to phytoestrogens, soy also contains potential anticarcinogens including protease inhibitors, phytosterols and saponins (Barnes 1990). In addition, many phytoestrogens, like coumestrol and genistein also possess a weak anti-oxidant activity, however, this latter effect is not believed to be their major mechanism of action (Mitchell 1998).

7.7.3 Conclusions

Taking the Asian observation as a starting point, one might conclude that phytoestrogens are protective against some diseases and pose no risk to fertility. However, Asians have been consuming these diets for centuries, and soy-related fertility problems may simply have been bred out of the population, making it difficult to draw direct conclusions.

Secondly, other phytochemicals have been identified with hormonal activity. The flavonoids apigenin and naringenin, and the cinnamic acid derivative syringic acid have been shown to possess weak progestational activity. In contrast, beta-carotene, chlorophylline and taxifolin are weak anti-androgens (Rosenburg 1998).

Following a recent meeting of the Senate Commission on the Evaluation of Food Safety (Cassidy 1998), it was proposed that phytoestrogens have the potential to act as:

★ estrogen agonists, which may reduce osteoporosis and heart disease in postmenopausal women, but may be adverse with respect to breast cancer.

★ antiestrogens which may reduce the risk of breast cancer.

★ developmental toxicants, that could potentially disrupt sexual differentiation by altering sex-specific patterns of development.

Although the epidemiological evidence for a protective role for phytoestrogens is strong, the mechanistic understanding of these agents is under-developed. We cannot rule out an association between plant products and increasing breast cancer in the west.

7.8 Increased breast cell proliferation alone may not mean increased breast cancer

Cumulative lifetime exposure to bioavailable estradiol links most known risk factors for breast cancer. Although estradiol induces cell proliferation in breast cells, the hallmark of cancer is DNA damage leading to mutation (Dipple 1995), with a number of specific mutations being highly associated with breast cancers (Callahan 1989). Estradiol-induced cell proliferation alone is unlikely to be solely responsible, which has lead to the hypothesis of a bifunctional genetic-hormonal pathway to breast cancer (Davis 1997). The most widely appreciated and investigated mechanism of estrogen action is the induction of cell proliferation (Preston-Martin 1990), and as we have demonstrated, numerous exogenous agents can also act as proliferaters. However it is also apparent that some E2 metabolites can cause DNA damage through redox cycling processes which generate reactive oxygen species (Liehr and Roy 1990).

7.8.1 Estradiol is metabolised to form reactive species

The metabolism of estradiol is a complex process involving several cytochrome P450 isozymes. Oxidation takes place primarily at C-2, but also C-4 to form catechols, at C-17 yielding estrone and at C-16 forming 16α-estrone. Each of these transformations represent intermediates in the generation of quinones and semiquinones, which can serve as substrates for redox cycling and the generation of ROS.

Increased levels of 16α-estrone correlate with mammary tumour incidence in humans and mice, and it has been shown to be genotoxic in the mouse (Liehr 1997). In addition it can bind covalently to ER causing a prolonged cell proliferation. Increased levels of catechol estrogens have also been seen in breast cancer patients (Adlercreutz 1986), with 4-OH E2 being both a persistent ER agonist (Barne 1983) and a carcinogen in the hamster kidney tumour model (Liehr et al. 1986). Recently it has become apparent that pesticides such as DDT and lindane alter the normal cellular metabolism of E2, increasing production of the ER agonist 16α-estrone, while reducing the production of antiestrogenic 4-OH metabolites (Bradlow 1995).

It is thought that the genotoxicity of these agents comes from the ability of semiquinone intermediates to react with molecular oxygen and form superoxide radicals (Liehr 1990), with DNA damage manifesting itself as DNA strand breaks and 8-hydroxylation of guanine bases. In MCF-7 breast cancer cells, Nutter et al. (1993) demonstrated the ROS forming abilities of 3,4-estrone-*o*-quinone. While more recently, research within our centre has shown that 10 nM E2 can induce strand breaks in MCF-7

cells, as detected using single cell gel electrophoresis (Rajapakse, personal communication).

Similar findings have been made with the synthetic estrogen DES. It is a well recognised carcinogen in humans and rodents, which has been shown to covalently bind DNA (Gladek 1989) and may also generate ROS (Wang 1994).

7.8.2 Food mutagens

Metabolites of E2 are not the only genotoxic agents with the potential to cause mutation in breast cancer cells. So-called "food mutagens" form a major class of genotoxic xenobiotic substances (Sugimura 1996). These include nitrosamines from fermented products, polycyclic aromatic hydrocarbons from heated food and heterocyclic amines from cooked meat and fish. Most of them are metabolically oxidized by cytochrome P450 isoforms and esterified. This yields the ultimately reactive forms which produce DNA adducts through electrophile and nucleophile reactions (Miller 1977). It is therefore possible that the DNA damaging potential of these agents leads to the mutational changes which result in breast cancer.

Experimental evidence for this is beginning to come to light following the discovery of genotoxic agents in human milk. Martin et al. (1999) have recently observed the ability of breast milk extracts to induce mutational responses in bacterial genotoxicity tests. In addition, they report measurable levels of DNA damage in viable exfoliated mammary cells, supporting the idea that DNA damage, not cell proliferation, is the major etiological factor in breast cancer.

7.8.3 A bifunctional genetic-hormonal pathway to breast cancer

The hypothesis therefore proposed is that there is an intimate interplay between agents which induce DNA damage (potential mutations) and those which increase cell proliferation (reduced repair time). This has the potential to lead to fixing of mutations within the genome and ultimately neoplastic transformation of cells.

E2 itself may well be such an agent. As we have noted, metabolites of E2 are able to cause measurable DNA damage as well as increase cellular proliferation. If this latter event pushes damaged cells through the cell cycle before repair has occurred, then mutations may become fixed and be passed on to subsequent generations. Mutations in key genes could in turn lead to neoplastic transformation of those cells, resulting in cancer.

With an added genotoxic burden from food mutagens, coupled with an increased cell turnover caused by estrogen-like xenobiotics, mutational events in breast cells may become more frequent. This could then explain the observed increases in breast cancer over the last 50 years.

7.8.4 Are we examining the relevant endpoint ?

The lack of epidemiological evidence for a major role for xenoestrogens in breast cancer, coupled with the necessity of mutational events, calls into question the relevance of ER activation as an endpoint in the study of breast cancer.

Recently, Enan and Matsumura (1998) have observed that *o,p'*-DDT and β-HCH are able to activate c-Neu tyrosine kinase at very low, physiologically relevant concentrations (0.1-1 nM). This event was not antagonised by tamoxifen and therefore

was thought not to be mediated via ER. c-Neu encodes an EGFR-like transmembrane protein which is increased in a large number of breast cancers, particularly those with a poor prognosis and a short relapse time (Slamon et al. 1987). This has strong implications for the role of these two agents and merits further study.

Further research is also required into the causes of mutation in breast cancer. A number of groups report the ability of estrogenic agents to induce transformed foci in cultured breast cancer cells, and this too may prove to be a more reliable marker of breast carcinogens (Zacharewski 1997).

In addition, rather than being driven by a systematic evaluation of individual candidate compounds, we may be better advise to examine the chemical components present in human serum or breast lipids. If this mixture itself proved to be carcinogenic then it may be more profitable to deconstruct this cocktail in order to ascertain the relevant agents.

7.9 Conclusions

Although we still cannot fully explain or address the rising incidence of breast cancer, by beginning to model the effects of mixtures of xenoestrogens, we have taken a large step forward in understanding the environmental causes of this disease.

References

Adlercreutz H. Phytoestrogens: Epidemiology and a possible role in cancer protection. Env Hlth Persp 103(suppl 7):113-122 (1995).

Adlercreutz H., Höckerstedt K., Bannwart C., Bloigu S., Hamalainen E., Fostis T. and Ollus A. Effect of dietary components including lignans and phytoestrogens, on enterohepatic circulation and liver metabolism of estrogens, and on sex hormone binding globulin (SHBG). J Steroid Biochem 27:1135-1144 (1987).

Adlercreutz H., Fostis T., Bannwart C., Hamalainen E., Bloigu S. and Ollus A. Urinary estrogen profile determination in young Finnish vegetarian and omnivorous women. J Steroid Biochem 24:289-296 (1986).

Ahlborg U.G., Lipworth L., Titus-Ernstoff L., Ghung-Cheng H., Hanberg A., Baron J., Trichopoulos D. and Adami H-O. Organochloride compounds in relation to breast cancer, endometrial cancer and endometriosis: An assessment of the biological and epidemiological evidence. Crit Rev Tox 25:463-531 (1995).

Altenburger R., Bödeker W., Faust M. and Grimme L. Evaluation of the isobologram method for the assessment of mixtures of chemicals. Ecotox Env Safe 20:98-114 (1990).

Altenburger R., Backhaus T., Boedeker W., Faust M., Scholze M. and Grimme L.H. Predictability of the toxicity of multiple chemical mixtures to *vibrio fischeri*: Mixtures composed of similarly acting chemicals. Env Tox Chem 19:2341-2347 (2000).

Andersen H., Andersson A., Arnold S., Autrup M., Beresford N., Bjerregaard P., Christiansen L., Gissel B., Hummel R., Jørgensen E., Korsgaard B., Guevel R., Leffers H., McLachlan J., Møller A., Nielsen J., Olea N., Oles-Karasko A., Pakdel F., Pedersen K., Perez P., Skakkebæk N., Sonnenschein C., Soto A., Sumpter J., Thorpe S., Grandjean P. Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. Env Hlth Persp 107(Supp 1):89-108 (1999).

Arnold S., Klotz D., Collins B., Vonier P., Guillette Jr L. and McLachlan J. Synergistic activation of the estrogen receptor with combinations of environmental chemicals. Science 272:1489-1492 (1996a).

Arnold S.F., Robinson M.K., Notides A.C., Guillette Jr. L.J. and McLachlan J.A. A yeast estrogen screen for examining the relative exposure of cells to natural and xenoestrogens Env Hlth Persp 104:544-548 (1996b).

Ashby J., Lefevre P.A., Odum J., Harris C.A., Routledge E.J. and Sumpter J.P. Synergy between synthetic estrogens ? Nature 385:494 (1997).

Auger J., Kunstmann K., Czyglik F. and Jarrell J. Decline in semen quality among fertile men in Paris during the past 20 years. New Eng J Med 332:281-285 (1995).

Backhaus T., Altenburger R., Boedeker W., Faust M., Scholze M. and Grimme L.H. Predictability of the toxicity of a multiple mixture of dissimilarly acting chemicals to *vibrio fischeri*. Env Tox Chem 19:2348-2356 (2000).

Barne E., MacLusky N. and Naftolin F. Kinetics of catechol-estrogen receptor dissociation: a possible factor underlying the differences in catecholestrogen biological activity. Steroids 41:643-656 (1983).

Barnes S., Grubbs C., Setchell K. and Carlson J. Soybeans inhibit mammary tumours in models of breast cancer. In: Mutagens and Carcinogens in the Diet (Pariza M., Aeschbacher H., Eton J. and Sato S. eds.). New York: Wiley-Liss pp. 239-253 (1990).

Barrett J. Phytoestrogens: Friends or foes ? Env Hlth Persp 104:478-482 (1996).

Berenbaum M.C. The expected effect of a combination of agents: the general solution. J Theoret Biol 114:413-431 (1985).

Berenbaum M.C. What is synergy ? Pharmacol Rev 41:93-141 (1989).

Bergeron J.M., Crews D. and McLachlan J.A. PCBs as environmental estrogens: Turtle sex determination as a biomarker of environmental contamination. Env Hlth Persp 102:780-782 (1994).

Berthois Y., Katzenellenbogen J. and Katzenellenbogen B. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. Proc Natl Acad Sci USA 83:2496-2500 (1986).

Bernstein L., Henderson B.E. and Hanisch R. Physical exercise activity reduces the risk of breast cancer in young women J Natl Cancer Inst 86:1403-1405 (1994).

Berrino F., Muti P., Micheli A., Bolelli G., Krogh V., Sciajno R,. Pisani P., Panico S. and Secreto G. Serum sex hormone levels after menopause and subsequent breast cancer. J Natl Cancer Inst 88:292-296 (1996).

Bitman J., Cecil H., Harris D. and Fries G. Estrogenic activity of *o,p'*-DDT in the mammalian uterus and avian oviduct. Science 162:371-372 (1968).

Bittner J.J. The causes and control of mammary cancer in mice. Harvey Lect 42:221-246 (1948).

Bliss C.I. The toxicity of poisons applied jointly. Ann Appl Biol 26:585-615 (1939).

Bradlow H., Davis D., Lin G., Sepkovic D. and Tiwari R. Effects of pesticides on the ratio of 16α/2-Hydroxyestrone: A biologic marker of breast cancer risk. Env Hlth Persp 103(suppl 7):147-150 (1995).

Bullock B., Newbold R. and McLachlan J. Lesions of testis and epididymis associated with prenatal diethylstilbestrol. Env Hlth Persp 77:29-31 (1988).

Bunone G., Briand P-A., Miksiek R. and Picard D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 15:2174-2183 (1996).

Burrow M., Weldon C., Tang Y., Navar G., Krajewski S., Reed J., Hammond T., Clejan S. and Beckman B. Differences in susceptibility to tumour necrosis factor α-induced apoptosis among MCF-7 breast cancer cell variants. Cancer Res 58:4940-4946 (1998).

Callahan R. and Campbell G. Mutations in human breast cancer: An overview. J Natl Cancer Inst 81:1780-1786 (1989).

Campbell P. and Hutchinson T. Wildlife and endocrine disrupters: Requirements for hazard identification. Env Tox Chem 17:127-135 (1998).

Carlsen E, Giwercman A., Keiding N. and Skakkebaek N. Evidence for decreasing quality of semen during past 50 years. BMJ 305:609-613 (1992).

Cassidy A. Risks and benefits of phytoestrogen rich diets. In: Hormonally active agents in food (Eisenbrand G. et al. eds) Wiley-VCH pp. 91-117 (1998).

Chen C., Hurd C., Vorojeikina D., Arnold S. and Notides A. Transcriptional activation of the human estrogen receptor by DDT isomers and metabolites in yeast and MCF-7 cells. Biochem Pharmacol 53:1161-1172 (1997).

Colborn T., vom Saal F. and Soto A. Developmental effect of endocrine disrupting chemicals in wildlife and humans. Env Health Persp 101:378-385 (1993).

Coldham N., Dave M., Sivapathasundaram S., McDonnel D., Connor C. and Sauer M. Evaluation of a recombinant yeast cell estrogen screening assay. Env Hlth Persp 105:734-742 (1997).

Cole M., Jones C. and Todd I. A new antiestrogenic agent in late breast cancer: an early pre-clinical appraisal of ICI 46, 474. Br J Cancer 25:270-275 (1971).

Davis D., Telang N., Osborne M. and Bradlow H. Medical hypothesis: Bifunctional genetic-hormonal pathways to breast cancer. Env Hlth Persp 105(suppl 3):571-576 (1997).

Davis D.L., Bradlow H.L., Wolff M., Woodruff T., Hoel D.G. and Anton-Culver H. Medical hypothesis: Xenoestrogens as preventable causes of breast cancer Env Hlth Persp 101:372-377 (1993).

De Cupis A., Schettini G. and Favoni R. New vs old fashioned estradiol antagonists in mammary carcinoma: 'In vitro' and 'in vivo' pharmacological approaches. Pharm Res 39:335-344 (1999).

Dipple A. DNA adducts of chemical carcinogens. Carcinogenesis 16:437-441(1995).

Dorgan J., Longcope C., Stephenson H., Falk R., Miller R., Franz C., Kahle L., Campbell W., Tangrea J. and Schatzkin A. Serum sex hormone levels are related to breast cancer risk in postmenopausal women. Env Hlth Persp 105(suppl 3):583-585 (1997).

Drescher K. and Bödeker W. Assessment of the combined effects of substances: The relationship between concentration addition and independent action. Biometrics 51:716-730 (1995).

Dudley M., Sheeler C., Wang H. and Khan S. Activation of the human estrogen receptor by the antiestrogens ICI 182, 780 and tamoxifen in yeast genetic systems: Implications for their mechanism of action. PNAS 97:3696-3701 (2000).

Enan E. and Matsumura F. Activation of c-Neu tyrosine kinase by o,p'-DDT and β-HCH in cell-free and intact preparations from MCF-7 human breast cancer cells. J Biochem Mol Tox 12:83-92 (1998).

Essex C. Phytoestrogens and soy based infant formula. BMJ 313:507-508 (1996).

Falck F., Ricci A., Wolff M., Godbold J. and Deckers P. Pesticides and polychlorinated biphenyl residues in human breast lipids and their relation to breast cancer. Arch Env Hlth 47:143-146 (1992)

Farber E. Cancer development and its natural history. A cancer prevention perspective. Cancer 62(suppl 8):1676-1679 (1988).

Farrow S. Falling sperm quality: fact or fiction ? BMJ 309:1-2 (1994).

Faust M., Altenburger R., Backhaus T., Bödeker W., Scholze M. and Grimme L. Hazard assessment of multiple chemical mixtures. J Eur Qual (in press).

Favoni R. and De Cupis A. Steroidal and nonsteroidal estrogen antagonists in breast cancer: basic and clinical appraisal. Trend Pharm Sci 19:406-415 (1998).

Feigelson H.S. and Henderson B.E. Estrogens and breast cancer. Carcinogenesis 17:2279-2284 (1996).

Forman D. and Moller H. Testicular cancer. Cancer Surveys 19/20:323-341 (1994).

Fraser T.R. Title unavailable. BMJ 2:485-487 (1872).

Fry D.M. Reproductive effects in birds exposed to pesticides and industrial chemicals. Env Hlth Persp 103(suppl 7):165-171 (1995).

Fry D.M. and Toone C.K. DDT-induced feminisation of gull embryos. Science 213:922-923 (1981).

Gaido K., Leonard L., Lovell S., Gould J., Babai D., Portier C. and McDonnel D. Evaluation of chemicals with endocrine modulating activity in a yeast-based steroid hormone receptor gene transcription assay. Tox Appl Pharmacol 143:205-212 (1997a).

Gaido K., McDonnell D., Korach K. and Safe S. Estrogenic activity of chemical mixtures: Is there synergism ? Act Chem Ind Inst Tox 17:1-7 (1997b).

Gladek A. and Liehr J. Mechanism of genotoxicity of diethylstilbestrol in vivo. J Biol Chem 264:16857-16852 (1989).

Gooch J. and Yee D. Strain-specific differences in formation of apoptotic DNA ladders in MCF-7 breast cancer cells. Cancer Lett 144:31-37 (1999).

Grant A. From "Big yellow taxi". A&M records USA (1994).

Greco W., Bravo G . and Parsons J. The search for synergy: A critical review from a response surface perspective. Pharmacol Rev 47:331-385 (1995).

Green S., Walter P., Kumar V., Krust A., Bornert J., Argos P. and Chambon P. Human estrogen receptor cDNA: sequence, expression and homology to v-*erb*-*A*. Nature 320:134-139 (1986).

Guillette Jr. L.J., Gross T.S., Masson G.R., Matter J.M., Percival H.F. and Woodward A.R. Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Env Hlth Persp 102:680-688 (1994).

Hammond B., Katzenellenbogen B.S., Krauthammer N. and McConnel J. Estrogenic activity of the insecticide chlordecone (Kepone) and interaction with uterine estrogen receptors. Proc Natl Acad Sci USA 76:6641-6645 (1979).

Hansen L.G. and Jansen H.T. Environmental estrogens. Science 266:526 (1994).

Harris C.A., Henttu P., Parker M.G. and Sumpter J.P. The estrogenic activity of phthalate esters *in vitro.* Env Hlth Persp 105:802-811 (1997).

Hartmann A., Blaszyk H., Kovach J. and Sommer S. The molecular epidemiology of p53 gene mutations in human breast cancer 13:27-33 (1997).

Helzlsouer K., Alberg M., Bush T., Longcope C., Gordon G. and Comstock G. A prospective study of endogenous hormones and breast cancer. Cancer Detect Prevent 18:79-85 (1994).

Henderson B.E. and Bernstein L. Endogenous and exogenous hormonal factors in breast cancer. In Diseases of the breast, Harris J.R., Morrow M., Lippman M.E. and Helman S. (eds.), Lippincott-Raven, Pilladelphia pp185-200 (1996).

Henderson B.E., Ross R. and Bernstein L. Estrogens as a cause of human cancer: The Richard and Hinda Rosenthal foundation award lecture. Cancer Res 48:246-253 (1988).

Henderson B., Ross R., Judd H., Krailo M. and Pike M. Do regulatory ovulatory cycles increase breast cancer risk. Cancer 56:1206-1208 (1985).

Hertz R. The estrogen problem - restrospect and prospect. In: Estrogens in the environment II (J. McLachlan ed.), New York: Elsevier, pp 1-11(1985).

Hilakivi-Clarke L. Estrogens, BRCA1 and breast cancer. Cancer Res 60:4993-5001 (2000).

Hunter D., Hankinson S., Laden F., Colditz G., Manson J., Willett W., Speizer F. and Wolff M. Plasma organochlorine levels and the risk of breast cancer. New Eng J Med 337:1253-1259 (1997).

Ingvarsson S. Molecular genetics of cancer progression. Semminars Cancer Biol 9:277-288 (1999).

Jaiyesimi I., Buzdar A., Decker d. and Hortobagyi G. Use of tamoxifen for breast cancer: twenty-eight years later. J Clin Oncol 13:513-529 (1995).

Jobling S., Reynolds T., White R., Parker M.G. and Sumpter J.P. A variety of environmentally persistent chemicals, including some phthalate plasticisers, are weakly estrogenic. Env Hlth Persp 103:582-587 (1995).

Jones C., Payne J., Wells D., Delhanty J., Kortenkamp A. and Lakhani S. Comparative genomic hybridisation reveals extensive variation amongst different MCF-7 cell stocks. Cancer Genet Cytogenet 117:153-158 (2000).

Jones P., Barker V., Irwin A. and Earl L. Modulation of MCF-7 cell proliferative responses by manipulation of assay conditions. Tox In Vitro 11:769-773 (1997).

Jordan V., Koch R. and Bain R. Prolactin synthesis by cultured rat pituitary cells: An assay to study estrogens, antiestrogens and their metabolites in vitro. In: Estrogens in the environment II: Influences on development (McLachlan J. Ed.). New York: Elsevier pp. 221-234 (1985).

Jordan V. and Morrow M. An appraisal of strategies to reduce the incidence of breast cancer. Stem Cells 11:252-262 (1993).

Kallioneimi A., Kallioneimi O., Piper J., Tanner M., Stokke T., Chen L., Smith H., Pinkel D., Gray J. and Waldman F. Detection and mapping of amplified DNA sequences in breast cancer by comparative cytogenetic hybridisation. Proc Natl Acad Sci USA 91:2156-2160 (1994).

Kallioneimi O., Holli K., Visakorpi T., Koivula T., Helin H. and Isola J. Association of c-erbB-2 protein over-expression with high rate of cell proliferation, increased risk of visceral metastasis and poor long-term survival in breast cancer. Int J Cancer 49:650-655 (1991).

Katzenellenbogen B., Kendra K., Norman M. and Berthois Y. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. Cancer Res 47:4355-4360 (1987).

Katzenellenbogen J. The structural pervasiveness of estrogenic activity. Env Health Persp 103(suppl 7):99-101 (1995).

Kelce W., Stone C., Laws S., Gray L., Kemppainen J. and Wilson E. Persistent DDT metabolite *p,p'*-DDE is a potent androgen receptor antagonist. Nature 375:581-583 (1995).

Kelsey J.L. and Bernstein L. Epidemiology and prevention of breast cancer. Ann Rev Pub Hlth 17:47-67 (1996).

Kendall R., Dickerson R., Giesy J. and Suk W. Principles and processes for evaluating endocrine disruption in wildlife. SETAC press, Pensacola, FL. pp 491 (1998).

Klotz D., Beckman B., Hill S., McLachlan J., Walters M. and Arnold S. Identification of environmental chemicals with estrogenic activity using a combination of *in vitro* assays. Env Health Persp 104:1084-1089 (1996).

Kortenkamp A. and Altenburger R. Synergisms with environmental estrogens: A reevaluation using the method of isoboles. Sci Tot Env 221:59-73 (1998).

Krieger N., Wolff M.S., Hiatt R.A., Rivera M., Vogelman J. and Orentreich N. Breast cancer and serum organochlorines: a prospective study among white, black and asian women. J Natl Cancer Inst 86:589-599 (1994).

Krishnan V., Heath H. Bryant H. Mechanism of action of estrogens and selective estrogen receptor modulators. Vitam Horm 60:123-47 (2000).

Kuiper G., Enmark E., Pelto-huikko M., Nilsson S. and Gustafsson J-A. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925-5930 (1996).

Kuiper G., Lemmen J., Carlsson B., Corton J., Safe S., Van der Saag P., Burg B. and Gustafsson J-A. Interaction of estrognic chemicals and phytoestrogens with estrogen receptor beta. Endocrinol 139:4252-4263 (1998).

Kulling S. and Metzler M. Induction of micronuclei, DNA strand breaks and HPRT mutations in cultured Chinese hamster V79 cells by the phytoestrogen coumestrol. Food Chem Tox 35:605-13 (1997).

Lakhani S., Chaggar R., Daviees S., Jones C., Collins N., Odel C., Stratton M. and O'Hare M. Genetic alterations in 'normal' luminal and myoepithelial cells of the breast. J Pathol 189:496-503 (1999).

Lee E. Tumour suppressor genes and their alterations in breast cancer. Semminars Cancer Biol 6:119-125 (1995).

Liehr J. Hormone-associated cancer: Mechanistic similarities between human breast cancer and estrogen-induced kidney carcinogenesis in hamsters. Env Hlth Persp 105(supp 3):565-569 (1997).

Liehr J. and Roy D. Free radical generation by redox cycling of estrogens. Free Radic Biol Med 8:415-423 (1990).

Liehr J., Gang W.F., Sirbasku D. and Ulubelen A. Carcinogenicity of catechol estrogens in Syrian hamsters. J Steroid Biochem 24:353-356 (1986).

Loewe S. and Muischneck H. Über Kombinationswirkungen 1. Mitteilung: Hiffsmittel der Fragestellung. Naunyn-Schmeidebergs Arch Exp Pathol Pharmakol 77:313-326 (1926).

López-Carillo L., Blair A., López-Cervantes M., Cebrián M,. Rueda C., Reyes R., Mohar A. and Bravo J. Dichlorodiphenyltrichloroethane serum levels and breast cancer risk: A case control study from Mexico. Cancer Res 57:3728-3732 (1997).

Martin F., Cole K., Weaver G., Williams A., Millar B., Grover P. and Phillips D. Genotoxicity of human milk extracts and detection of DNA damage in exfoliated cells recovered from breast milk. Biochem Biophys Res Commun 257:319-326 (1999).

McLaclan J. Synergistic effect of environmental estrogens: Report withdrawn. Science 277:463 (1997).

Miller J. and Miller E. Ultimate chemical carcinogens as reactive mutagenic electrophiles. In: Origins of human cancer, Book B (Hiatt H., Watson J. and Winsten J. eds.). Cold Spring Harbour, NY. pp. 605-627 (1977).

Mitchell J., Gardner P., McPhail D., Morrice P., Collins A. and Duthie G. Antioxidant efficacy of phytoestrogens in chemical and biological model systems. Arch Biochem Biophys 360:142-148 (1998).

Muir C., Waterhouse J., Mack T., Powell J. and Whelan S. Cancer incidence in five continents. International Agency for Research on Cancer (IARC Scientific publication No. 88), vol. 5. Lyon, France (1987).

Nagel S., Vom Saal F., and Welshons W. The effective free fraction of estradiol and xenoestrogens in human serum measured by whole cell uptake assays: Physiology and delivery modifies estrognic activity. Proc Soc Exp Biol Med 217:300-308 (1998).

Nandi S., Guzman R.C. and Yang J. Hormones and mammary carcinogenesis in mice, rats and humans: A unifying hypothesis. Proc Natl Acad Sci USA 92:3650-3657 (1995).

Nimrod A.C. and Benson W.H. Environmental estrogenic effects of alkylphenol ethoxylates. Crit Rev Tox 26:335-364 (1996).

Nutter L., Ngo E. Abul-Hajj Y. Characterisation of DNA damage induced by 3,4-estrone-*o*-quinone in human cells. J Biol Chem 266:16380-16386 (1993).

Olea N., Pulgar R., Prerez P., Olea-Serrano F., Rivas A., Novillo-Fertell A., Pedraza V., Soto A. and Sonnenschein C. Estrogenicity of resin-based composites and sealants used in dentistry. Env Health Persp 104:298-305 (1996).

Osborne C., Zhao H. and Fuqua S. Selective estrogen receptor modulators: structure, function and clinical use. J Clin Oncol 18:3172-3186 (2000).

Parker M. Structure and function of estrogen receptors. Vitamins and Hormones 51:267-287 (1995).

Payne J., Jones C., Lakhani S. and Kortenkamp A. Improving the reproducibility of the MCF-7 cell proliferation assay for the detection of xenoestrogens. Sci Tot Env 248:51-62 (2000).

Pike M., Bernstein L. and Spicer D. Exogenous hormones and breast cancer risk. In Neiderhuber J. (ed.) Current therapy in oncology. BC Decker, St Louis pp292-302 (1993).

Pöch G. Combined effects of drugs and toxic agents. Modern evaluation in theory and practice. Springer. Wein (1993).

Preston-Martin S., Pike M., Ross R., Jones P. and Henderson B. Increased cell division as a cause of human cancer. Cancer Res 50:7415-7421 (1990).

Price K. and Fenwick G. Naturally occurring estrogens in foods - A review. Food Adit Contam 2:73-106 (1985).

Purdom C., Hardiman P., Bye V., Eno N., Tyler C and Sumpter J. Estrogenic effects of effluents from sewage treatment works. Chem Ecol 8:275-285 (1994).

Rajapakse N., personal communication.

Ramamoorthy K., Wang F., Chen I-C., Norris J.D., McDonnell D.P., Leonard L.S., Gaido K.W., Bocchinfuso W.P., Korach K.S. and Safe S. Estrogenic activity of a dieldrin/toxaphene mixture in the mouse uterus, MCF-7 human breast cancer cells, and yeast-based estrogen receptor assays: No apparent synergism. Science 275:405 (1997).

Risebrough R. Endocrine disruption: Awkward questions for the environmental community. SETAC-Europe news 10:12-13 (1999).

Rohan T.E and Bain C.J. Diet in the etiology of breast cancer. Epidemiol Rev 9:120-145 (1987).

Rosenburg R., Grass L., Jenkins D., Kendall C. and Diamandis E. Modulation of androgen and progestrerone receptors by phytochemicals in breast cancer cell lines. Biochem Biophys Res Commun 248:935-939 (1998).

Routledge E. and Sumpter J. Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen. Env Tox Chem 15:241-248 (1996).

Saceda M., Lippman M., Chambon P., Lindsey R., Ponglikitmongkol M., Puente M. and Martin M. Regulation of the estrogen receptor in MCF-7 cells by estradiol. Mol Endocrinol 2:1157-1162 (1988).

Safe S. Environmental and dietary estrogens and human health: Is there a problem ? Env Hlth Persp 103:346-351 (1995).

Sathyamoorthy N., Wang T. and Phang J. Stimulation of pS2 expression by diet derived compounds. Cancer Res 54:957-961 (1994).

Schatz R., Soto A. and Sonnenschein C. Estrogen-induced cell multiplication: Direct or indirect effect on rat uterine cells ? Endocrinology 115:501-506 (1984).

Semenza J.C., Tolbert P.E., Rubin C.H., Guillette Jr. L.J. and Jackson R.J. Reproductive toxins and alligator abnormalities at Lake Apopka, Florida. Env Hlth Persp 105:1030-1032 (1997).

Sharpe R. and Skakkebaek N. Are estrogens involved in falling sperm counts and disorders of the male reproductive tract ? Lancet 341:1392-1395 (1993).

Shekhar P.V., Werdell J. and Basrur V.S. Environmental estrogen stimulation of growth and estrogen receptor function in preneoplastic and cancerous human breast cell lines. J Natl Cancer Inst 89:1774-1782 (1997).

Singer P.L. Occupational oligospermia. JAMA 140:1249 (1949).

Skehan P., Storeng R., Scudiero D., Monks A., McMahon J., Vistica D., Warren J., Bokesch H., Kennedy S. and Boyd M. New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82:1107-1112 (1990).

Slamon S.D., Godolphin W., Jones L., Holt J., Wong S., Keith D., Levin W., Stuart S., Udove J., Ullrich A. and Press M. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244:707-712 (1987).

Soto A., Chung K. and Sonnenschein C. The pesticides endosulfan, toxaphene and dieldrin have estrogenic effects on human estrogen-sensitive cells. Env Hlth Persp 102:380-383 (1994).

Soto A., Sonnenschein C., Chung K., Fernandez M., Olea N. and Olea Serrano F. The E-SCREEN assay as a tool to identify estrogens: An update on estrogenic environmental pollutants. Env Hlth Persp 103(suppl 7):113-122 (1995).

Soto A., Fernandez M., Luizzi M., Oles Karasko A. and Sonnenschein C. Developing a marker of exposure to xenoestrogen mixtures in human serum. Env Hlth Persp 105(suppl 3):647-654 (1997).

Soule H., Vazquez J., Long A., Albert S. and Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst 51:1409-1413 (1973).

Steinmetz R., Young P., Caperell-Grant A., Gize E., Madhukar B., Ben-Jonathan N. and Bigsby R. Novel estrogenic action of the pesticide residue β-hexachlorocyclohexane in human breast cancer cells. Cancer Res 56:5403-5409 (1996).

Strauss L., Santti R., Saarinen N., Streng T., Joshi S. and Makela S. Dietary phytoestrogens and their role in hormonally dependent disease. Toxicol Lett 102-103:349-354 (1998).

Sugimura T., Nagao M. and Wakabayashi K. Carcinogenicity of food mutagens. Env Hlth Persp 104(Suppl 3):429-433 (1996).

Sumpter J. and Jobling S. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Env Hlth Persp 103(suppl 7):173-178 (1995).
Swann S., Elkin E. and Fenster L. Have sperm densities declined ? A reanalysis of global trend data. Env Hlth Persp 105:1228-1232 (1997).

Thomas J. Falling sperm counts. Lancet 346:635 (1995).

Tirkkonen M., Tanner M., Karhu R., Kallioneimi A., Isola J. and Kallioneimi O. Molecular cytogenetics of primary breast cancer by CGH. Genes Chromosom Cancer 21:177-184 (1998).

Toniolo P. Endogenous estrogens and breast cancer risk: The case for prospective cohort studies. Env Hlth Persp 105(Suppl 3):587-592 (1997).

Toniolo P.G., Levitz M., Zeleniuch-Jacquotte A., Banerjee S., Koenig K.L., Shore R.E., Strax P. and Pasternack B.S. A prospective study of endogenous estrogens and breast cancer in postmenopausal women. J Natl Cancer Inst 87:190-197 (1995).

Toppari J., Larsen J., Christiansen P., Giwercman A., Grandjean P., Guillette Jr. L.J., Jegou B., Jensen T.K., Jouannet P., Keiding N., Leffers H., McLachlan J.A., Meyer O., Muller J., Rajpert-De Meyts E., Sheike T., Sharpe R., Sumpter J. and Skakkebaek N.E. Male reproduction and environmental chemicals. Env Hlth Persp 104(suppl 4):741-803 (1996).

Van 't Veer P., Lobbezoo I., Martin-Moreno J., Guallar E., Gómez-Aracena J., Kardinaal A., Kohlmeier L., Martin B., Strain J., Thamm M., van Zoonen P., Baumann B., Huttunen J. and Kok F. DDT (dicophane) and postmenopausal breast cancer in Europe:case-control study. BMJ 315:81-85 (1997).

Venitt S. and Parry J. In, Mutagenicity testing (Rickwood D. and Hames B.eds.), IRL press, Oxford, p 146 (1984).

Villalobos M., Olea N., Brotons J., Olea Serrano M., Ruiz de Almodovar J. and Pedraza V. The E-Screen assay: A comparison of different MCF-7 cell stocks. Env Hlth Persp 103:844-850 (1995).

Vos C., Cleton-Jansen A., Berx G., de LeeuwW., ter Haaar N., va Roy F., Cornelisse C., Peterse J. and van de Vijer M. E-cadherin inactivation in lobular carcinoma *in situ* of the breast: an early event in tumourigenesis. Br J Cancer 76:1131-1133 (1997).

Walz E. Isoflavon- and saponin-glocuside in *Soja hispida*. Justus Leibigs Annal Chem 498:118-155 (1931).

Wang C. and Kurzer M. Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. Nutr Cancer 28:236-247 (1997).

Wang T., Sathyamoorthy N. and Phang J. Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis 17:271-275 (1996).

Wang M-Y. and Liehr J. Identification of fatty acid hydroperoxide cofactors in the cytochrome P450-mediated oxidation of estrogens to quinone metabolites: role and balance of lipid peroxides during estrogen induced carcinogenesis. J Biol Chem 269:284-291 (1994).

Watanabe T., Inoue S., Ogawa S., Ishii Y., Hiroi H., Ikeda K., Orimo A. and Muramatsu M. Agonist effect of tamoxifen is dependent on cell type, ERE-promoter context and estrogen receptor subtype: Functional differences between estrogen receptors α and β. Biochem Biophys Res Commun 236:140-145 (1997).

Weise T. and Kelce W. An introduction to environmental estrogens. Chemistry & Industry 18 August, 648-653 (1997).

Welch R.M., Levin W. and Conney A.H. Estrogenic activity of DDT and its analogues Tox Appl Pharmacol 14:358-367 (1969).

Whorton D., Krauss R.M., Marshall S. and Milby T.H. Infertility in male pesticide workers Lancet Dec 17, 1259-1261 (1977).

Willard S. and Frawley L. Phytoestrogens have agonistic and combinatorial effects on estrogen-responsive gene expression in MCF-7 human breast cancer cells. Endocrine 8:117-121 (1998).

Wolff M.S., Toniolo P.G., Lee E.W., Rivera M. and Dubin N. Blood levels of organochlorine residues and risk of breast cancer. J Natl Cancer Inst 85:648-652 (1993).

Wolff M. and Toniolo P. Environmental organochlorine exposure as a potential etiological factor in breast cancer. Env Hlth Persp 103(suppl 7):141-145 (1995).

Wolff M., Collman G., Barrett J. and Huff J. Breast cancer and environmental risk factors: Epidemiological and experimental findings. Annu Rev Pharmacol Toxicol 36:573-596 (1996).

Wolff M. and Weston A. Breast cancer risk and environmental exposure. Env Hlth Persp 105(suppl 4):891-896 (1997).

Zacharewski T. *In vitro* bioassays for assessing estrogenic substances. Env Sci Technol 31:613-623 (1997).

Zava D., Blen M. and Duwe G. Estrogenic activity of natural and synthetic estrogens in human breast cancer cells in culture. Env Hlth Persp 105(suppl 3):637-645 (1997).

Appendix 1

Yeast media

Yeast estrogen screen medium

Preparation and storage of components

All glassware critically cleaned before use and spatulas washed twice in ethanol.

Minimal Medium

Add 13.61g KH_2PO_4, 1.98g $(NH_4)_2SO_4$, 4.2g KOH pellets, 0.2g $MgSO_4$, 1 ml $Fe_2(SO_4)_3$ solution (40 mg / 50 ml H2O), 50 mg L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine and 375 mg L-serine to 1 L ultra high quality water (UHQ).

Place on heated stirrer to dissolve.

Dispense 45 ml aliqouts to glass bottles, sterilise (121 °C, 10 min) and store at room temp.

Vitamin solution

Add 8 mg thiamine, 8 mg pyridoxine, 8 mg pantothenic acid, 40 mg inositol and 20 ml biotin solution (2 mg / 100 ml H2O) to 180 ml UHQ water.

Sterilise via filtration (0.2 µm) 10 ml aliquots to glass containers.

Store at 4 °C.

D-(+)-Glucose

Prepare a 20% w/v solution and sterilise 20 ml aliquots (121 °C, 10 min).
Store at room temperature.

L-Aspartic acid

Prepare a solution of 4 mg / ml and sterilise 20 ml aliquots (121 °C, 10 min).
Store at room temperature.

L-Threonine

Prepare a solution of 24 mg / ml and sterilise 20 ml aliquots (121 °C, 10 min).
Store at 4 °C

Copper (II) sulphate

Prepare a 20 mM solution and sterilise via filtration (0.2 µm) 5 ml aliquots to glass containers.
Store at room temperature.

Chlorophenol red-β-D-galactopyranoside (CPRG)

Prepare a 10 mg / ml solution and sterilise via filtration (0.2 µm) 5 ml aliquots to glass containers.
Store at 4 °C.

Preparation of growth medium

Add 5 ml glucose solution, 1.25 ml L-aspartic acid solution, 0.5 ml vitamin solution, 0.4 ml L-threonine and 125 µl copper (II) sulphate solution to 45 ml minimal medium.

Preparation of assay medium

Add 0.5ml CPRG to 50 ml fresh growth medium.

Appendix 2

Presentations and publications

Presentations and publications

As well as being the subject of regular internal peer review, this work has been put forward as a number of presentations and publications.

Presentations

Payne J.P., Mclean J. and Kortenkamp A. An improved protocol for the detection of estrogenic agents using the MCF-7 cell proliferation assay. 9th Annual Meeting of SETAC Europe, 25-29 May 1999, Leipzig, Germany.

Payne J.P. and Kortenkamp A. Predicting and assessing the effect of a four component mixture of xenoestrogens. 9th Annual Meeting of SETAC Europe, 25-29 May 1999, Leipzig, Germany.

Kortenkamp A., Payne J.P. and Altenburger R. The potential impact of weakly estrogenic environmental pollutants on the effects of endogenous estrogens. 9th Annual Meeting of SETAC Europe, 25-29 May 1999, Leipzig, Germany.

Silva E., Ives J., Payne J.P. and Kortenkamp A. Determination of estrogen-induced gene expression in MCF-7 human breast cancer cells. 9th Annual Meeting of SETAC Europe, 25-29 May 1999, Leipzig, Germany.

Payne J.P., McLean J., Hasskamp J. and Kortenkamp A. Detecting environmental estrogens using the MCF-7 cell proliferation assay. One day meeting of the UKEMS Molecular Epidemiology Group. Environmental estrogens-do they pose a human risk? 6th May 1998, London, UK.

Payne J.P., Hasskamp A. and Kortenkamp A. Combination effects of binary mixtures of estrogenic agents in vitro. 8th Annual Meeting of SETAC Europe, 14-18 April 1998, Bordeaux, France.

Publications

Jones C., Payne J., Wells D., Delhanty J., Kortenkamp A. and Lakhani S. Comparative genomic hybridisation reveals extensive variation amongst different MCF-7 cell stocks. Cancer Genet Cytogenet 117:152-158 (2000).

Payne J., Jones C., Lakhani S. and Kortenkamp A. Improving the reproducibility of the MCF-7 cell proliferation assay for the detection of xenoestrogens. Sci Tot Env 248:51-62 (2000).

Payne J., Rajapakse N., Wilkins M. and Kortenkamp A. Prediction and assessment of the effects of four xenoestrogens. Env Hlth Persp 108:983-987 (2000).

Payne J., Scholze M. and Kortenkamp A. Mixtures of four organochlorines enhance human breast cancer cell proliferation. Env Hlth Persp (in press).

One further manuscript based on the work of Chapter 6 is currently being prepared.