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Evaluation of a novel method of predicting estrogen activity of a group of structurally diverse compounds

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EVALUATION OF A NOVEL METHOD OF PREDICTING ESTROGEN ACTIVITY OF A GROUP OF STRUCTURALLY DIVERSE COMPOUNDS

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ABSTRACT

The number of environmental chemicals found to have some level of endocrine activity has led to concern about the possible effects these compounds could have on human health and the health of other species, populations, and possibly whole ecosystems. The United States Environmental Protection Agency has been charged with testing a large number of these compounds, called endocrine-disrupting chemicals or hormonally active agents for hormonal activity. Limited testing resources have led to a call for alternate methods of screening, possibly for use in prioritizing this list to assist in efficient allocation of resources for further testing. This study describes a computational method, the categorical structure activity relationship (cat-SAR) program, which has demonstrated high predictivity for the estrogen-like activity of a set of diverse chemical structures. The data set for this model was taken from a set of 122 compounds assayed for estrogenicity with the ESCREEN assay, an *in vitro* assay for estrogenicity. Two endpoints were modeled. The model for relative proliferative potency demonstrated an 82% correct prediction rate, while the relative proliferative effect achieved an 86% correct rate of prediction in model validation. Preliminary evaluation of fragments upon which the models were based suggested a sound mechanistic basis. The models also compared similarly to previous ESCREEN models developed using a different methodology. Based on the results described herein, the cat-SAR method would be a useful approach in screening compounds for estrogen activity as well as for investigating their mechanism of action.

CHAPTER 1. INTRODUCTION

As reported by the Committee on Hormonally Active Agents in the Environment, appointed by the National Research Council in 1995, observed cases of developmental and reproductive abnormalities in certain animal populations exposed to varying levels and mixtures of synthetic chemicals have heightened concerns about the potential for some xenobiotics to interfere with normal endocrine function (Kavlock 1996). Endocrine disruptors, also called hormonally active agents, are recognized as exogenous compounds known to disrupt endocrine signaling pathways. The United States Environmental Protection Agency (EPA) has defined an endocrine disruptor as "an exogenous chemical substance or mixture that alters the structure or function(s) of the endocrine system and causes adverse effects at the level of the organism, its progeny, populations, or subpopulations of organisms, based on scientific principles, data weight-of-evidence, and the precautionary principle" (EPA 1998). The mechanisms for endocrine disruption include direct binding and activation of receptors of endogenous endocrine signaling agents, blocking receptors without activation, or interfering with normal hormone levels or metabolism (Witorsch 2000).

The EPA has been given the mandate, under the Food Quality Protection Act of 1996, to assess the endocrine disruptive activity of over 87,000 chemicals (EPA 1998). To this end, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was formed to make recommendations to the EPA on how to develop screening strategies. EDSTAC presented its final report in 1998, laying out a recommended approach to the problem (EPA 1998). The EPA has a stated goal to develop quantitative structure-activity relationship (QSAR) tools for pre-screening these chemicals for further testing (Timms 2002).

This study has two purposes. The first of these is to contribute to the development and evaluation of a new structure-activity relationship (SAR) modeling method. Called the categorical structural activity relationship (cat-SAR) method, this approach uses two-dimensional chemical fragments to model binary response data for a particular biological activity. The method produces categorical predictions of biological activity (active/inactive). Cat-SAR is mechanistically and functionally transparent, evaluating all possible fragments in the learning set. This study evaluates the performance of a cat-SAR model of estrogen activity, in this case a compound's ability to induce estrogen-like MCF-7 breast tumor cell proliferation, for a set of structurally diverse structures.

The second objective of the study is to evaluate fragments contributing to the model in light of known estrogen receptor (ER) binding information. The mechanistic validity of the model was studied by comparing the fragments used by the model to the known receptor-binding characteristics of the estradiol pharmacophore.

CHAPTER 2. LITERATURE REVIEW

2.1 Environmental estrogens

Endocrine disruptors include estrogens, antiestrogens, androgens, antiandrogens, aryl hydrocarbon receptor agonists and antagonists, and others. The majority of the research to date, however, has been focused on compounds with estrogenic (estrogen like), and to a lesser extent antiestrogenic activity. Diverse environmental chemicals have been shown to have the potential to interact with human estrogen receptors. The list of potentially estrogenic compounds includes phytoestrogens, alkylphenols, polychlorinated biphenyls, drugs, pesticides, industrial chemicals, and others (Katzenellenbogen 1995). The diversity in chemical structure of estrogenic compounds may be due, at least in part, to the relative promiscuity of mammalian estrogen receptors (Elsby 2000, Barnes 2001). The majority of these compounds are significantly weaker than endogenous 17β-estradiol (E_2) in eliciting a estrogenic response (Safe 1994).

Phytoestrogens and synthetic estrogens are often considered to be separate classes. Phytoestrogens are produced by plants for a variety of signaling purposes, both endogenous and exogenous (Moggs 2004). Endogenously, these purposes include triggering production of scents or colors. The exogenous effects of these phytoestrogens often involve signaling bacteria, fungi, and other plants. One notable function of phytoestrogens is their use by legumes to entice symbiotic fungi and bacteria to take up residence in their root nodules. (Peters 1988). Some of these signaling agents also have effects in nontarget organisms (Fox 2004.) Some studies indicate associations between levels of synthetic estrogens and reproductive tract abnormalities, altered sexual differentiation and other effects. These studies seem to imply synthetic estrogens may have a larger effect than the generally-higher levels of phytoestrogens in the subjects' diets (Moggs 2004). Other studies, however, have found similar associations in children of

vegetarians, who have a higher than normal level of phytoestrogens in their diets (North 2000, Cassidy 1994).

Criticism has been leveled at the hypothesized health threats posed by endocrine disruptors. Much of this criticism is based on comparisons of "natural" dietary estrogens (largely phytoestrogens) and synthetic estrogens like the insecticide dichloro-diphenyl-trichloroethane (DDT), various polychlorobiphenyls (PCB's) and others. As Stephen Safe argued in 1994, relative potencies of natural estrogens and xenoestrogens vary widely. Daily uptake and body burdens also vary widely. In an attempt to determine "estrogen equivalents" based on available potency data, Safe demonstrated that exposure to synthetic estrogens would be expected to have relatively insignificant biological effect when compared to dietary exposure to natural estrogens (Safe 1994).

2.2 Estrogen physiology

Laboratory studies confirming the existence and major activities of endogenous estrogens were done in the early twentieth century, beginning with experiments by Adler, in Vienna, and Fellner, Herrmann and Iscovesco in France showing the ability of sow ovary extracts to cause uterine growth in rabbits and guinea pigs (Simmer 1971). Endogenous estrogens are important in the development of the female genital tract, and of secondary sex characteristics, including the growth and differentiation of breast tissue. They also have an effect on regulation of menstrual cycles, on the course of pregnancy, and in lactation (Couse 1999). Estrogens are also known to function as vasodilators (Emmens 1943), decrease levels of low-density lipoprotein (LDL), increase high-density lipoprotein (HDL) (Oliver 1956), intercept free radicals, and have other effects which reduce the risk of atherosclerosis. They also help to maintain bone density in post-menopausal women (Lauritzen 1999).

Estrogens are aromatized in fatty tissue from endogenous androgens. In plasma, most endogenous estrogen is bound to the sex hormone binding globulin (SHBG), which is produced by the liver. In cells, estrogens act on nuclear receptors of two known types, estrogen receptor alpha (ERα) and estrogen receptor beta (Erβ), each of which is coded for by a single gene found on different chromosomes. In humans, the ERα gene is found on chromosome 6 and ERβ is found on chromosome 14 (Philipp 1942, Lauritzen 1999).

During pregnancy, E_2 and estrone increase to 10 times, and estriol to 100 times their pre-pregnancy levels. The additional estrogen produced by the fetal portion of the placenta (Philipp 1942, Lauritzen 1999). Estrogens are also aromatized from androgens in the brain, where they have specific effects on fetal brain development and sex imprinting (Lauritzen 1999).

2.3 Effects of exposure to environmental hormones

Evidence from as early as the 1940's has indicated exposure to hormonally active xenobiotics can lead to a wide variety of physiological effects. Examples of these effects include cancer (Marselos 1992, 1993), reproductive tract abnormalities (Gill 1979, Gusti 1995, Henderson 1976), altered sexual development (Cohen 1946, vom Saal 1992, Gray 1992, Lauritzen 1999), decreased sperm production (Guzelian 1982), and others.

Studies have shown that *in utero* exposure to strong estrogens can lead to reproductive effects, such as increased prostate size (Nonneman 1992, Timms 1999). While this seems to be true for high doses of potent estrogens such as E_2 and diethylstilbestrol (DES), this is less certain for relatively weak estrogens. In one study, low doses of bisphenol A and DES exposure *in utero* both failed to have a significant effect on prostate size for CF1 mice (Ashby 1999). The effects of adult exposure to low doses of weak estrogens are also unclear, as are the interactions between the hormonally active compounds themselves.

2.4 Estrogens and breast cancer

Of all the observed and hypothesized effects of exposure to xenobiotic estrogens, the most controversial may be the potential link to increasing rates of breast cancer (Davis 1993). Breast cancer is the most commonly diagnosed cancer for women. The US government estimates approximately 217,440 new cases of breast cancer for 2004, over 215,000 of these in women (Ries 2004). Mortality due to breast cancer increased from the 1960's till the late 1980's, when it began to decrease in most developed western countries (Parkin 2001). Incidence, however, has continued to increase in the United States, rising at an age-adjusted rate of approximately 0.6% annually in women since 1992. During the same period, mortality rates for women with breast cancer have fallen at approximately 2.4% annually (Ries 2004).

Breast cancer risk increases with age, age of menarche, personal history of breast cancer, family history of breast cancer, body mass index, physical activity, radiation therapy, breast density, hormone replacement therapy, radiation therapy of the chest area, and alcohol consumption. White women have higher rates of breast cancer than African Americans, Asians, and Latinas (National Cancer Institute 2004). At least some researchers believe exposure to reproductive hormones may underlie many of these risk factors, specifically the increased risk due to alcohol consumption, physical inactivity, and obesity (Brody 2003).

Breast tissue is not fully differentiated at the time of birth, and does not become fully differentiated until a woman's first full-term pregnancy. This fact is thought to underlie the increased breast cancer risk associated with earlier menarche, and with age of first childbirth. It is thought that breast tissue is more susceptible during rapid growth of breast tissue during puberty, pregnancy, and perinatal and postnatal periods (Russo 1996). Animal studies have indicated a higher incidence of chemically-induced breast tumors in sexually immature subjects

(Dunnick 1995). This susceptibility seems to decrease after a female's first full-term pregnancy (Russo 1996).

A number of studies have found a correlation between the concentration of estrogens and incidence of breast cancer. One such study, a meta-analysis of a number of published breast cancer studies, found that breast cancer risk significantly increased both with an increase in body mass index (BMI), and with levels of E_2, estrone, and other endogenous estrogens. When controlled for other known breast cancer risk factors, only the number of full-term pregnancies and age at first full-term pregnancy had any effect on the association. Both of these increased the magnitude of the BMI-breast cancer association. When controlling for levels of estrogens, the relative risk of breast cancer associated with BMI was largely mitigated. No increased risk was found associated with levels of several androgens which were also evaluated, including testosterone, and androstenedione. These results support the hypothesis that the increase in breast cancer associated with increasing BMI is largely due to increased levels of estrogens (Key 2003).

Endogenous estrogens have been found not to be mutagens or genotoxic carcinogens. They are known, however, to act as growth hormones in target tissues. Thus, they may act as mitogens for some tumors originating in those tissues. In this respect, estrogens are often considered to be growth promoters of existing cancerous and precancerous cells (Lauritzen 1999). Studies have shown that treatment of mammary tissues with chemical carcinogens will not produce tumors in the absence of estrogens, and that treatment with excess estrogens will lead to an increase in tumor production even without carcinogen exposure (Russo 1996). Estrogens, then, appear to be necessary for the development of mammary tumors, and higher levels of endogenous estrogens are known to be a risk factor for mammary tumors.

While it seems clear that levels of endogenous estrogens are associated with increased risk of breast cancer, the relationship to levels of xenoestrogens, phytoestrogens, and others is less well known. The New York University Women's Health Study, for example, showed a correlation between breast cancer and serum levels of DDE, a metabolite of DDT known to have endocrine-disruptive activity (Wolff 1993). This may be questionable, however, as DDE is not generally considered to be estrogenic (Safe 1994). Other studies have failed to corroborate this finding (Ashby 1997, Datson 1997, Krieger 1994, Savitz 1994). Another study focusing on a GIS assisted assessment of exposure to DDT, a pesticide with known estrogenic activity, found no correlation between pesticide application patterns and breast cancer (Brody 2004).

Evidence for potential health effects from xenobiotics estrogens has also come from studies of wildlife populations living in heavily contaminated areas, such as the classic studies of reproductive disorders found in Lake Apopka alligators exposed to high levels of chemicals from an industrial accident (Guilette 1994, Guilette 1995). Suspicion that these effects may carry over to human populations (Colborn 1993), due in part to similarities of these effects to those observed in children of mothers exposed to diethylstilbestrol during pregnancy (Gusti 1995, Henderson 1976, Gill 1979), provided impetus for the effort to determine whether these effects may be applicable to human populations.

2.5 Testing for estrogen activity

The 1996 Food Quality Protection Act provided a mandate for the EPA to develop a strategy for testing a large, diverse set of environmental chemicals for endocrine disruptive activity (EPA 1996.) Under this mandate, the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) was formed by the EPA in order to make recommendations for the development and implementation of a screening program. Approximately 87,000 potentially

estrogenic chemicals are being considered for this testing (ACS 2003). This would require a great deal of time and funding to accomplish with current estrogenicity assays.

The gold standard test for estrogenicity is an *in vivo* rodent assay which measures estrogen induced changes in uterine tissue mass (Korach 1995). Standardization has proven difficult with this test, and replication of results has been variable between laboratories (Ashby 1997, Odum 1997.) *In vitro* studies have shown potential for faster, less costly testing, as well as and easier standardization. Some of these include competitive estrogen receptor binding assays (EORTC 1987, Fang 2000, Thorpe 1987), E-SCREEN MCF-7 breast cancer cell proliferation assay (Soto 1992), and recombinant yeast estrogen screens (Gaido 1997, Routledge 1996). Computational methods, such as Structural Activity Relationship (SAR) and Quantitative Structure Activity Relationship (QSAR) modeling, represent another way to narrow the search for these agents (Gail 1992). Use of computational methods is especially attractive, where feasible, because they could significantly reduce the cost as well as the time required by traditional methods.

2.6 *In silico* toxicology

Computational prediction of chemical toxicity, according to Richard, lies at the intersection of a number of disciplines. Toxicology prediction requires the integration of biology, chemistry, statistics, and the "domain of computation" (Richard 1999). Perhaps partly due to this necessary integration, predictive computational toxicology has been a difficult, and often elusive goal. A wide variety of methods have been used in attempts to predict toxicological endpoints, but all have at a certain basic structure in common. Each has three parts to an equation which makes up the model: the part based on the chemical and/or biological activity of the model

components, the biological activity of those components for the modeled endpoint, and the relationship between those two (Benfenati 1997).

One of the difficulties encountered in toxicology prediction is the diversity of toxicologically relevant information. One reviewer uses an onion analogy to illustrate the schematic layers of such information (Johannsen 1990). At the center of the onion lies the chemical structure of interest. Moving outward, each successive layer represents both increased biological complexity, and direct relevance to human health. Some of these factors, roughly in order of increasing complexity, include: physiochemical properties, DNA and protein binding, DNA damage and repair, receptor binding, mutagenicity, cell proliferation, acute toxicology, cancer, and, finally, risk assessment. While there are numerous other kinds of available information about toxicology, and toxicological endpoints, it is enough to note that the relationship between structure and activity is, at least potentially, very complex.

Richard defines two major domains of toxicity prediction models: activity-activity relationship models and structure-activity relationship models (Richard 1999). Hybrid systems, which incorporate some aspects of each, have also been developed. Activity-activity relationship (AAR) models usually try to extrapolate from one layer of chemical and biological information to a more complex layer. These models can use both biological activity and chemical properties to predict toxicological responses. Structure-activity relationship (SAR) models, however, extrapolate from the very center of the problem, the structure of the compound itself, to the desired toxicological endpoint.

From a slightly different point of view, Richard also divides predictive toxicology models between those which approach toxicology from a top-down, empirical approach, and those that attempt to build upon existing knowledge. The top-down approach relies on statistical methods

or other kinds of automated algorithms to make generalizations based on model data alone, without human input in classifying chemicals. The bottom-up approach, characterized by the so-called "expert systems," approaches the problem using human judgement, chemical classifications, as well as other chemical and mechanistic data. MultiCASE, HQSAR, and cat-SAR, all of which will be discussed further, take a top-down, statistical approach. Rule-based expert systems, including the commercially available OncoLogic and DEREK, take the second approach (Richard 1998).

Structure-activity relationship models include any model which uses molecular fragments and structural alerts to predict for a given biological endpoint. SAR models are further differentiated as those which are based on categorical data, usually predictions of "active" or "inactive" for a given biological endpoint, and those which can produce quantitative and/or potency predictions. This second group, a subset of SAR, is known as QSAR modeling. These are mathematical models which quantitatively describe the relationships between chemical structure and biological activity (Cronin 2003, Jaworska 2003).

The form taken by molecular structural data also differentiates various SAR modeling approaches. One approach to structural data is that taken by three-dimensional SAR methods, including comparative molecular field analysis (CoMFA) and CoMSIA. The other, characterized by methods such as HQSAR, MultiCASE and cat-SAR, is based on two-dimensional characterizations of molecular structure. Three-dimensional CoMFA and CoMSIA models both describe a three-dimensional space, with regions of positive and negative relationships to the modeled endpoint for each descriptor. These descriptors are based on biological data, and so these models, in general, tend to be hybrid approaches with respect to SAR/AAR classification. A National Center for Toxicological Research dataset, describing the RBA for a diverse group of

compounds for rat uterine cytosol (Klopman 1994, NCTR 2004), has been successfully modeled using CoMFA as well as using two-dimensional holographic quantitative structural activity relationships (HQSAR). While CoMFA models have proven themselves to be predictive within the model space occupied by the learning set, they generally require congeneric sets of chemicals. They also tend to have difficulty predicting values for chemicals outside their domain. The large amount of structural variability among the compounds which have been shown to have estrogenic activity can lead to difficulties in using this approach to predict activity of diverse sets of environmental xenobiotics.

Two-dimensional models, including cat-SAR, HQSAR, MultiCASE and others, eliminate the need for the often-complex structural alignments required for good three-dimensional models. The elimination of this alignment issue makes these methods excellent for rapid analysis, while also facilitating the use of larger and more diverse datasets. These diverse datasets would otherwise be difficult or impossible to align on a single template. These methods also avoid the need for the numerous molecular descriptors usually required by three-dimensional QSARs, requiring only the two-dimensional structure and the biological endpoint data for prediction. Two-dimensional models have been shown, at least in some cases, to have comparable predictive ability to three-dimensional models for the same dataset (Lowis 1997).

It has been noted that there seems to be relationship between learning set chemical diversity and the complexity of the biological response variable. The more complex the biological response in study, the more learning set variability a QSAR model can tolerate (Wold 1983). For a specific mechanism, such as estrogen receptor binding, the learning set often needs to be relatively homogeneous (Eriksson 2003), though this may be dependent on the type of QSAR used.

AAR, and to some extent hybrid models have at least one theoretically significant advantage over SAR models. For AAR models, there is generally less distance, in terms of biological complexity, between the information which comprises the model and the predicted endpoint. This leaves fewer intervening processes which must be accounted for by the model. However, AAR models require much more complex, detailed information. This is more expensive, time consuming, and in general much less useful for compounds for which there is little preexisting toxicological information (Richard 1999). It is precisely those little-studied compounds, however, which would seem to be the best candidates for a computational approach. Further, it may also be noteworthy that as one adds complex biological information to a model, uncertainty present in the data itself tends to increase. Uncertainty in pure SAR models, on the other hand, is found only in one side of the relationship—the model's endpoint. Along with uncertainty in model data, and complexity of biological interactions, another problem to be considered (at least in some cases) is the large uncertainty inherent in extrapolating from one species to another.

2.7 Uses of SAR models

Pharmaceutical research has increasingly turned to SAR methods for preliminary toxicity testing (Dearden 2002). This is unsurprising considering the substantial cost of a standard two-year rodent assay, as well as the social and ethical issues associated with animal testing. Approximately 16% of new drugs fail during animal toxicity testing, and 14% are lost due to adverse effects in humans (Kennedy 1997). Drug development cycles usually run for 10 to 12 years, and can cost up to $500 million (Dearden 2002). With these numbers in mind, clearly even a modestly increased ability to catch potential toxicity problems earlier in the development cycle could translate to large savings in time and resources. Several SAR/QSAR methods have indeed

been adopted by pharmaceutical researchers, with varying success rates (White 2003, Mekenyan 2002).

Many computational toxicology efforts have focused on chemical carcinogenicity. A number of reasons for this have been offered, including: large resource and time investments in traditional two-year rodent carcinogenicity assays, increasing social pressure for decreasing our dependence on animal testing, a great deal of quality data available about rodent carcinogenicity, and the number of chemicals which have not yet undergone extensive toxicological testing (Richard 2002).

There is a fundamental difficulty, however, with computational prediction of carcinogenicity. Any such approach must recognize that there is a vast and complex set of mechanistically distinct biological processes involved (Richard 1999). The challenge for predicting carcinogenicity is to develop a top-level model which incorporates as many of these separate domains as possible into one predictive package (Richard 2000).

A number of commercially available programs have been developed for toxicity prediction. MultiCASE, developed by Klopman and Rosenkranz, (Klopman 1992) and TOPKAT (Enslein 1994) are two approaches characterized by a top-down statistical approach. DEREK (Sanderson 1991), OncoLogic (Woo 1995), and HazardExpert (Darvas 1999) take rules-based, expert system approaches to the problem of toxicity prediction. Both DEREK and OncoLogic have rules based on human knowledge, obtained from expert input. OncoLogic, dedicated to the prediction of carcinogenicity, has an extensive rule set with thousands of rules for some chemical classes. It also contains rules for features which modulate the activity suggested by other significant features. DEREK, on the other hand, is made up of a simpler rule set triggered by the

presence of structural alerts it calls “toxiphores,” with no specification of severity and no modulating features (Richard 2000).

2.8 Regulatory use

Computational toxicology is often considered better for identifying potential hazards than at ruling them out. In other words, toxicology models tend to be better at predicting activity than at predicting inactivity. However, those predictive approaches with a sound mechanistic basis can sometimes be used to argue for a compound’s lack of a particular hazard (Richard 1998).

The majority of SAR and QSAR use by regulatory agencies has resulted from the Toxic Substances Control Act (Cronin 2003). The Interagency Testing Committee (ITC) was formed under TSCA to be an independent advisory committee to the EPA. Its purpose is to identify and prioritize chemicals in need of testing. The EPA has historically used SAR methods largely for prescreening new chemicals under the Premanufacture Notification Review under TSCA, at an early stage in a chemical’s development cycle (Richard 1998). Some SAR methods, including OncoLogic, were developed specifically for this sort of setting. To date, the regulatory community has not made extensive use of SAR methods as the final word in these assessments. The EPA is, however, under increasing pressure to screen for an ever-growing set of toxic effects. SAR methods may be used in the future to assist in the best application of limited resources to testing (Richard 1998). In cooperation with the ITC, the EPA and Food and Drug Administration (FDA) account for the majority of SAR use worldwide. In Europe and the rest of the world SAR use tends to be less widespread, to date. This is likely to change in the future. (Cronin 2003).

2.9 The ESCREEN assay

The primary models for this study are based on the ESCREEN dataset, a set of 122 compounds tested for estrogenic activity. ESCREEN is an *in vitro* test for estrogen-like hormonal activity (Soto 1992, 1994, 1995, Sonnenschein 1995, 1998). As noted earlier, the standard for estrogen activity is its proliferative effect on the female genital tract. The ESCREEN assay is based on a biologically similar activity, its ability to induce proliferation of estrogen-sensitive MCF-7 human breast cancer cells. These cells demonstrate estrogen-dependent growth as tumors in athymic (lacking T lymphocytes) mice (Soule 1980, Soto 1985). Growth of these estrogen-sensitive cells *in vitro* is inhibited by charcoal-dextran stripped human serum (Soto 1984). This serum is collected from venous blood of adult volunteers, centrifuged for clarification, heat-inactivated at 56° C for half an hour, and stored at -20°C until use. The serum is treated with a carcoal-dextran suspension, and filtered (Soto 1992). This removes over 99% of the sex steroids present in serum (Soto 1985). This stripped serum inhibits growth of estrogen-dependent cells. Only added estrogens, whether natural or synthetic, are known to overcome this inhibition. The ESCREEN determines estrogenicity by measuring an exogenous compound's ability to overcome this inhibition by inducing the MCF-7 cells to divide.

Cloned MCF-7 cells are plated and allowed to attach in a seeding medium of 5% fetal bovine serum in DME. They are then washed and treated with experimental medium, mixed as 10% heat-inactivated, charcoal-dextran stripped human serum in phenol red-free Dulbecco's modified Eagle medium (DME). A range of test compound concentrations are applied and the cells are incubated until termination on the 6^{th} day, during their late exponential growth phase (Soto 1992).

Estrogenic activity, as determined by the ESCREEN assay, is reported as two related values. These are relative proliferative potency (RPP) and relative proliferative effect (RPE). Relative proliferative potency is defined as the ratio between the concentration of E_2 required to achieve maximum cell yield and the concentration of the test substance needed to achieve an equivalent effect. An RPP of 1, then, would indicate the proliferative potency of the substance is equivalent to that of E_2. Values greater than 1 indicate increased potency, while those less than 1 indicate decreased potency relative to E_2. Many compounds would not achieve proliferation equivalent to E_2 at any testable concentration. Another measure, then, is necessary to determine a compounds maximum proliferative performance. Relative proliferative effect (RPE) is reported as 100 times the ratio of the highest cell yield achieved by the test chemical to the yield achieved by E_2. RPE can be understood as the maximum proliferative effect of the test chemical as a percentage of the maximum achieved by E_2, with 100 indicating a full agonist (Soto 1992).

CHAPTER 3. MATERIALS AND METHODS

3.1 Data set

A set of compounds assayed with the ESCREEN test for estrogenicity were chosen as the learning set for this study (Soto 1992, Soto 1995, Fang 2000). Two endpoints for this assay were reported in the literature. These were relative proliferative potency (RPP) and relative proliferative effect (RPE.) Each endpoint was modeled and evaluated separately. Cat-SAR uses categorical data (active vs. inactive.) For each endpoint, chemicals in the learning set were categorized as either active or inactive based ESCREEN values. For simplicity, any a positive result was considered to designate an active compound.

3.2 Program overview

Cat-SAR is a predictive SAR method developed by this laboratory which uses structural data to predict biological activity. It has been used previously to model respiratory sensitizers (Cunningham 2005). Cat-SAR uses Tripos's Sybyl implementation of HQSAR for generation of the fragment matrices (Tripos 2002). In HQSAR, an established two-dimensional QSAR method, each molecule is divided into all possible fragments of specified numbers of atoms. The fragments themselves can be defined on the basis of several criteria. These criteria include atom type, bonds, and connections (a measure of atomic hybridization states). Molecular fragments generated using HQSAR can be linear, branched, or cyclic. These fragments are then hashed into numbered "bins," and partial least squares analysis is applied to the matrix of fragment-containing bins and the biological data, resulting in a quantitative association between fragment patterns and biological activity. The method tested here doesn't use pseudo-randomized "bins" of fragments. This avoids the need for complex statistical analysis and, perhaps more importantly, allows for a more direct analysis of the particular contribution of any particular

fragment to the model. In this way it should be more feasible to describe a cat-SAR model's results in terms of specific structures than to do so for a given HQSAR model.

After generating a list of all possible fragments for each structure in a learning set, a Sybyl script was used to associate each fragment with all learning set structures in which it was found. This resulted in a matrix in which the rows are the original chemicals, and the columns corresponded to each fragment in the model. Table 1 shows an abbreviated illustration of a similar matrix. For each chemical, the fragments it contains were tabulated across its row. For each fragment, then, chemicals containing it are read down its column.

Table 1: Example Chemical-Fragment Matrix

	Frag. 1	**Frag. 2**	**Frag. 3**	**Frag. 4**
Chem. A	X	X	X	
Chem. B		X		
Chem. C	X			X

Two matrices of chemicals and fragments were generated for each endpoint (RPE and RPP values). The first matrix for each endpoint was generated using atoms, bonds, and connections as fragment criteria (labeled hereafter as ABC). The second set of fragments was generated using hydrogens as a fourth criterion (ABCH).

Matrices were developed initially using Sybyl HQSAR default values for fragment sizes. This generated fragments of between 4 and 7 heavy atoms (excluding hydrogen). After further testing, however, it was determined that more significant models could be obtained using a setting which generated all possible fragments ranging in size from 3 to 7 atoms. After evaluating the resulting models, a fifth matrix was created using the ABC criteria containing all possible fragments from 3 to 12 atoms in size. Other available options for fragment generation criteria were not evaluated in this study.

Cat-SAR models are built by a straightforward comparison of structural fragments found in active and inactive chemicals in the learning set. Chemicals in a cat-SAR learning set are given categorical designations of either active or inactive. Fragments are evaluated by the program based on the number of active and inactive chemicals in which they are found. In order to assess each fragment's contribution, several user-selected rules are applied. These rules are used to generate list of fragments considered to be significant in describing the biological activities considered for the learning set.

The first rule specifies the minimum number of learning set chemicals in which a given fragment must be found in order to be considered significant. A minimum cutoff of three was selected for the test models. This cutoff value determines that only fragments which are found in at least three compounds will used to make predictions. The choice of three is based on previous experience, and has been used to develop predictive models previously (Cunningham 2005). The purpose of this rule is to exclude rare or unique fragments, which would be less useful in determining overall patterns of relationship between structure and activity. If the cutoff were too large, important features of the chemicals in the learning set might be missed. If it were too small, the model would not be able to differentiate between true patterns and singular outliers.

Another measure of a fragment's predictive utility is the proportion of active or inactive compounds in which it can be found. In the test models, the structures were evaluated only using fragments which were found in either active or inactive compounds at a predetermined ratio. For the test models, several ratios were evaluated, including 0.65, 0.75, and 0.85. A ratio of 0.75 has been found to produce useful results. At this cutoff, for example, a fragment found in ten of the learning set structures would have to be found in at least 8 active fragments to be used as a

positive indicator of estrogen activity. The same applies to fragment's association with inactive compounds. The cat-SAR program treats "active" and "inactive" fragments equally.

The learning set described herein does not contain an equal number of active and inactive compounds. Since the ratios expected by chance, in this case, would not be the same for both positive and negative chemicals, it requires these cutoff levels to be corrected to account for the expected random distribution of negative and positive compounds in which a given fragment would be found. In a set with ten active and twenty inactive compounds, for example, a random distribution of a given fragment would be expected to favor active compounds at a ratio of two to one. Cutoff ratios were balanced to account for this. The RPE model contained 72 compounds defined as active, and 50 defined as inactive. The percent cutoff was balanced based on these numbers by determining the proportion of active vs. inactive compounds in which it would be expected to occur by chance. For the RPE model, this was equal to 59 percent active and 41 percent inactive. The percent cutoff was then balanced by adding and subtracting 0.09 from the active cutoff and inactive cutoff respectively. The purpose of this second rule is to ensure that those fragments which were found to be strongly associated with either activity or inactivity would be used in the model. It was reasoned that fragments found in roughly equal proportions of active and inactive chemicals would not be useful in modeling a particular biological activity.

The rules used to determine the predictive utility of each fragment are not predetermined. The "best" set of rules must be determined empirically by testing the predictive ability of the model it generates. This is likely to vary significantly between learning sets as well as between models for different biological endpoints. The number of significant fragments remaining after these rules are applied is generally a function of the size and complexity of the learning set. For

the ESCREEN models tested here, the fragments numbered in the thousands, though this includes many structurally similar fragments.

After the fragment set had been pruned via the process outlined above, those remaining were used to model the biological activity of the learning set. Each significant fragment is associated with a probability of activity and of inactivity, based only on the ratio of active and inactive learning set compounds in which it is found.

3.3 Model validation

A leave-one-out cross-validation was performed to assess the performance of each model. To perform this validation, the program leaves one compound out of the learning set and recreates a model from this reduced set. The resulting model is then used to predict the activity of the compound which was left out. This is repeated once for each compound in the learning set. The performance of the overall model is assessed by its ability to correctly predict each compound. Sensitivity of the model is defined as the ratio of known positives correctly predicted to the total number of positive compounds in the learning set. Specificity is the ratio of correctly predicted negatives to overall negatives, and overall correct prediction (OCP) is the overall rate of correct prediction for this validation. At least one author has noted, however, that internal validations of this sort tend to lead to inflated assessments of model performance. Performance against outside test sets is often somewhat lower (Golbraikh 2002).

3.4 Model prediction

Cat-SAR predicts the activity of a compound, both for internal validation and for prediction of unknowns, by computing the average probability of activity for all significant fragments it contains. For a given compound, its probability of activity is equal to the average probability of its active and inactive fragments. As described earlier, a fragment's probability of

activity, or of inactivity, is based on the number of active and inactive learning set compounds in which it is found. Note, however, that if the rules governing significant fragments exclude all fragments found in a compound, no determination will be made. An example of a compound's fragments and model prediction is shown in Table 6. Each significant fragment is considered to have an activity equal to the ratio of active and inactive learning set compounds in which it is found. The prediction for a given compound's activity is based simply on the average activity and inactivity of each of its significant fragments.

During the development of cat-SAR, several potential pitfalls were recognized when considering the effect model parameters and structure of the learning sets would have on model results. It was recognized, first, that not all learning sets would contain equal numbers of active and inactive chemicals. Second, the final number and distribution of significant fragments may not be equal even for those which did. Third, average probabilities of a compound's significant fragments do not indicate true probabilities, in a strict sense. Finally, depending on the model's intended use, the end user may want to adjust for rates of false positives and false negatives. With these in mind, the choice of what "probability of activity" indicates true activity, or inactivity, is situational. The chosen cutoff determines the divide which separates active compounds from inactive. At a cutoff of 0.5, for example, all compounds with a probability of activity of greater than 0.5 would be predicted to have biological activity. The actual cutoff for a cat-SAR model is chosen empirically based on performance.

The cat-SAR validation program reports a range of cutoffs, and the predictive results at each. This range begins at a cutoff of 0.00, at which all compounds containing significant fragments are predicted to be positive, and ends at 1.00, where all are predicted to be negative.

The program considers every possible cutoff in this range in increments of 0.01. An example portion of this output is shown below, in Table 2.

Table 2. Example portion of a cat-SAR validation output

Cutoff	*Predicted active (correct/total)*	*Predicted inactive (correct/total)*	*Sensitivity*	*Specificity*	*OCP*
0.720	35/42	44/54	0.833	0.815	0.823
0.730	28/42	45/54	0.667	0.833	0.760
0.740	23/42	46/54	0.548	0.852	0.719
0.750	21/42	47/54	0.500	0.870	0.708

Sensitivity: Percent of active compounds correctly predicted to be active
Specificity: Percent of inactive compounds correctly predicted to be inactive
OCP: Overall correct prediction percent

There is currently no algorithm which determines the "best" choice among these cutoff values. Whether any such algorithm would be appropriate is not certain, as the best choice among the available cutoff values depends on the particular goals the end user has for the model in question. This was largely an exploratory study, and a number of different model configurations were evaluated. For these purposes, the best choice was largely determined by maximizing the level of overall correct predictions. However, the balance between sensitivity and specificity was also considered in order to avoid model "lopsidedness." This affected the cutoff used in at least one case. Two of these models were chosen, based on these criteria, for further evaluation.

3.5 Model comparisons

The predictions of a pair of ESCREEN models were further evaluated for consistency with models of other toxicological endpoints. The purpose of this evaluation was to demonstrate the potential, or lack of potential of environmental estrogens to exhibit other toxicological activities. This evaluation used the "Chemical Diversity Approach" method. This method uses a constructed list of 10,000 chemicals chosen to approximate a representative sample of chemicals present in the environment (Pollack 1999). These 10,000 chemicals were randomly chosen from structural libraries and from the National Cancer Institute's repository of potential cancer

chemotherapeutic agents. The evaluated models are used to predict the biological activity of this set of chemicals, and results of this prediction are compared to results obtained by other validated models. A comparison of endpoints predicted by two models can then be evaluated. If the endpoints of two models are expected to be independent, the number of test chemicals predicted active in both models would be statistically equivalent to that expected by chance. Significantly greater overlap of the two endpoints than expected by chance indicates a similarity, or other relationship between the modeled endpoints. Significantly less overlap than expected from chance might indicate an antagonistic or exclusionary relationship between the two endpoints. This approach has had success in estimating the number of potential *Salmonella* mutagens in the environment (Rosenkranz 2001).

For one part of this comparison, a model of *Salmonella* mutagenesis was constructed using data from the National Toxicology Program (NTP) *Salmonella* mutagenesis database (National Toxicology Program 2004). Comparisons were also made against two rat carcinogenesis models developed with cat-SAR. These models were constructed using the carcinogenic potency database (CPDB), a standardized compilation of the results of long-term animal carcinogenesis studies. (Carcinogenic Potency Project 2004). The first of these models was based on a learning set of 100 rat mammary gland carcinogens and 100 rat noncarcinogens. The second was based on a learning set of 100 rat mammary gland carcinogens and 100 compounds which were found to be rat carcinogens in tissues other than the mammary gland. Finally, comparisons were also made to a previously developed model of the same ESCREEN dataset which was constructed using the MultiCASE system (Cunningham 2004).

CHAPTER 4. RESULTS AND DISCUSSION

4.1 Predictive performance

The predictive ability of the ESCREEN models were evaluated using the validation process described previously. The results for several models are reported in Table 3. All the evaluated models demonstrated good predictive ability during model validation. Cat-SAR is still under development, and so a number of model configurations were explored. For each endpoint, RPE and RPP respectively, several combinations of percent cutoff and fragment generation criteria were evaluated. Some apparent trends emerged when evaluating the models. It is still uncertain whether these trends (if they are real) are global trends for the cat-SAR, or whether they are specific to this dataset or modeled activity. The first was the increase in correct prediction rates as percent cutoff was increased. These percent cutoff levels, as described earlier, were used to remove from consideration fragments which were not strongly associated with either activity or inactivity. Three percent cutoffs were used for each endpoint, and each was balanced to correct for the distribution of inactive chemicals. These cutoffs were 0.65, 0.75, and 0.85 percent. When balanced for the RPE endpoint, percent cutoff values of 0.65, 0.75, and 0.85 corresponded to: 0.74 active/0.56 inactive, 0.84/0.66, and 0.94/0.76. The same was done for the RPP models, as can be seen in Table 3.

As mentioned, the models demonstrated a higher overall correct prediction rate at more restrictive fragment cutoff percentages. However, this more restrictive cutoff led to a lower retention of significant fragments, as reported in the significant fragments, significant active fragments, and significant inactive fragments columns in Table 3. As might be expected, this also led to an increase in the number of compounds which contained no significant fragments, and for which no prediction could be made. In short, increases in percent cutoff tend to lead to

Table 3. Predictive performance of ESCREEN estrogen response models

Model	Total frags	Sign. frags	Sig. active fragments	Sig. inactive fragments	Opt. Pred. Cutoff	Sensitivity	Specificity	OCP
RPE Models 122 compounds 72 active 50 inactive								
abc (0.65)						**0.833**	**0.830**	**0.832**
0.74/0.56	6001	1176	903	273	0.75	(60/72)	(39/47)	(99/119)
abc (0.75)						**0.889**	**0.822**	**0.863**
0.84/0.66	6001	1036	824	213	0.72	(64/72)	(37/45)	(101/117)
abc (0.85)						**0.966**	**0.813**	**0.911**
0.94/0.76	6001	875	723	152	0.16	(56/58)	(26/32)	(82/90)
abch (0.65)	1373					**0.889**	**0.700**	**0.811**
0.74/0.56	2	2890	2217	673	0.76	(64/72)	(35/50)	(99/122)
abch (0.75)	1373					**0.847**	**0.837**	**0.843**
0.84/0.66	2	2467	1941	526	0.86	(61/72)	(41/49)	(102/121)
abch (0.85)	1373					**0.938**	**0.756**	**0.867**
0.94/0.76	2	1986	1611	375	0.43	(60/64)	(31/41)	(91/105)
RPP Models 122 compounds 50 active 72 inactive								
abc (0.65)						**0.600**	**0.656**	**0.631**
0.56/0.74	6001	1052	822	230	0.64	(30/50)	(40/61)	(70/111)
abc (0.75)						**0.833**	**0.815**	**0.823**
0.66/0.84	6001	719	554	165	0.72	(35/42)	(44/54)	(79/96)
abc (0.85)						**0.857**	**0.851**	**0.854**
0.76/0.94	6001	502	343	159	0.82	(36/42)	(40/47)	(76/89)
abch (0.65)	1373					**0.600**	**0.739**	**0.681**
0.56/0.74	2	2275	2138	587	0.67	(30/50)	(51/69)	(81/119)
abch (0.75)	1373					**0.756**	**0.818**	**0.793**
0.66/0.84	2	2020	1599	421	0.73	(34/45)	(54/66)	(88/111)
abch (0.85)	1373					**0.622**	**0.796**	**0.717**
0.76/0.94	2	1457	1050	407	0.82	(28/45)	(43/54)	(71/99)

higher prediction rates, but fewer predictions overall. Depending on a user's specific goals for a cat-SAR model, a balance can be found with acceptable levels of both. In this case, both endpoints seemed to perform well for our purposes at a cutoff of 0.75. At this cutoff, the RPE model made predictions for 117 of 122 compounds. Of these, 111 were correctly identified by the model as either active or inactive, for an overall correct prediction rate of 0.863. At the 0.65 cutoff, the model only increased the number of compounds predicted by two, for a total of 119 out of 122. However, both the number and the rate of overall correct predictions were lower at this cutoff. At a cutoff of 0.85, the rate of correct predictions increased somewhat, but the model only made predictions for 90 of 122 compounds. This was 27 fewer than for the 0.75 cutoff. Similar results were found with the RPP endpoint. It was decided that the 0.75 cutoff provided the best balance between number of predictions and rate of correct predictions.

The models based on atoms, bonds, and connections (ABC) had higher correct prediction rates than those which added hydrogen atoms to the fragment generation criteria (ABCH). Adding hydrogen atoms led to a significant increase in the number of fragments generated, as well as the number of significant fragments retained by the models. Unsurprisingly, the ABCH models also made predictions for a greater number of the 122 compounds. It was decided, however, that the slightly greater number of predictions made by the ABCH models was not enough to make up for the lower overall rate of correct predictions. The ABC models were then selected for further evaluation.

This left two models for further consideration, an RPE ABC model using a fragment percent cutoff of 0.75, and an RPP model using the same parameters. The predictions made by these two models can be seen in Table 4. Of these, the RPE model slightly outperformed the RPP model both in terms of OCP (by 4%) and in terms of number of predictions made (117 for RPE

against 96 for RPP). Overall, the RPE model correctly identified 86% of the compounds during the leave-one-out validation process. The RPP model correctly identified 82% of the same set.

The only difference between these two endpoints, for the purposes of the cat-SAR model, were 23 compounds classified as active in the RPE model but inactive in the RPP model, and one RPP active compound which was inactive for the RPE endpoint. In Table 4, these are 16-hydroxyestrone through 6-bromonapthol-2. Aside from 16-hydroxyestrone, all these were compounds tested negative for the ESCREEN RPP endpoint but had small positive results for RPE. The RPP model predicted all but two of these compounds correctly to be inactive, and the RPE model correctly predicted all 23 to be positive. This differs from the results previously obtained for the same dataset when modeled using MultiCASE. Of these MultiCASE models, the RPE model predicted all 23 correctly to be active, but the RPP model also incorrectly predicted approximately half of these compounds to have biological activity (Cunningham 2004). As the MultiCASE RPE model was more predictive than the RPP model, and assuming high predictivity is indicative of mechanistically meaningful models (Cunningham 1998), this was interpreted as implying these compounds contained structural features consistent with estrogen activity (Cunningham 2004). The cat-SAR results, however, do not seem to agree with this previous assessment. This may indicate the cat-SAR model is more sensitive to subtle differences in a dataset than MultiCASE. Also unlike the MultiCASE models, the cat-SAR RPE model was only slightly more predictive than the RPP model. Some of this difference between RPP model performance for MultiCASE and cat-SAR can be explained by cat-SAR correctly identifying 21 of these 23 correctly to be negative for the RPP endpoint, with the MultiCASE model only correctly predicting 11.

Table 4. Model validation for ESCREEN estrogen response models

	RPP			RPE		
Chemical	**Experimental**	**Prob**	**Prediction**	**Experimental**	**Prob**	**Prediction**
Carbaryl	-	0.00	-	0	0.00	-
Chlorothalonil	-	0.00	-	0	0.00	-
Chlorpyrifos	-	0.00	-	0	0.02	-
Thiram	-	0.00	-	0	0.04	-
Ziram	-	0.00	-	0	0.04	-
Picloram	-	0.00	-	0	0.19	-
Dacthal	-	0.00	-	0	0.29	-
1-Naphthol	-	0.00	-	0	*	*
Atrazine	-	0.01	-	0	0.01	-
Propazin	-	0.01	-	0	0.01	-
Simazine	-	0.01	-	0	0.01	-
Maneb or zineb	-	0.04	-	0	0.04	-
Cyanazine	-	0.11	-	0	0.06	-
Hexazinone	-	0.19	-	0	0.04	-
Butylate	-	0.27	-	0	0.04	-
Malathion	-	0.37	-	0	0.10	-
Diazinon	-	0.54	-	0	0.00	-
Trifluralin	-	0.60	-	0	0.03	-
Metalochlor	-	0.62	-	0	0.09	-
Bendiocarb	-	0.62	-	0	0.15	-
2,3,7,8-TCDD	-	0.62	-	0	0.00	-
Parathion	-	0.63	-	0	0.00	-
Alachlor	-	0.63	-	0	0.10	-
2,4-Dichlorophenoxyacetic acid	-	0.64	-	0	0.33	-
2,4-DB Acid	-	0.64	-	0	0.28	-
Chlordimeform	-	0.66	-	0	0.05	-
4-Butooxyphenol	-	0.67	-	0	0.28	-
Phenol	-	0.67	-	0	*	*
4-Hexyloxyphenol	-	0.67	-	0	0.28	-
Dibutyl phthalate	-	0.68	-	0	0.33	-
Carbofuran	-	0.69	-	0	0.32	-
Octachlorostyrene	-	0.69	-	0	0.72	-
Diamyl phthalate	-	0.70	-	0	0.33	-
Dinoseb	-	0.70	-	0	0.55	-
Lindane	-	0.70	-	0	0.89	+
Butylated hydroxytoluene	-	0.71	-	0	0.69	-
Rotenone	-	0.72	-	0	0.60	-
Methoprene	-	0.72	+	0	0.95	+
Dinonyl phthalate	-	0.73	+	0	0.33	-

Table 4 continued

5,6,7,8-Tetrahydronaphthol-2	-	0.75	+	0	0.87	+
Mirex	-	0.76	+	0	0.95	+
Kelthane	-	0.77	+	0	0.99	+
Styrene	-	0.86	+	0	0.86	+
Dimethyl isophthalate	-	*	*	0	0.32	-
Dimethyl terephthalate	-	*	*	0	0.32	-
Tetrachloroethylene	-	*	*	0	0.86	+
1,2-Dichloropropane	-	*	*	0	*	*
2-Naphthol	-	*	*	0	*	*
Hexachlorobenzene	-	*	*	0	*	*
2,2',3,3',5,5'-Hexachlorobiphenyl	-	0.75	+	1	1.00	+
2,3,3',4,5-Pentachlorobiphenyl	-	*	*	1	1.00	+
3,5-Dichloro-4-hydroxybiphenyl	-	0.69	-	1.5	1.00	+
4-Monochlorobiphenyl	-	*	*	2.1	1.00	+
2,3',5-Trichlorobiphenyl	-	*	*	2.2	1.00	+
3,5-Dichlorobiphenyl	-	*	*	2.7	1.00	+
2,3,5,6-Tetrachlorobiphenyl	-	*	*	3.1	1.00	+
2,6-Dichlorobiphenyl	-	*	*	3.4	1.00	+
Decachlorobiphenyl	-	0.75	+	3.5	1.00	+
2,5-Dichlorobiphenyl	-	*	*	3.7	1.00	+
Chlordene	-	0.70	-	4	0.97	+
Gibberellic acid	-	0.83	+	4	0.88	+
2,3,4,5,6-Pentachlorobiphenyl	-	*	*	4.4	1.00	+
2-Monochlorobiphenyl	-	*	*	4.4	1.00	+
2,3,4,4'-Tetrachlorobiphenyl	-	*	*	4.7	1.00	+
2',3',4',5,'5-Pentachloro-2-hydroxybiphenyl	-	0.65	-	4.8	0.99	+
4-Ethylphenol	-	0.69	-	5	0.84	+
Chlordane	-	0.71	-	5	0.96	+
3,5-Dichloro-2-hydroxybiphenyl	-	0.64	-	5.4	0.99	+
2,3,6-Trichlorobiphenyl	-	*	*	5.8	1.00	+
Heptachlor	-	0.70	-	8	0.97	+
4-Propylphenol	-	0.70	-	17	0.84	+
6-Bromonaphthol-2	-	0.79	+	38	1.00	+
t-Butylhydroxyanisol	0.00006	0.66	-	30	0.18	-
2',5'-Dichloro-2-hydroxybiphenyl	0.0001	*	*	13	1.00	+
2',3',4',5'-Tetrachloro-3-hydroxybiphenyl	0.0001	*	*	35.3	1.00	+
2,3,4,5-Tetrachlorobiphenyl	0.0001	*	*	39.2	1.00	+
1-Hydroxychlordene	0.0001	0.64	-	40	0.97	+

Table 4 continued

Toxaphene	0.0001	0.75	+	51.9	0.94	+
Dieldrin	0.0001	0.75	+	54.89	0.96	+
Methoxychlor	0.0001	0.73	+	57	0.48	-
2,2',3,3',6,6'-Hexachlorobiphenyl	0.0001	*	*	61.6	1.00	+
2,2',4,5-Tetrachlorobiphenyl	0.0001	*	*	61.6	1.00	+
2',5'-Dichloro-3-hydroxybiphenyl	0.0001	*	*	69.9	1.00	+
p,*p*'-DDT	0.0001	0.73	+	71	0.85	+
2,4,4',6-Tetrachlorobiphenyl	0.0001	*	*	75.7	1.00	+
2,3,4-Trichlorobiphenyl	0.0001	*	*	77	1.00	+
Endosulfan	0.0001	0.74	+	81.25	0.96	+
o,*p*'-DDD	0.0001	0.73	+	84	0.85	+
Kepone	0.0001	0.76	+	84	0.93	+
o,*p*'-DDT	0.0001	0.73	+	86.14	0.85	+
4-*tert*-Butylphenol	0.0003	0.73	+	71	0.84	+
4-*sec*-Butylphenol	0.0003	0.74	+	76	0.84	+
Bisphenol A	0.0003	0.75	+	82	0.84	+
4,4'-Dihydroxyiphenyl	0.0003	0.86	+	84	1.00	+
4-Hydroxybiphenyl	0.0003	0.86	+	87	1.00	+
Butylbenzylphthalate	0.0003	0.46	-	90	0.23	-
4-*iso*-Pentylphenol	0.0003	0.33	-	93	0.33	-
4-*tert*-Pentylphenol	0.0003	0.74	+	105	0.85	+
Tamoxifen	0.001	0.69	-	11	0.54	-
2,2',5-Trichloro-4-hydroxybiphenyl	0.001	0.33	-	37.8	1.00	+
2',5'-Dichloro-4-hydroxybiphenyl	0.001	0.86	+	71.2	1.00	+
2',3',4',5'-Tetrachloro-4-hydroxybiphenyl	0.001	0.86	+	92	1.00	+
Coumestrol	0.001	0.73	+	93	0.89	+
Bisphenol A dimethacrylate	0.003	0.75	+	84	0.48	-
4-Nonylphenol	0.003	0.73	+	100	0.87	+
2',4',6'-Trichloro4-hydroxybiphenyl	0.01	0.86	+	99.8	1.00	+
4-Octylphenol	0.03	0.73	+	100	0.87	+
5-Octylphenol	0.03	0.76	+	100	0.89	+
16-Hydroxyestrone	0.1	0.80	+	0	0.95	+
Pseudo diethylstilbestrol	0.1	0.73	+	100	0.95	+
Equilenin	1	0.81	+	82	0.89	+
Zearalenone	1	0.82	+	88	0.46	-
Zearalenol	1	0.78	+	93	0.50	-
Estrone	1	0.80	+	95	0.87	+
Allenolic acid	1	0.65	-	105	0.75	+
Estriol	10	0.81	+	95	0.90	+

Table 4 continued

Indenestrol	10	0.74	+	100	0.97	+
Ethynylestradiol	100	0.80	+	92	0.91	+
17β-estradiol	100	0.81	+	100	0.90	+
Moxestrole	1000	0.81	+	110	0.89	+
11β-chloromethylestradiol	1000	0.81	+	110	0.90	+
Diethylstilbestrol	1000	0.83	+	112	0.97	+
RPE Models	**122 chems 72 pos, 50 neg. model cutoffs: 3, 0.84 pos, 0.66 neg**					
RPP Models	**122 chems 50 pos, 72 neg. model cutoffs: 3, 0.66 neg, 0.84 pos**					

Model comparisons based on ABC fragment criteria.
RPP model cutoff at 0.75/0.75 active/inactive, balanced as 0.66/0.84.
RPE model cutoff at 0.75/0.75 active/inactive, balanced as 0.84/0.66.
Prob: Model generated probability of activity.
Pred: Model prediction at selected cutoff of 0.72. ("*" indicates the model did not predict this compound)

The RPE model proved slightly more predictive than the RPP model, and was chosen for further mechanistic evaluation. The structural fragments that went into predicting several learning set compounds during model validation are shown in Figures 1 to 3 and Tables 5 to 7. During model validation, these compounds do not contribute their own fragments to the model. Because of this, they do not influence the prediction of their own activity. Table 5 and Figure 1 show the fragments which went into the cat-SAR prediction of E_2. Many of these fragments show a large degree of structural similarity. There is a significant degree of redundancy present in these fragments, and this issue will be addressed in future development of the cat-SARmethod. Also note that in some cases the same molecular fragment could be found more than once in the same molecular structure. Based on the average activity of these fragments, E_2 was predicted to have a probability of activity of 0.901. The optimal cutoff for prediction of this RPE model was 0.72 (Table 3), so E_2 was correctly predicted to be positive.

4.2 Mechanistic Analysis

Effects of E_2, the most active endogenous estrogen, and all its mimics and derivatives are mediated by intracellular estrogen receptors (ER) (Anstead 1997). Binding of E_2 to the ER is understood to involve all four rings and both terminal OH groups. The phenolic OH (located on the A ring) contributes approximately 1.9 kcal/mol of binding energy as a hydrogen bond donor, while the 17β-hydroxyl group contributes about a third as much, about 0.6 kcal/mol, probably as a hydrogen bond acceptor. As mentioned, all four rings are involved in receptor binding, as the receptor seems to surround the E_2 molecule. The aromatic ring contributes approximately 1.5 kcal/mol through polar interactions. Binding to the estrogen receptor is generally inhibited by the addition of polar substituents, and by larger hydrophobic substitutions in a number of positions (Anstead 1997).

The significant model fragments for E_2 are shown in Table 5 and illustrated in Figure 1, showing their large degree of structural similarity. None of the fragments in E_2 were associated with a significant probability of inactivity. The majority of these fragments can be assigned to three general areas of the E_2 structure. Those grouped at the top of the figure all contain the 17β hydroxyl group and varying portions of both the C and D rings. A smaller number of these fragments also contain some portion of the B ring. The second group, fragments 34 to 57 (to the left in Figure 1) are also mostly comprised of C and D ring atoms, with some contributions from the B ring. The major difference between first and second groups is that none of the second contains the 17β OH. One of these fragments, however, stands out in that it contains a single aromatic carbon shared by the A and B rings. The third group, to the right in the figure and made up of fragments 62-139, is made up largely of fragments containing at least one aromatic carbon, mostly from the shared carbons of the A and B rings. These fragments also contain large portions

Table 5. Cat-SAR fragments used in the prediction of E_2

Frag Name	# Act	#Inact	Total	% Act	%Inact
frag3	8	1	9	0.889	0.111
frag4	6	0	6	1.000	0.000
frag6	8	1	9	0.889	0.111
frag7	7	0	7	1.000	0.000
frag8	6	0	6	1.000	0.000
frag9	8	0	8	1.000	0.000
frag11	6	0	6	1.000	0.000
frag12	6	0	6	1.000	0.000
frag13	7	0	7	1.000	0.000
frag14	6	0	6	1.000	0.000
frag15	7	1	8	0.875	0.125
frag16	6	1	7	0.857	0.143
frag17	6	0	6	1.000	0.000
frag18	7	1	8	0.875	0.125
frag19	6	0	6	1.000	0.000
frag20	4	0	4	1.000	0.000
frag23	8	0	8	1.000	0.000
frag24	4	0	4	1.000	0.000
frag25	4	0	4	1.000	0.000
frag26	8	0	8	1.000	0.000
frag27	7	0	7	1.000	0.000
frag28	8	0	8	1.000	0.000
frag29	8	1	9	0.889	0.111
frag34	13	1	14	0.929	0.071
frag36	13	1	14	0.929	0.071
frag37	16	3	19	0.842	0.158
frag39	15	2	17	0.882	0.118
frag40	5	0	5	1.000	0.000
frag41	6	0	6	1.000	0.000
frag43	5	0	5	1.000	0.000
frag44	9	1	10	0.900	0.100
frag45	11	2	13	0.846	0.154
frag46	12	2	14	0.857	0.143
frag48	9	1	10	0.900	0.100
frag50	18	3	21	0.857	0.143
frag51	6	0	6	1.000	0.000
frag52	8	1	9	0.889	0.111
frag55	6	0	6	1.000	0.000
frag56	5	0	5	1.000	0.000
frag57	11	2	13	0.846	0.154
frag62	6	1	7	0.857	0.143
frag65	8	1	9	0.889	0.111
frag66	8	1	9	0.889	0.111
frag70	6	1	7	0.857	0.143
frag71	7	1	8	0.875	0.125
frag72	7	1	8	0.875	0.125
frag73	6	1	7	0.857	0.143
frag74	6	1	7	0.857	0.143

Table 5 continued

frag76	6	1	7	0.857	0.143
frag77	7	1	8	0.875	0.125
frag78	6	1	7	0.857	0.143
frag79	7	1	8	0.875	0.125
frag83	6	1	7	0.857	0.143
frag87	6	1	7	0.857	0.143
frag88	6	1	7	0.857	0.143
frag89	6	1	7	0.857	0.143
frag91	6	1	7	0.857	0.143
frag92	6	1	7	0.857	0.143
frag93	6	1	7	0.857	0.143
frag94	6	1	7	0.857	0.143
frag99	6	1	7	0.857	0.143
frag100	6	1	7	0.857	0.143
frag104	6	1	7	0.857	0.143
frag132	6	1	7	0.857	0.143
frag133	6	1	7	0.857	0.143
frag134	6	1	7	0.857	0.143
frag137	6	1	7	0.857	0.143
frag139	6	1	7	0.857	0.143
frag160	16	3	19	0.842	0.158

Validation Summary for 17β-estradiol (E_2)

Activity	Mean %Act	Mean %Inact	Frag Count
1	0.901	0.099	69

of the B and C rings, with smaller contributions from the D ring. Both the second and third groups can mostly be associated with the nonpolar steroid structure of E_2. The final group, consisting of a single fragment (fragment 160) is the only fragment to contain the phenolic OH and a significant portion of the A ring.

Table 6 and Figure 2 show the fragments which went into the cat-SAR prediction of indenestrol, a metabolite of diethylstilbestrol. Like the model fragments for E_2, none of these were significantly associated with inactivity. Also like E_2, though perhaps to a lesser extent, these fragments demonstrate the mechanistic strength of the model with respect to known ER binding characteristics. Figure 2 shows the regions of indenestrol which are roughly equivalent to the A to D rings of E_2. The fragments used by the cat-SAR model to predict indenestrol were

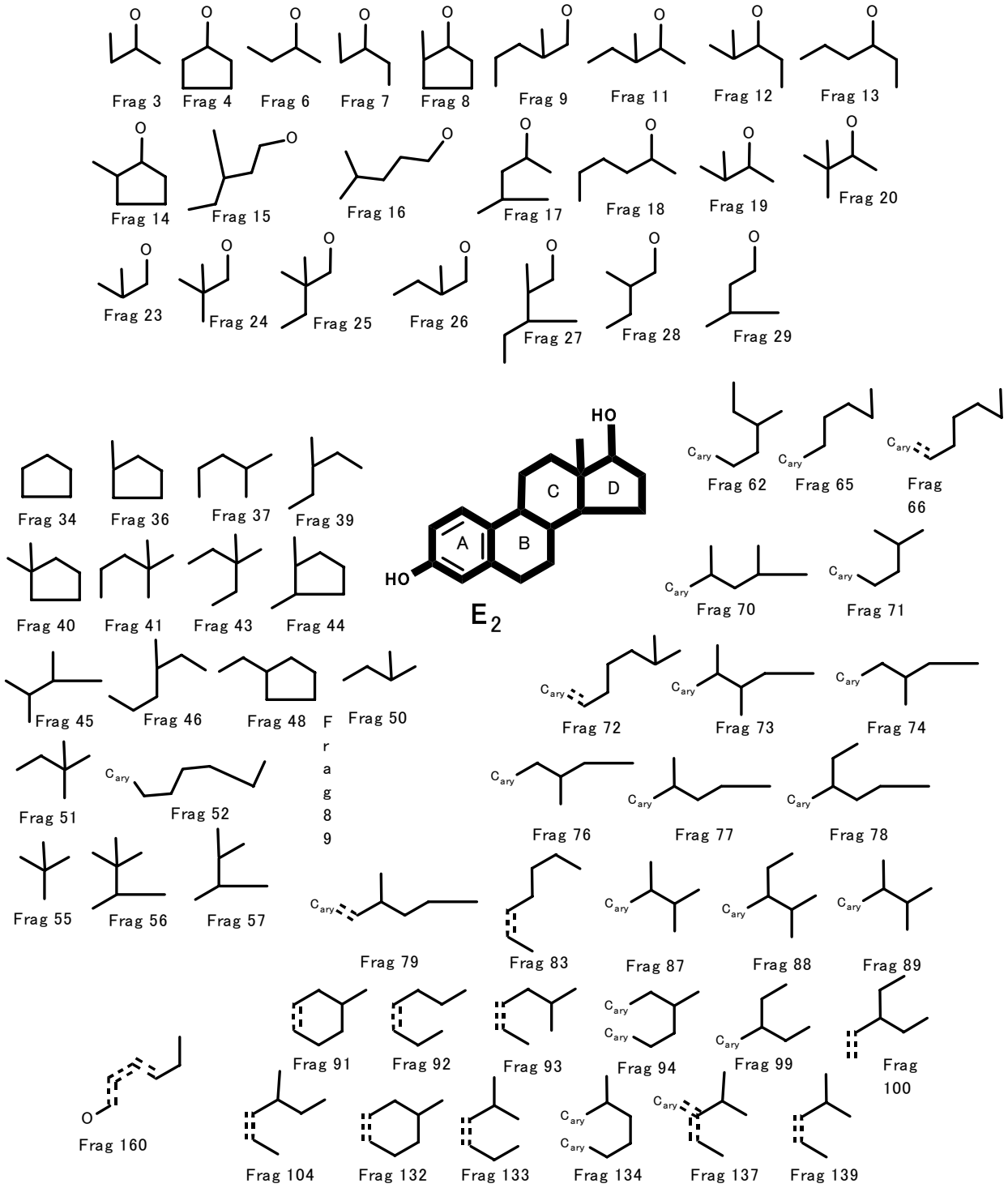

Figure 1. Cat-SAR fragments contributing to the prediction of 17β-estradiol

divided into two major groups. Nearly all significant fragments from both of these groups included the double bond in the five member ring equivalent to the C ring of E_2. The second, and larger group of fragments was distinguished by those which contained portions of the phenol ring

equivalent to E_2's A ring. Only one of these, however, contained the terminal OH on this ring, and none contained the terminal OH on the D ring equivalent.

Table 6. Cat-SAR fragments contributing to the prediction of indenestrol

Frag Name	# Act	#Inact	Total	% Act	%Inact
frag472	7	0	7	1	0
frag588	14	2	16	0.875	0.125
frag629	8	0	8	1.000	0.000
frag747	15	2	17	0.882	0.118
frag752	8	0	8	1.000	0.000
frag3366	3	0	3	1.000	0.000
frag3373	3	0	3	1.000	0.000
frag3374	5	0	5	1.000	0.000
frag3381	3	0	3	1.000	0.000
frag3382	5	0	5	1.000	0.000
frag3387	3	0	3	1.000	0.000
frag3390	3	0	3	1.000	0.000
frag3391	5	0	5	1.000	0.000
frag3393	4	0	4	1.000	0.000
frag3399	3	0	3	1.000	0.000
frag3402	3	0	3	1.000	0.000
frag3403	5	0	5	1.000	0.000
frag3405	4	0	4	1.000	0.000
frag3411	3	0	3	1.000	0.000
frag3419	3	0	3	1.000	0.000
frag3420	5	0	5	1.000	0.000
frag3423	4	0	4	1.000	0.000
frag3429	3	0	3	1.000	0.000
frag3438	3	0	3	1.000	0.000
frag3439	5	0	5	1.000	0.000
frag3440	4	0	4	1.000	0.000
Validation Summary for Indenestrol					
Activity		Mean %Act	Mean %Inact	Frag Count	
1		0.97	0.03	26	

Malathion, an organophosphorous ester widely used as an insecticide, was evaluated to provide an example of a negative cat-SAR prediction, as shown in Table 7 and Figure 3. Only one of the ten fragments was associated with any probability of activity. This fragment forms a separate group in Figure 3. The rest, all representing segments of the organophosphorous portion of the molecule, are strongly associated with inactivity. Malathion was predicted as having a mean probability of activity of 0.1.

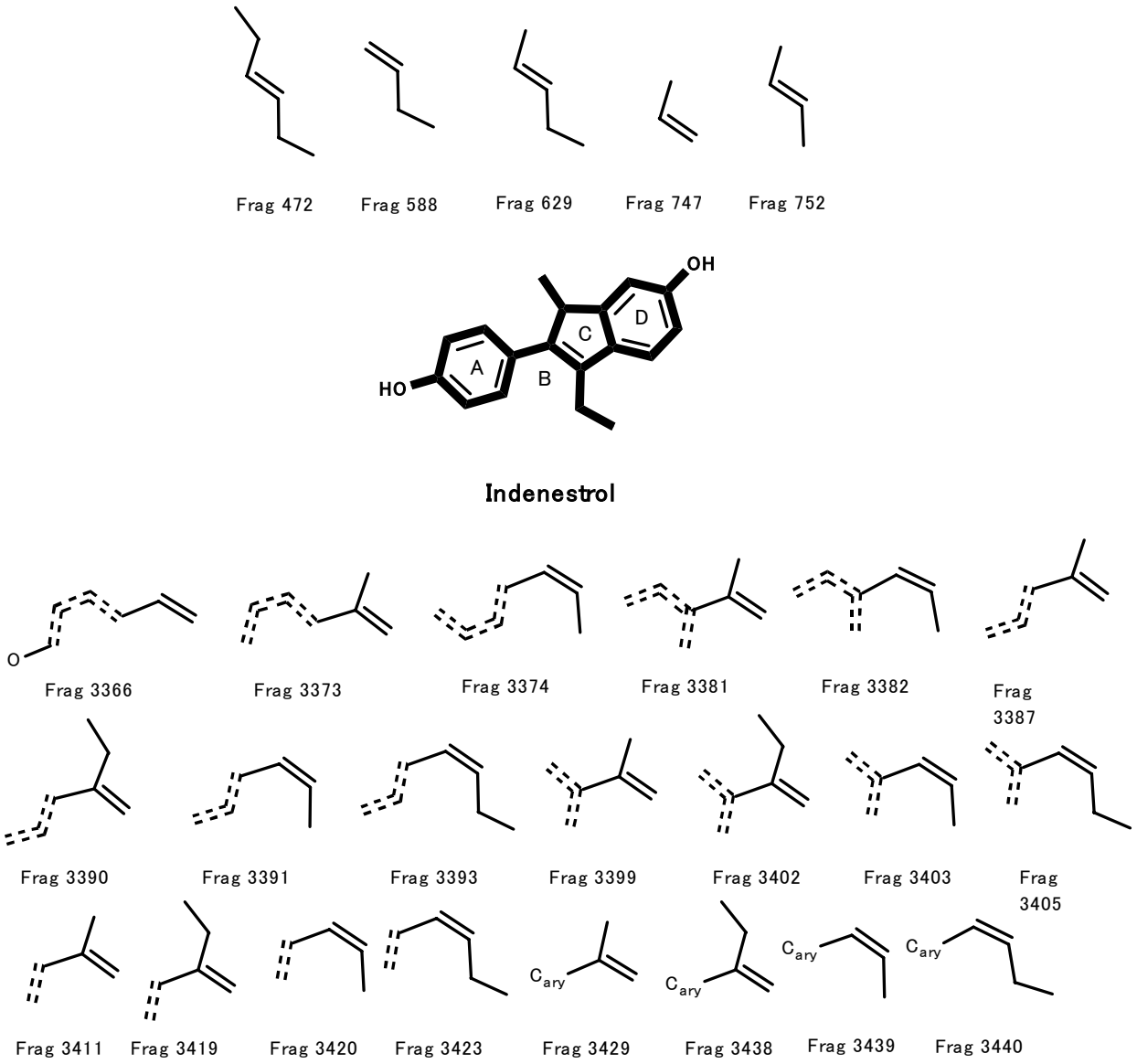

Figure 2. Cat-SAR fragments contributing to the prediction of indenestrol

In order to compare the relationship of the ESCREEN endpoints to that of other models, the "chemical diversity approach" was used. As mentioned earlier, a chosen cat-SAR RPE model was used to predict the activities of a set of 10,000 chemicals. This set was selected randomly from a several structure libraries, and was designed to provide a rough model of the diversity of chemicals encountered in the environment. The model was compared to a previous RPE model made using the MultiCASE software, a cat-SAR model for *Salmonella* mutagens and to two different rodent mammary carcinogen models described previously. These are shown in Table 8.

Table 7. Cat-SAR fragments contributing to the prediction of malathion

Frag Name	# Act	#Inact	Total	% Act	%Inact
Frag652	3	0	3	1	0
frag2412	0	3	3	0	1
frag2413	0	3	3	0	1
frag2414	0	3	3	0	1
frag2418	0	3	3	0	1
frag2422	0	3	3	0	1
frag2429	0	3	3	0	1
frag2437	0	3	3	0	1
frag2438	0	3	3	0	1
frag2450	0	3	3	0	1

Validation Summary for Malathion

Activity	Mean %Act	Mean %Inact	Frag Count
0	0.1	0.9	10

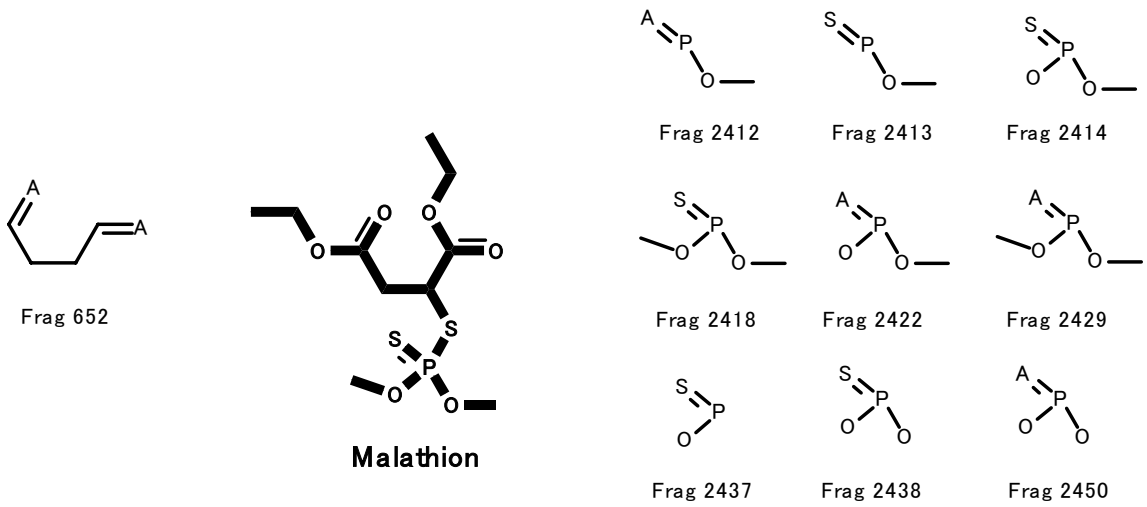

Figure 3. Cat-SAR fragments contributing to the prediction of malathion

Table 8. Mechanistic relationships of the ESCREEN RPE model to other models, and other toxicological endpoints.

Analysis	Observed	Expected	Δ	p-value	100Δ/Expected
MultiCASE ESCREEN RPE	827	499	328	<0.0001	65.73%
Salmonella Mutagenicity	684	1094	-410	<0.0001	-37.48%
CPDB rat mammary carcinogens vs. rat noncarcinogens	446	799	-353	<0.0001	-44.20%
CPDB rat mammary carcinogens vs. rat nonmammary carcinogens	933	935	-2	0.961	0.21%

Observed: Number of compounds simultaneously identified as active in the RPE model and the given endpoint.
Expected: Product of the individual prevalences of compounds identified to be RPE active, and that for the given endpoint (this would be the overlap expected if they were completely independent)
p-value: Difference of two means test.
Δ: Difference of observed and expected
100Δ/Expected: Percent difference from expected

As expected, there was a relatively high degree of overlap with the MultiCASE ESCREEN RPE model predictions. The MultiCASE model had a validation sensitivity of 0.88,specificity of 0.86, and an overall rate of correct prediction (OCP) of 0.86. The cat-SAR sensitivity, specificity, and OCP values were 0.89, 0.82, and 0.86 respectively. These two models, based on the same dataset but developed with very different methodology, still showed a high degree of similarity in their results. As MultiCASE is considered to be an established SAR method, this provides further evidence for the validity of the cat-SAR approach. When the cat-SAR RPE model was compared to a model of *Salmonella* mutagenicity, on the other hand, a negative relationship was found. This could be interpreted as indicating an antagonistic relationship between these two endpoints--that those chemicals with estrogen activity are less likely than chance to be mutagenic. This might indicate that there is something about the structure of estrogen-active compounds which is not compatible with mutagenicity, or vice versa.

When evaluated against the predictions of the first rat CPDB model, rat mammary carcinogens vs. rat noncarcinogens, another strong negative relationship was found. This could

be evidence of a similar antagonistic relationship between the two biological endpoints, mammary carcinogenesis and estrogenicity. This result may be understandable in light of recent evidence that environmental estrogens are not, in fact, mammary carcinogens (Safe 1997). Of course, the carcinogenic action of estrogens has long been associated with their proliferative effects and not with any mutagenic effects. This result, then, may be a function of the selected rat dataset, which may be heavily biased toward mutagenic carcinogens. Note also that when evaluated against the second rat model, a model developed to compare rat mammary carcinogens to rat carcinogens which do not specifically cause mammary tumors, there is no apparent relationship between the endpoints.

CHAPTER 5. SUMMARY AND CONCLUSIONS

5.1 Summary

The cat-SAR modeling system was successfully used to create several predictive models for estrogenicity based on the ESCREEN dataset. ESCREEN is an *in vitro* test of a compound's capacity to stimulate growth in MCF-7 breast tumor cells, and the dataset is made up of a relatively diverse set of compounds. A number of models were developed and tested based on the ESCREEN dataset. One set of models was developed for the RPE endpoint, a measure of maximum proliferative potency, and one set for the RPP endpoint, a measure of a compounds proliferative potency relative to that of E_2. The models tested here for each of these endpoints varied only in the criteria used for fragment generation and the percent cutoff variable. Each endpoint was modeled using atoms, bonds, and connections for fragment generation, and again using the inclusion of hydrogen atoms as a fourth criterion. The percent cutoff variable was one of those primarily responsible for determining the number of fragments which went into constructing the model. More restrictive values led to higher rates of correct predictions, but also resulted in an increase in the number of compounds for which the model made no prediction. Models were validated using a leave-one-out method of cross-validation. One model for each endpoint was chosen for further study. Both of these based on a percent cutoff of 0.75. These were chosen because demonstrated the desired balance between prediction rate and number of predictions made.

The RPE model made predictions for 117 of the 122 compounds, correctly predicting 101 of these for an 86% accuracy rate. The model demonstrated 89% sensitivity and 82% specificity. This was considered well balanced for both positive and negative predictions, as it did not maximize the rate of either correct positive or negative predictions at the cost of either higher

rates of false positives or false negatives. The RPP model made predictions for 96 of 122 compounds, correctly predicting 79 for an 82% rate of accuracy. Sensitivity of the RPP model was 83% and specificity was 81%. These models exhibited similar predictivity to models created with the same dataset using the MultiCASE system, an accepted SAR method.

5.2 Conclusions

Cat -SAR's successful prediction for this dataset provides an early indication of its potential use in screening large numbers of structurally diverse compounds for further testing. The high degree of concordance with a model based on the same learning set, but using a different methodology, would seem to indicate that the cat-SAR method seems to perform well when compared to at least one other established method. The cat-SAR approach has also been used to develop predictive models for *Salmonella* mutagens, skin sensitizers, and rodent carcinogens.

Cat-SAR is a mechanistically transparent method, in that it evaluates all model fragments for their potential association with the modeled endpoint. The fragments contributing to the prediction of each compound can then be evaluated to determine their mechanistic validity. The fragments contributing to the prediction of E_2 fit reasonably well with what is known about E_2's estrogen receptor binding characteristics. This sort of mechanistic information can be useful in providing testable hypotheses regarding estrogenic activity. This is particularly relevant given the EPA's broad definition of an endocrine disruptor, wherein it is unlikely that one mechanism will completely describe chemical-induced estrogenic activity

What seems to be an antagonistic relationship was found between the predictions for a set of 10,000 chemicals cat-SAR made for the ESCREEN RPE endpoint and those made by a *Salmonella* mutagenesis model. This relationship was not found when evaluating two MCASE

models for the same endpoints (Cunningham 2004). It is unclear whether this result truly describes the relationship of *Salmonella* mutagens and estrogens, or whether it is somehow a function of the cat-SAR methodology. A similar antagonistic relationship was found between the cat-SAR estrogen model and a cat-SAR model for a set of rat mammary carcinogens and rat noncarcinogens. This antagonism was not found, however, between estrogens and a model of rat mammary carcinogens and rat carcinogens which were not specifically mammary carcinogens. Similar analyses of MCASE models did not find these results (Cunningham 2004).

5.3 Future directions

Future work may proceed in a number of directions. New models could be developed to keep pace with future additions to the ESCREEN dataset. Initial efforts have already been made to test models made with fragments which could include larger numbers of atoms. To date, fragments of three to twelve atoms in length have been modeled using cat-SAR. Some difficulty has been encountered, however, with the computing requirements for the very large numbers of fragments which can be generated in this way. However, these larger fragments might provide additional insight into the mechanistic sense of the model. This might especially be true for estrogen receptor binding characteristics, as the E_2 pharmacophore is larger than seven atoms in length.

The cat-SAR method itself might be applied to other endpoints as well. It has already been used to model rodent carcinogens, skin sensitizers, *Salmonella* mutagens and estrogens. It might also be useful in pharmacological study, possibly as an aid to drug discovery efforts or as an aid to determining the mechanism behind a given biological activity. It may also be useful to look further into the apparent antagonistic relationship between the estrogen model and that for *Salmonella* mutagenicity.

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