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REGULATORY RESEARCH PERSPECTIVES

Impact on Public Health

Development of Quantitative Structure-Activity Relationships (QSARs) and Their Use for Priority Setting in the Testing Strategy of Endocrine Disruptors

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Abstract: Considerable scientific, regulatory and popular press attention has been devoted to the Endocrine Disrupting Chemicals (EDCs), of which estrogenic chemicals figure prominently. A large number of potential estrogenic EDCs are associated with products regulated by the Food and Drug Administration (FDA), including plastics used in food packaging, phytoestrogens, food additives, pharmaceuticals, cosmetics, etc. Recent legislation mandates the U.S. Environmental Protection Agency (EPA), a sister regulatory agency, to develop a screening and testing program for potential EDCs in drinking water and food additives. Under the legislation, a large number of chemicals will undergo various *in vitro* and *in vivo* assays for their potential estrogenicity, as well as other hormonal activities. There is a crucial need to set priority for these chemicals to reduce the cost and speed the screening and testing process. At the FDA National Center for Toxicological Research (NCTR), quantitative structure-activity relationships (QSARs) is a major component of the Endocrine Disruptor Knowledge Base (EDKB) project – a prototype Toxicological Knowledge Base. By integrating experimentation and modeling, a series of QSAR models have been developed and validated in the project. These models are integrated into a "Four-Phase" scheme, with each successive phase eliminating unlikely estrogen receptor (ER) binders, resulting in a priority listing of chemicals for regulatory application. The system performance has been validated using several data sets with known estrogenic activity and, subsequently, applied to three environmental data sets, identified by the EPA. It has also been used to assess estrogenic activity of chemicals of concern at other Centers within the FDA, namely the Center for Food Safety and Applied Nutrition (CFSAN) and the Center for Drug Evaluation and Research (CDER). The rigorous validation of the integrated system is ongoing via the interagency agreement (IAG) between EPA and NCTR. The approach presented here for estrogen is anticipated to be equally applicable for other receptor-mediated, endocrine disrupting mechanisms, e.g., androgen receptor binding, and other toxicity endpoints.

Introduction

While the toxicological sciences have advanced steadily over past decades owing to constant improvement in experimental techniques and instrumentation, and in the general understanding of biological mechanisms, the overall paradigm is largely unchanged. Specifically, models for risk assessment are predominantly dependent on slow and expensive testing in laboratory animals, since epidemiological data are insufficient and testing in humans is not possible (except for drugs during the advanced stages of develop-

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ment). A fundamental tenet of this paradigm is that effects in laboratory animals can be extrapolated to humans (or other animals). Intrinsic variability in the response of the test species, and the number of animals tested, limits the resolving power of the affordable protocols to determine a No Observed Adverse Effect Level (NOAEL), the dose which provides no statistically significant increase in adverse effects above the control value. This, together with the need to extrapolate to humans, results in the use of safety factors, generally in the range of two or more orders of magnitude to determine an allowable dose for non-genotoxic chemicals.

In 1995, the National Center for Toxicological Research (NCTR) reoriented its strategic goals to begin to alter the paradigm of toxicological research, taking direct aim at increasing regulatory efficiency by reducing the time, expense and animal use in the regulation process. One primary strategic goal was the development of knowledge bases, that were defined to be computer-based systems that unify applicable literature and data and provide computational models to predict a chemical's toxic potential, predict experimental needs and improve regulatory risk assessment capability. The inspiration for the Estrogen Knowledge Base (EKB) program came from a center wide challenge issued by then NCTR Director, Dr. Bernard Schwetz, to develop a knowledge base with the capability to extend the predictability of existing data. Scientists within the Division of Reproductive and Developmental Toxicology suggested that estrogens might provide an appropriate area to develop a prototype. Knowing that NCTR scientists had been engaged in estrogen related research for more than two decades, Dr. Schwetz posed the questions whether they could recognize an estrogen receptor ligand, solely based on chemical structure, and whether models based on existing data could be used to develop models to make such a prediction. The answers were "no" and, "let's try," respectively. The "let us try" subsequently developed into a concept to develop a prototype knowledge base to predict estrogenicity, and a grant from FDA's Office of Women's Health enabled the acquisition of the required computer hardware and software.

The earliest models, based on 13 chemicals tested at NCTR, proved very inadequate. Despite the clear statement in endocrinology textbooks that a single nuclear receptor protein that exhibited high ligand specificity mediated most estrogenic effects, it became quickly apparent that the estrogen receptor binds chemical structures of surprisingly broad diversity. Next, we used the extensive data published in the literature to build models based on 50-chemical training sets. While these models were much improved, we learned that better models were possible given a proper database.

During the course of our work on the early models, a major scientific and regulatory issue developed surrounding environmental chemicals, termed endocrine disrupting chemicals (EDCs), suspected of disrupting endocrine function by mimicking natural hormones in experimental animals, wildlife and humans. There was a constantly growing concern among the scientific community, government regulators and the public that EDCs in the environment were adversely affecting human and wildlife health (*1, 2*). Adverse outcomes had been observed in experimental animals and wildlife; potential effects in humans included reproductive and developmental toxicity, carcinogenesis, immunotoxicity, and neurotoxicity, among others (*3*). The scientific debate surrounding EDCs grew contentious, in part owing to the fact that some suspected EDCs are high production volume, economically important chemicals. The public and regulatory concerns led to government regulatory actions and expanded research across Europe, Japan and North America (*4, 5*). In 1996, the U.S. Congress mandated that the Environmental Protection Agency (EPA) develop a strategy for screening and testing a large number of chemicals found in drinking water, food additives and other sources for their endocrine disruption potential (*4*).

In response to Congressional action, the EPA established the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), which includes scientific expertise from government, academia and industry. EDSTAC recommended a two-tier (Tier 1 screening and Tier 2 testing) strategy to screen and test for estrogenic, androgenic and thyroid endpoints for a large number of chemicals. To accomplish this, chemicals will be screened (Tier 1) using a multiple-endpoint strategy that includes more than 20 different *in vitro* and *in vivo* assays recommended by EDSTAC (*6*). Although more than ~87,000 chemicals were initially selected for evaluation, many were polymers or otherwise unlikely to bind to steroid receptors, leaving about ~58,000 chemicals for evaluation in Tier 1. The number that will progress to the testing step (Tier 2) (*7*) is not known. Processing all chemicals through both tiers, if required, will require many years and extensive resources. Hence, the EPA has adopted an approach requiring priority setting before Tier 1 (www.epa.gov/scipoly/oscpendo/), and where QSAR predictions are likely to prove of particular utility.

The EKB multidisciplinary team of researchers developed a plan to meet the newly important challenge for precise and validated predictive models that was reviewed and endorsed by the NCTR Scientific Advisory Board (SAB) in 1997. The most important finding of the early model building (*8-12*) effort was that, despite decades of testing for estro-

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genicity, the existing data were inadequate to construct robust QSAR models (SETAC chapter) (*12*). Accordingly, the EKB plan called for developing a model training data set by conducting ER binding measurements in a validated assay. The chemicals to be tested would be chosen by the computational chemists to obtain a training set spanning the broad range of chemical structures of ER ligands, both agonists and antagonists. Furthermore, there needed to be several types of models ranging from fast and easy classification methods to rule out inactive chemicals, to highly accurate but time-intensive, three-dimensional QSAR models to quantify binding affinity for active chemicals. As the scope of the EKB project expanded in the late 1990s, to include all Endocrine Disrupting Compounds, we renamed the prototype knowledge base the Endocrine Disruptor Knowledge Base (EDKB). A follow-up second SAB review occurred in 2000, which supported placing the EDKB database on the WEB (http://edkb.fda.gov). As the program developed, additional funding was provided by the OWH, in 1998 and 2000, by a Cooperative Research and Development Agreement (CRADA) with the American Chemistry Council (ACC), formerly the Chemical Manufacturer's Association (CMA), and again in 2001, with continued support to 2005, by EPA.

The objective of the EDC priority setting is to rank order a large number of chemicals for more resource-intensive and costly Tier 1 evaluations from most important to least important. There are a number of criteria that can be used for this purpose, such as production volume, persistence and fate in the environment, human exposure levels, etc. Most of the 58,000 chemicals required for assay have no biological data. Both QSARs and transcription-based high throughput pre-screening (HTPS) were recommended by the EDSTAC as the primary source of biological effect information for priority setting. However, in a pilot study undertaken by EPA, the HTPS system did not perform well (*13*), such that the EDKB models could prove critical in EDC screening and testing program.

The compounds assayed at NCTR were selected based on providing uniform coverage of the diverse chemical structure space of chemicals that bind the receptors, as well as coverage of an activity range extending down to a million-fold below that of the endogenous hormones. The model training set designed for chemical structure diversity comprises 130 ER binders and 100 non-binders (*14, 15*). The large number of inactive chemicals, included in the training sets, enables models to be trained to distinguish active from inactive compounds. Many SAR, QSAR and chemometric predictive models were developed using the many powerful commercial software packages that are now routinely applied in drug discovery and development. In the end, the rigorous, three-dimensional, QSAR method of Comparative Molecular Field Analysis (CoMFA) was selected for quantitative prediction of receptor binding affinity (*16*). Three different types of models (structural alerts, pharmacophores and classification methods) were combined (*17*) to make qualitative prediction (i.e., active or inactive) of ER binding activity.

In the sections that follow, we present results of integrating the qualitative and quantitative predictive models into a sequential "Four-Phase" scheme (*18, 19*) according to the strength of each type of model. Hierarchical sequencing of the models allows faster models to be used to eliminate the majority of inactive chemicals with an extremely low rate of false negatives. The more time-consuming but more precise models can be used to refine predictions for an increasingly smaller number of remaining chemicals. The application of the more refined models further eliminates true negatives, as well as false positives, from earlier models. Results are presented suggesting that the use of this scheme could eliminate from testing about 90% of the chemicals of potential concern in the national screening and testing program. Should the ER-binding models be used for priority setting, the program begun as a prototype effort will have matured to one of the most significant uses of QSAR in the regulatory application.

Currently, the EDKB team is completing models for prediction of binding to the androgen receptor (AR). A validation program is also now underway via an Interagency Agreement between FDA/NCTR and EPA. A large number of chemicals will be tested blind by the models, for both ER and AR binding, and the predictions then compared to assay results from an outside contract laboratory. The validation results will define whether and how model predictions are used in priority setting in the EPA's Endocrine Disruptor Screening and Testing Program.

Quantitative Structure-Activity Relationships (QSARs)

QSAR modeling employs statistical approaches to correlate and rationalize variations in the biological activity of a series of chemicals with variations in their molecular structures. The molecular structure is often represented by a set of quantities commonly known as structural descriptors. QSARs have been applied extensively in a wide range of scientific disciplines including chemistry, biology and toxicology (*20, 21*). In both drug discovery and environmental toxicology (*22*), QSAR models are now regarded as a scientifically credible tool for predicting and classifying the biological activities of untested chemicals. As we enter the

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informatics era, QSAR has become essential in the drug discovery process as a screening and enrichment tool to eliminate, from further development, those chemicals lacking drug-like properties (*23-25*) or those chemicals predicted to elicit a toxic response. This developing scenario portends the spread of QSAR, beyond the pharmaceutical industry, to human and environmental regulatory authorities for use in toxicology (*16, 19, 26-30*). The EDSTAC considers QSAR as an important part of its priority setting process (*31*). QSARs can be used to evaluate untested chemicals to provide biological data for use in priority setting (*32-36*).

The basic assumption in every QSAR model is that a chemical's physical and chemical properties and its biological activities are predicted by its structure (*37*). Since structural descriptors of a chemical can be determined by computational means more efficiently than its biological activity using *in vitro* or *in vivo* approaches, a statistically validated QSAR model is capable of predicting the biological activity of a new chemical in lieu of the time-consuming and labor-intensive processes of chemical synthesis and biological evaluation. Applied judiciously, QSAR can save substantial amounts of time, money and human resources. A major advantage of QSARs, to priority setting, is the efficiency of scale when applied to a large number of chemicals. When several endpoints are analyzed simultaneously, the efficiency of scale of computation is even more pronounced.

The first step in developing a QSAR model is acquisition of a training set of chemicals that have known activities. Second, descriptors representing molecular structure of individual chemicals (i.e., hydrophobicity, structural fragments, charged surface area, the number of hydrogen bonds, solubility, and etc.) are calculated. Then, a correlation between descriptors and activity for the training set is evaluated by employing various statistical approaches to determine the most statistically significant relationship (the QSAR model). A proper validation is required to ensure the model's predictive value for the chemicals not used in the training set. With adequately validated performance, such models can be used to predict activities of untested potential EDCs.

Obtaining a good quality QSAR model heavily depends on many factors, in the approach, particularly on quality of biological data, descriptor selection and statistical methods:

(1) Quality of biological data – It is desirable that data come from the same assay protocol, and care is taken to avoid inter-laboratory variability. Any bad data points may corrupt the proper correlation of structure and activity. Rules of thumb for a good QSAR data set are: 1) the dose-response relationship is smooth; 2) the potency (or affinity) is reproducible; 3) the activity range spans two or more orders of magnitude from the least active to the most active chemical in the series; 4) the number of chemicals used to build the QSAR model is sufficiently large to ensure statistical stability; 5) activities of the chemicals are evenly distributed across the range of activity; and 6) the chemicals selected for the training set possess enough structural diversity to span the range of "chemistry space" associated with the biological activity under study. It is important to note that most toxicity data do not meet all these criteria because of the nature of toxicological research, in which care should be taken in interpreting QSAR results.

(2) Descriptor selection - There are many types of chemical structure descriptors available from commercial software. Obtaining a statistically robust model is very much dependent on how well the selected descriptors can encode the variation of activity with structure. The more that is known at the molecular level about the biological mechanism of action of the chemicals, the better the chemist is able to select among the wide variety and types of specific structural descriptors. Commercially available molecular modeling programs often include statistical tools to help in evaluating which descriptors best encode structure-activity variation. Some of these tools include the genetic algorithm (GA) in its various incarnations, which employs the evolutionary rules of natural selection to select the optimal (i.e., fittest) subset of descriptors amongst its wide set for a particular problem.

(3) Statistical methods – It is also critical that the QSAR method selected to develop the structure-activity correlation be suitable. Although the relationship between a structural descriptor and biological activity may be linear or non-linear, it is still common practice today to employ linear approaches such as multiple (or multivariate) linear regression (MLR) or partial least-squares (PLS) regression to construct the QSAR model. For non-linear modeling, the Polynomial Neural Network (PNN) offers an alternative that combines the best features of Artificial Neural Networks (ANNs) and MLR/PLS by providing the inherent non-linearity of the ANN with the desired analytical regression equation furnished by MLR and PLS (*38*). The most common scenario encountered in practice is for the number of possible descriptors to exceed the number of chemicals, a situation that can lead to chance correlation. Fortunately, soft modeling methods, such as PLS, reduce the risk of encountering chance correlation by transforming the

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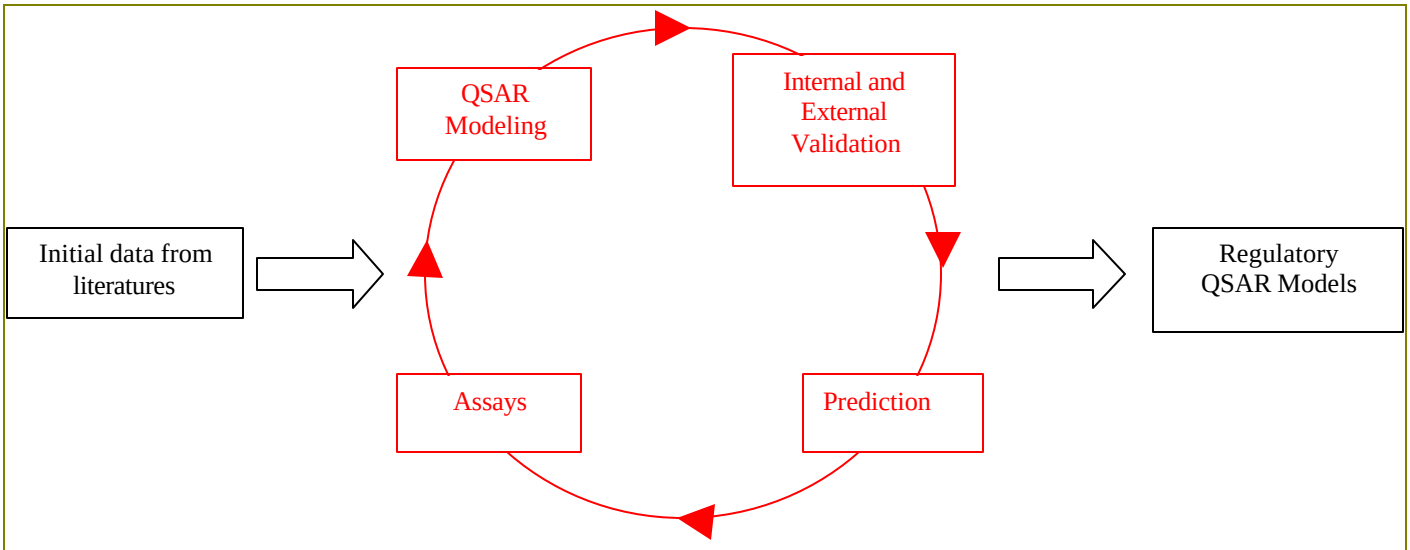

Figure 1. Depiction of the recursive process used by NCTR to develop QSAR models for predicting estrogen receptor binding. The process starts with an initial set of chemicals from literatures for QSAR modeling. Next, the preliminary QSAR models are used prospectively to define a set of chemicals that will further improve model's robustness and predictive capability. The new chemicals are assayed, and these data are then used to challenge and refine the QSAR models. The process output is the models for use in toxicological regulation. Validation of the model is critical, particularly with respect to confirming minimal false negative predictions.

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dimensionality of the regression problem from chemical-descriptor space to so-called chemical-principal components (PCs) space.

QSAR models are useful in research for purposes beyond prediction (*39*). Additional benefits that may accrue include: (1) leveraging existing structure-activity data; (2) providing insights into mechanism or identifying an alternative mechanism (e.g., metabolism) of action; (3) identifying important chemical structure characteristics; (4) suggesting new design strategies and synthetic targets; (5) narrowing the dose range for a planned assay; (6) assisting in generation of new hypotheses to guide further research; and (7) revealing chemicals that deviate from the QSAR model.

The NCTR Model Development Process

In the past few years, a number of QSAR models have been developed for ligand binding to the ER (*8-11, 40-44*). Most of these QSAR models were constructed using the Comparative Molecular Field Analysis (CoMFA). Although a predictive QSAR model is dependent on a number of factors, a training set with a broad representation over the chemistry space is critical to ensure its predictive capability for a large number of diverse chemicals. Unfortunately, most QSAR models for ER binding, developed previously, were based on data sets available in the literature, which to date had been small data sets with limited structural diversity (*8, 42, 43*). Although these models yield good statistical results and explain some structural determinants for ER binding, they have limited applicability in predicting the ER-ligand binding affinity of chemicals that, in fact, cover a wide range of structural diversity.

In order to obtain an adequate training set to develop a more robust QSAR model for regulatory purpose, a recursive process (Figure 1) has been adopted at NCTR by integrating assay and QSAR modeling to determine chemicals for the training set (*12, 45*) along with the model construction. The process is highly interdisciplinary, involving computational chemists, biologists and experimental toxicologists. As depicted in Figure 1, the process starts with an initial set of chemicals from literatures for QSAR modeling. Next, the preliminary QSAR models are used prospectively to define a set of chemicals that will further improve model's robustness and predictive capability. The new chemicals are assayed, and these data are then used to challenge and refine the QSAR models. Through this process, we identified and assayed ~230 chemicals for final model construction. This NCTR data set contains chemicals that were selected to cover the structural diversity of chemicals (Figure 2a) that bind to ER with an activity distribution ranging over six orders of magnitude (Figure 2b), which is an essential requirement for a robust predictive model for structurally diverse estrogens. The NCTR data set is a highly consistent data set for use in developing models for estrogens.

The assay used in the process to provide ER binding data is a rat uter-

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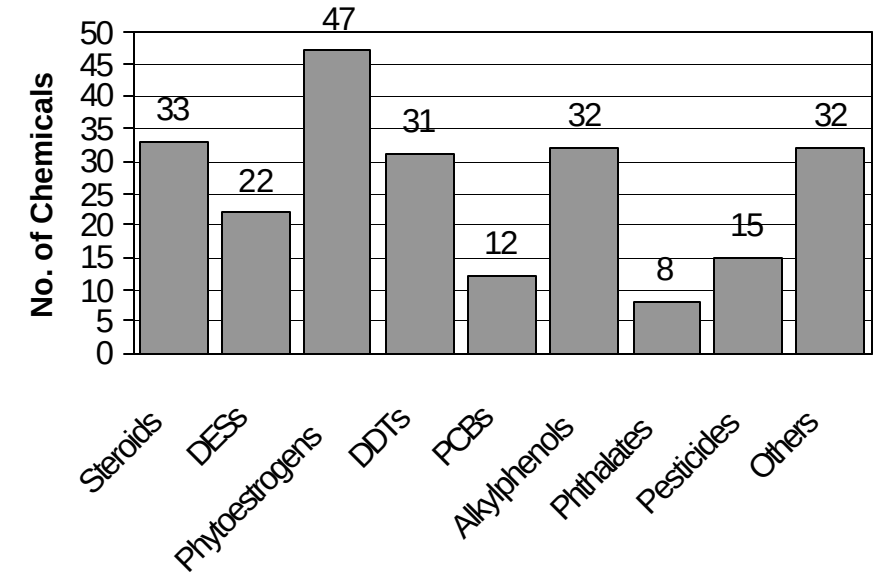
ine cytosol ER competitive binding assay that is considered the gold standard for *in vitro* ER assays (*14, 15*). We found a strong linear correlation for ER binding affinities among a diverse group of chemicals assayed with ER derived from our rat uterine cytosol and human ERa (*46*). Furthermore, the rat ER binding data also correlated strongly with the results from assays measuring estrogenicity using a downstream event, i.e., a yeast-based reporter gene assay and MCF-7 cell proliferation assay. Importantly, chemicals positive in uterotrophic responses (*in vivo* estrogenic activity) are also positive in the ER binding assay, indicating that binding affinity is a good predictor of *in vivo* activity with few false negatives observed (*47*). These findings demonstrate that ER binding is the major determinant for estrogenic EDCs, and the prediction of the rat ER binding affinity provides an important piece of information for priority setting.

In this process, a model validation step is specifically emphasized to ensure the model's predictive value for priority setting purposes (*12*). Internal and external validation were included in the process; each provides a different level of confidence for the model's predictivity. Generally, the model is first validated using leave-one-out cross validation. In this method, each chemical in the training set is systematically excluded once from the data set, after which its activity is predicted by a model derived from the remaining chemicals. This internal validation assesses the model's extrapolation within the training set. Sometimes, we employ leave-*N*-out cross-validation to achieve more robust internal validation; a procedure similar to leave-one-out, but in this one, we systematically exclude one group of chemicals after randomly dividing the training set into *N* groups. When additional data are available, the model is used to predict other chemicals not used in the training set, but which have known activities (the testing set). This external validation assesses the model's predictive capability for untested chemicals.



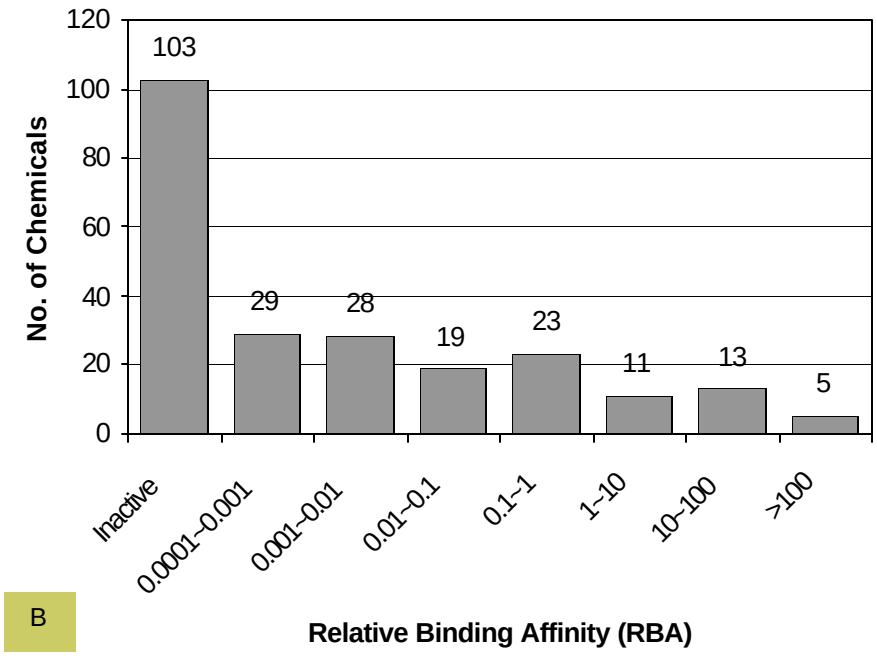


Figure 2. The distribution of (A) chemical classes and (B) binding activity for the NCTR data set. The estrogen receptor binding activity is represented as RBA (Relative Binding Affinity). The RBA for the endogenous ligand, 17ß-estradiol, was set to 100.

Several benefits accrue from the integration of the experimental and modeling efforts. Immediate feedback can be given to the experimentalists so that suspected problems can be rapidly investigated. Also, as the pre-

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dictive and diversity models evolve, the modelers can select the chemicals for subsequent testing, based on considerations of structural diversity and the activity range. While cross validation using the training set remains an important part of the model validation, each new data point directly from the lab become a challenge to the evolving model, the results of which can then be assessed by the joint team of the modeler and experimentalist.

Each time the model is challenged, the result is either further confirmation of its validity, identification of a limitation or an outlier prediction. Failure of the model will, in turn, provide important information. This may include identification of the need for new data based on a rational understanding of the dependence of activity on structure. Alternatively, it may help elucidate which of many mechanisms may play an important role in a specific chemical's response; for example, delineating agonists from antagonists, or defining where metabolism may be important. Regardless of the cause of model failure, in essence, a research hypothesis is spawned that should lead to new data and/or an improved training set, and an improvement to what is a living model.

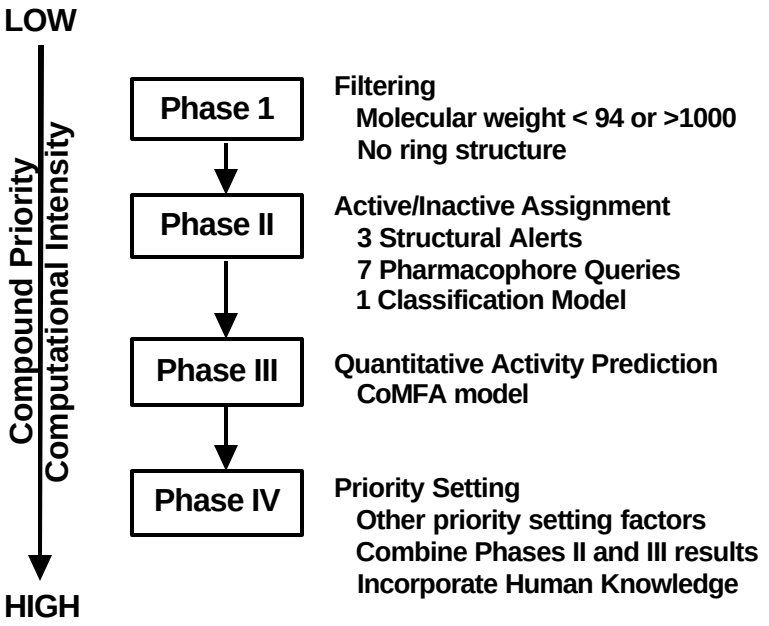

Figure 3. Overview diagram of the NCTR "Four-Phase" approach for priority setting. In Phase I, chemicals with molecular weight <94 or >1000 or containing no ring structure will be rejected. In Phase II, three types of approach (structural alerts, pharmacophores, and classification methods) with total 11 models are used to make a qualitative activity prediction. In Phase III, a 3D QSAR/ CoMFA model is used to make a more accurate quantitative activity prediction. Phase IV, an expert system is expected to combine information gained from Phase II and Phase III and other sources to make a decision on priority setting. Different phases are hierarchical; different methods within each phase are complementary.

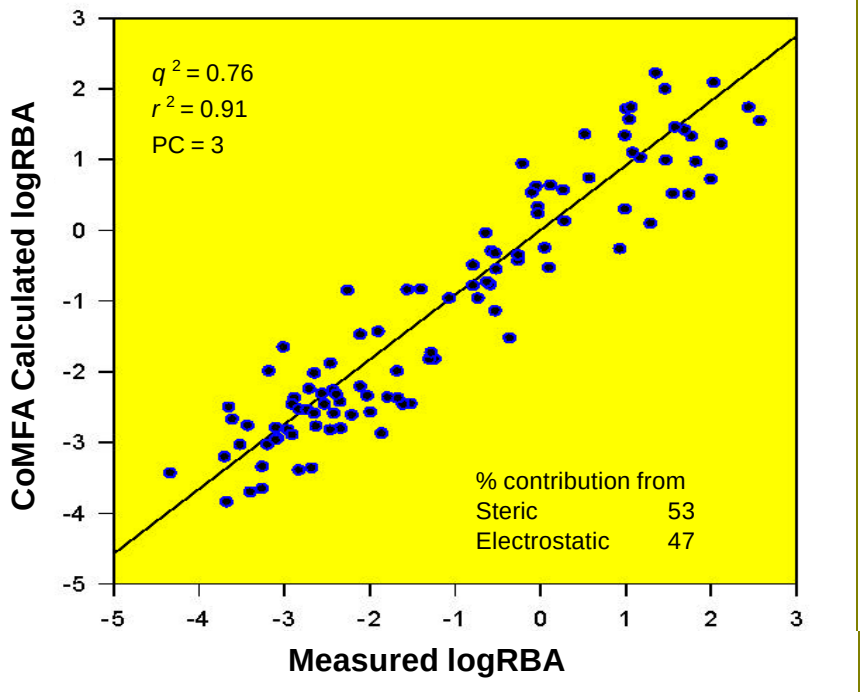

Figure 4. Plot of CoMFA-calculated log relative binding affinity (logRBA) versus observed logRBA for the NCTR data set. r^2= 0.91; q^2=0.76.

The NCTR "Four-Phase" System

Priority setting using QSAR is widely applied in the process of drug discovery. The objective of priority setting in pharmaceutical industry is to increase the chance of finding active chemicals or "hits" that are more likely to be developed into "leads". Hence, false positives are of great concern. In contrast, a good priority setting method for regulatory application should generate a small fraction of false negatives (chemicals predicted to fail to bind to their receptor, but which actually bind). False nega-

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tives constitute a crucial error because they will receive a relatively lower priority for evaluation, in Tier 1, and may remain in use for many years. Furthermore, the methods should provide reasonable quantitative accuracy for true positives, as those with higher affinities will generally be of higher priority. Based on these considerations, we have developed an integrated computational system that rationally combines different QSAR models into a sequential "Four-Phase" scheme according to the strength of each type of model (Figure 3). A progressive Phase paradigm is used as a screen to reduce the number of chemicals to be considered in each subsequent Phase. Therefore, these four phases work in a hierarchical way to incrementally reduce the size of a data set with increasing precision of prediction. Within each phase, different models have been selected to work complementarily in representing key activity-determining structure features to minimize the rate of false negatives. For predicting ER binding affinity, the models comprised of these four phases were:

- ***Phase I: Filtering*** – Two rejection filters, molecular weight < 94 or >1000 and no-ring structure, were used to significantly, and with high confidence, eliminate those chemicals considered unlikely to bind ER (*17*). These two filters were validated on ~2000 chemicals whose ER activities were available from the literature.
- ***Phase II: Active/Inactive Assignment*** – The chemicals passing through Phase I were assigned as YES/NO for ER binding using three different methods, i.e., structural alerts, pharmacophore searching and classification models. While structural alerts identify key 2D structural features associated with ER binding, pharmacophore search identifies 3D sub-structure important for ER binding. Classification models use pattern recognition to qualitatively categorize chemicals into active and inactive subsets on the basis of their similarity in physicochemical properties. In its current form, this Phase employs in parallel 11 models, three structural alerts, seven pharmacophores, and one classification model to discriminate active from inactive chemicals. To ensure a lower false negative rate in this Phase, a chemical predicted to be active, by any of these models, is subsequently evaluated in the Phase III, while only those predicted inactive, by all 11 models, will be eliminated for further evaluation. Since each method incorporates and weighs differently the various structural features that endow a chemical with the ability to bind the ER, the combined outputs derived from the three approaches are complementary in minimizing false negatives. Moreover, combining the outputs of these 11 models provides a rational means to rank order the chemicals in decreasing order of potential activity (*17*).
- ***Phase III: Quantitative Predictions*** – In this Phase, a CoMFA model is used to make a more accurate quantitative activity prediction for chemicals from Phase II. Chemicals with higher predicted binding affinity are given higher priority for further evaluation in Phase IV. The CoMFA model demonstrated good statistical reliability using both internal and external validation (*16*). A plot of the CoMFA-predicted versus experimental RBAs (as logs), computed for the training-set compounds, is given in Figure 4. The conventional r^2 and cross-validated q^2 are 0.91 and 0.76, respectively, indicating that the CoMFA model is both internally consistent and highly predictive. Figure 5 shows two different distributions: 1) the range of fold-differences for individual experimental data points and 2) the range of fold-differences for CoMFA predicted and experimental means. The predictions fall in a similar range to the experimental data points.

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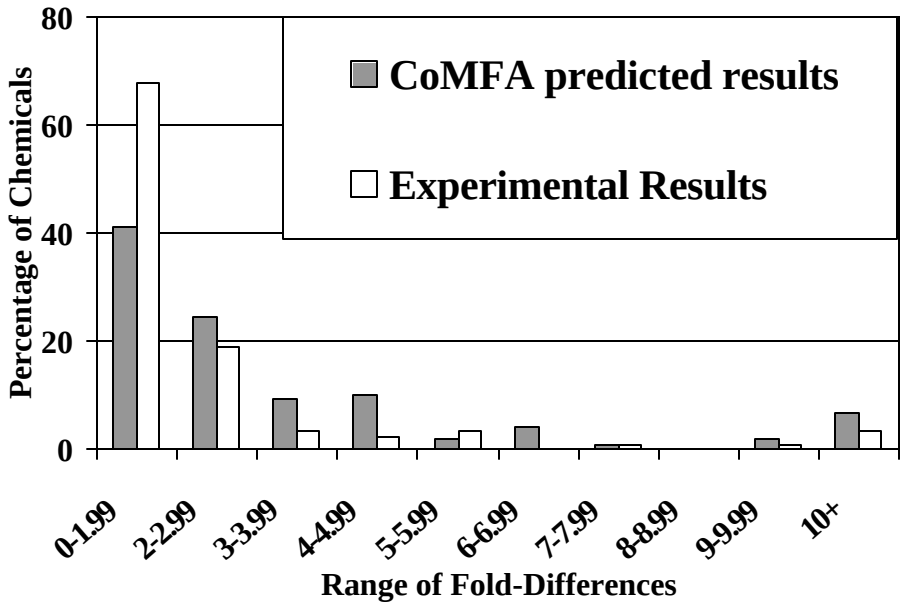

Figure 5. Fold differences for experimental measurements and CoMFA predicted results.

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- ***Phase IV: Rule-Based Decision-Making System*** - In this final stage of the integrated priority setting approach, we propose to use a knowledge-based system, or expert system, to make a priority setting decision. This is a multidisciplinary effort that includes computational chemists, toxicologists and environmental and regulatory scientists from different agencies. The system is useful only after incorporating accumulated human knowledge and expertise (i.e., rules). This system can make decisions on individual chemicals based on the rules in its knowledge base, which at this juncture include but are not limited to:
  1. Information gained at each phase of the integrated computational approach.
  2. Information on human exposure, environmental fate and other effects, and chemical production level.
  3. Chemical structure novelty, that is, when a structure is encountered that is dissimilar to all those that have been used to train and test the models.

The NCTR "Four-Phase" system has been validated by a number of existing data sets, including the NCTR ER binding data set (*14*), the E-SCREEN assay data (*48*), the yeast two-hybrid reporter gene assay data (*49*), and other data sets (*41, 50-54*). To date, the system has produced no false negatives, which is critical in priority setting for regulatory purposes. The same integrated scheme is being extended to include endpoints of other endocrine disrupting mechanisms (e.g., AR binding) at NCTR.

Regulatory Application

The NCTR "Four-Phase" system is a primary candidate priority setting tool for chemical entry to Tier 1 screening. The system was recently applied to three environmental data sets, recognized by EPA, as representative subsets of potential EDCs:

- HPV-Inerts data set – It contains 623 High Production Volume inerts (HPV-Inerts), which is a portion of the Toxic Substances Control Act (TSCA) Inventory. The EPA is including HPV-Inerts in version 2 of the Endocrine Disruption Priority Setting Database (EDPSD2), and there was a need to prioritize HPV-Inerts for further experimental evaluation. Of 623 chemicals, 166 chemicals were either mixtures or their structures were not available, thus excluding them from prediction. Therefore, 457 chemicals were predicted by this system.
- Walker data set - Walker *et al*. developed a database that contains a large and diverse collection of known pesticides and industrial chemicals, as well as some food additives and drugs (*55*). The database contains 92,964 Chemical Abstract Service (CAS) Registry numbers of chemicals that will probably have to be evaluated for their potential endocrine disruption. A final data set of 58,391 chemicals was processed by our system after eliminating those chemicals for which structures were not available (*55*) and/or 3D structures could not be generated (*17*).
- Validation data set – To validate the NCTR "Four-Phase" system, the EPA provided a list of 6,645 chemicals. The EPA randomly selected 200 chemicals from the list and 50 chemicals from those predicted to be active by the system for the list. These 250 chemicals are going to be assayed. By comparing the assay results with the prediction, we will be able to estimate (assess) the degree of false negatives, false positives and quantitative accuracy associated with the system. With this validation, we hope to establish QSARs as a priority setting tool for regulatory application.

Table 1 summarizes the priority setting results for these data sets using the NCTR "Four-Phase" system. When only the Phase I and II protocols are used, the system dramatically reduced the number of potential estrogens by some 80-85%, demonstrating effectiveness in eliminating these most unlikely ER binders from further expensive experimentation. The Phase III CoMFA model further reduces the data size by about 5-10%. Importantly, the quantitative

(Continued on page 10)

Table 1. Size reduction of three environmental data sets processed by the NCTR "Four-Phase" system

	HPV-Inerts	Walker	Validation
Original Data Size	457 (100%)	58,391 (100%)	6,645 (100%)
After Phase I and II	15.7%	12.0%	11.0%
After Phase III	9.8%	—	4.8%

(Continued from page 9)
binding affinity prediction from Phase III provides an important rank-order value for priority setting.

Concluding Remarks

EDCs have potential adverse effect on human beings and wildlife. The potential of chemicals to interfere with estrogen functions may be related to their ability to mimic estrogen and bind to ER. The potential to mimic estrogen and bind to ER can be quantitatively predicted using computational methods such as QSARs. QSARs are an important part of priority setting to determine which chemicals should be experimentally evaluated first in Tier 1 screening recommended by EDSTAC.

In conjunction with EPA, the EDKB project team at the FDA/NCTR has developed a number of QSAR models for prediction of chemical binding to the ER. These models are integrated into a "Four-Phase" scheme, which we have shown to demonstrate efficiency and accuracy for priority setting of potential estrogenic EDCs for use, by regulators, at EPA. We anticipate that the same scheme will be equivalently applicable to other mechanisms (e.g., androgen receptor binding) involved in endocrine disruption and other toxicity endpoints. The stringent requirement for developing models for toxicity mechanisms is appropriately designed training data set similar to the one employed here for the ER-binding models. Properly validated data allow the structural rules that govern activity to be determined and used to develop robust predictive models.

While the results presented here clearly show both the feasibility and utility of using QSARs for priority setting, it is important to note that predictions from any model are intrinsically no better than the experimental data employed for modeling. Any limitations of the assay used to generate the training data apply equally to the model's predictions. Moreover, false negatives and false positives depend on the defined cut-off value to distinguish active from inactive for the models only providing YES/NO prediction. As the cut-off value is lowered, it is likely that error will increase even for a well designed and executed assay, and false positives and false negatives will both increase. Similarly, more false prediction might be introduced for chemicals with activity close to the cut-off value. The issue for a large number of chemicals, of the rate of false positives and false negatives in predicted values, must be dealt with experimentally by running assays on a sufficiently large number of chemicals to characterize the rates. Therefore, our current model validation process with EPA is an important step to ensure the model's quality for regulatory application.

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Glossary

Quantitative structure-activity relationships (QSARs): Technique to quantitatively correlate for a set of chemicals **structural descriptors** encoding chemical structures or properties with a dependent variable representing biological activity using, for example, **regression** methods.

Structural Descriptors: Parameters that are used to characterize chemical structure. Categories of descriptors commonly used in **QSAR** include, but are not limited to, spatial, electronic, topological, information-content, thermodynamic, conformational, quantum mechanical, and shape descriptors.

Regression: Mathematical approaches to generate equations that correlate independent variables (e.g., **descriptors**) with dependent variables (e.g., biological activity). The equations can be used to predict values of one variable (e.g., biological activity) when given values of the others (e.g., **descriptors**). Many types of statistical regression techniques are used to develop **QSAR** equations.

Training set: The set of chemicals used to develop the **QSAR** equation for which the biological activity data are known.

Testing set: The set of chemicals for which biological activity are known that is used to challenge the QSAR models developed based on the **training set**.

Cross-validation (or internal validation): Statistical approaches that are often used to determine predictive effectiveness of a QSAR model develop based on a particular **training set**. For example, in the leave-one-out **cross-validation** method, each compound is systematically excluded once from the **training set**, after which its activity is predicted by a model derived from the remaining compounds. Therefore, the summary of differences between the actual and predicted activity data for each compound can be used to assess the predictive effectiveness. This process only assesses interpolation of the model within the **training set**. Thus, sometimes it is called **internal validation**. **Internal validation** is less rigorous than **external validation.**

External validation: A more convincing process to evaluate how well the QSAR equation generalizes for an external **testing set**. One common practice is to divide the original data into two groups, the **training set** and the **testing set**. The **training set** is used to derive a model, and the model is then used to predict the activities of the **testing set**. The summary of differences between the actual and predicted activity data for the **testing set** can be used to assess the predictive effectiveness of the QSAR model for those chemicals not included in the **training set**.

Endocrine Disrupting Compounds (EDCs): An exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes.

High Throughput Screening (HTPS): A general term referring to the automation, often employing robotics, of biological assays to achieve a high volume of separate tests.

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(Continued on page 14)

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Roger Perkins

Roger Perkins received his B.S. and M.S. in Nuclear Engineering Sciences at the University of Florida and has since had a diverse career centered in computational science. He currently is Director of the Scientific Computing Division of Northrop Grumman Health Solutions. He also is Program Manager of a diverse information technology contract at the FDA/NCTR located in Jefferson, Arkansas. This contract provides systems, network, software engineers, statistics, and computational science support. Mr. Perkins spent 10 years as a reactor physicist in the U.S. Fast Breeder Reactor Program. He was part of the management team at the inception of the National Science Foundation's San Diego Supercomputer Center, in 1985, prior to becoming Director of the Navy's Advanced Scientific Computational Center in 1990. He became manager of the information technology contract at NCTR, in 1994, where he has been directly involved in a range of computational science efforts.

Hong Fang received her Ph.D. in Chemistry from the University of Missouri-St. Louis, in 1995, and continued her post-doctoral work at the same university. She next worked for two years as a forensic scientist with the St. Louis Police Department. In 1997, she began a two-year postdoctoral assignment at the NCTR, in the Division of Reproductive and Developmental Toxicology working for Dr. Daniel Sheehan, in the Endocrine Disruptor Knowledge Base (EDKB) program. Dr. Fang's EDKB work ranged from design and conduct of assays to data analysis, and then using the experimental data to develop computer-based predictive models. In 1999, she joined the Northrop Grumman Health Solution's contract at NCTR as a Senior Computational Scientist, where she continued EDKB work developing models to predict estrogen and androgen activity. She is now developing models to predict chemically-induced carcinogenicity and has participated in development of the NCTR bioinformatics capability.

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(Continued on page 15)

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(Continued from page 14)
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William (Bill) Branham received a Master of Science degree in 1972 from West Virginia University. Following a four-year commitment to the Air Force, he came to the NCTR to work with Dr. Daniel Sheehan as part of the Hormone Research Program. During the 25 years of their working relationship, Mr. Branham published a series of papers describing the toxic effects of estrogens and antiestrogens on the development of the reproductive tract in the female rat. This work was, in part, responsible for increased precautionary FDA warnings to deter the prescription of antiestrogenic drugs

William (Bill) Branham

during pregnancy. Mr. Branham is currently working under the direction of Dr. Jim Fuscoe, in the Center for Functional Genomics, on a project to bring microarray technology to the NCTR.

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School located at the Oak Ridge National Laboratories. He did postdoctoral studies at Baylor University, in the laboratory of Bert O'Malley, investigating receptor-mediated regulation of cellular physiology. He joined the NCTR approximately 25 years ago and has spent the majority of that time investigating estrogen agonist and antagonist effects on uterine morpholgy and physiology. Several years ago, he initiated a collaboration to build knowledge bases at the NCTR beginning with the Estrogen Disruptor Knowledge Base. He retired from the FDA in the summer of 2001.

Jeanne Farr Anson is the NCTR Associate Director for the Office of Planning, Finance and Information Technology. In 1974, Ms. Anson began her career in FDA as a microbiologist studying unscheduled DNA

Jeanne Farr Anson

synthesis using tissue culture. She went on to study nuclear proteins to identify biomarkers of toxic stress using flow cytometric and autoradiographic techniques. At this juncture, she returned to school at night and obtained her Master's in Health Services Administration. She moved into management at NCTR, in 1990, and is active in promoting the computational biosciences for use in the development of knowledge bases (including development of a data warehouse) and predictive models. She serves as project officer for an IAG with EPA to validate androgen receptor binding as a screen for endocrine disrupting activity.

NCTR Mission Statement

The mission of the National Center for Toxicological Research is to conduct peer-reviewed scientific research that supports and anticipates the FDA's current and future regulatory needs. This involves fundamental and applied research specifically designed to define biological mechanisms of action underlying the toxicity of products regulated by the FDA. This research is aimed at understanding critical biological events in the expression of toxicity and at developing methods to improve assessment of human exposure, susceptibility and risk.

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