International Symposium on Environmental Endocrine Disrupters 2001

Saturday, December 15 - Monday, December 17, 2001

セッション3
2001年12月16日（日）

Session 3
Sunday, December 16, 2001

HTPS・QSAR
（ハイ・スループット・スクリーニング／構造活性相関）

High Throughput Pre-Screening (HTPS) and Quantitative Structure-Activity Relationship (QSAR) Techniques

HTPS and QSAR:
Screening Methods and Cutting Edge Sciences in Endocrine Disruptor Issue

Jun Kanno

National Institute of Health Sciences

The characteristics of Endocrine Disrupting Chemical (EDC) issue could be better understood when a concept of "Receptor-mediated toxicity" is introduced. Hormonally active chemicals are known to exist for long, and according to the redundancy of the corresponding nuclear receptor system, a substantial number of environmental chemicals are now recognized to be estrogenic. On the other hand, homeostatic regulations are protecting the organisms from the xenogenic estrogens, and the concept of receptor-mediated toxicity suggests that the traditional toxicology studies using intact organisms would hardly detect adverse effects. Although there are sensitive *in silico / in vitro* hormone activity assays and some specialized bioassays having an equivalent sensitivity, the development of testing systems for endocrine disruption as receptor-mediated toxicity is awaiting for further basic research.

The strategy for EDC issue of US EPA/EDSTAC and of our own, which would be adopted by some of the ministries, basically consists of two stages, the screening stage for the prioritization of chemicals according to their estrogenicity, and the testing stage for the detection of adverse effects by a system that can properly detect the receptor-mediated toxicity.

The "low dose effect" has been issued by a series of studies at a dose range below the no effect level of traditional toxicology methods where various irreversible effects are reported to exist in a particular assay system. However, such ranges were equivalent to the range of receptor binding assay or other bioassays and just lower than the detection levels of traditional toxicology methods.

Receptor-mediated toxicity is an outcome from a series of molecular events initiated by the ligand binding to a receptor, and from where the subsequent responses are not always identical in various aspects depending on the nature of each ligand molecule. The screening methods will evolve from the old simple ones to the one that can monitor a series of complex processes, when the molecular events downstream of the receptor-ligand interaction are dissolved and the high-through put methods for identification of such events are precisely developed by the cutting edge sciences. Eventually, the development of testing for endocrine disruption in organisms and the chemical screening to predict the receptor-mediated toxicity may progress in parallel. In the past, the regulatory screening and testing methods were developed based on a rather classic knowledge. Now, on the contrary, it is progressing more likely that newest science will be rapidly incorporated to the regulatory field. Current toxicology has just started to intensively interact with the molecular biology, which cannot be properly handled by traditional toxicology and its endpoints that has been convincing but too crude.

Priority Setting of Endocrine Disruptors Using QSARs

Weida Tong[1], Hong Fang[1], Huixiao Hong[1], Qian Xie[1], Roger Perkins[1],

William Branham[2], Daniel M Sheehan[3]

[1]Logicon ROW Sciences

[2]FDA's National Center for Toxicological Research (NCTR)

[3]Daniel M Sheehan and Associates

The U.S. Congress passed laws that resulted in the U.S. Environmental Protection Agency (EPA) developing and implementing a strategy for screening and testing chemicals for estrogen, androgen and thyroid endpoints. A two-tiered, multiple-endpoint strategy, which incorporates more than 20 different *in vitro* and *in vivo* assays, was recommended by EPA's Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC). As many as 87,000 chemicals may need to be screened for endocrine-disruption potential. The large number of chemicals and assays makes it difficult for each chemical to be run through these assay batteries in a reasonable time. There is a crucial need for priority setting to identify the chemicals most likely to possess endocrine disrupting activity for early entry into screening.

Priority setting using quantitative structure-activity relationships (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, minimizing false negatives is critical for regulatory purpose because chemicals labeled as inactive are dropped into a lower priority category. For this purpose, the Endocrine Disruptor Knowledge Base (EDKB) project team at the U.S. Food and Drug Administration's (FDA) National Center for Toxicological Research (NCTR) has 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. The system is being evaluated by the EPA to determine its appropriateness for priority setting of potential estrogenic endocrine disruptors. In Phase I, two simple rejection filters or rules are used to exclude those chemicals that are most unlikely to exhibit estrogenic activity. Phase II uses three different types of models (structural alerts, pharmacophores, and classification methods) to make a qualitative activity predictions. In Phase III, a CoMFA model is used to make a more accurate quantitative activity prediction for chemicals from Phase II. In Phase IV, an expert system is recommended to combine Phase II and Phase III predictions with exposure, fate and other data to set priorities. In this scheme, each Phase is used as a screen to reduce the number of compounds to be considered in the subsequent Phase. Therefore, these four Phases work in a hierarchical way to incrementally reduce the size of a dataset while simultaneously with increasing precision of predictions. Within each Phase, different complimentary models have been selected to represent key activity-determining structure features and to minimize the rate of false negatives.

The system has been validated by a number of datasets with known estrogenic activity. It is demonstrated that the system can reject over 80% of environmental chemicals for experimental testing with a low rate of false negatives as would be critical in priority setting for regulatory purpose. The same integrated scheme is currently being extended to include endpoints of other endocrine disrupting mechanisms (e.g., AR binding).

3D-SAR Analysis of EDs Based on Target Receptor Structure

Nobuo Tomioka, Asako Kubo, Akiko Itai

Institute of Medicinal Molecular Design, Inc.

Most of the EDs give rise to their activity by binding to a specific receptor in the body, substituting the intrinsic bioactive substances such as hormones. If we can establish a method to predict strengths of the binding of substances (ligands) to a target receptor by using a computer, it will become a powerful tool for predicting activity of EDs and for searching novel ED-candidates without experimental procedures.

Due to the recent advances of molecular biology and X-ray crystallography, several target receptors of EDs, such as the nuclear receptors of estrogens, androgens and thyroid hormone, have their steric structures of ligand-binding domain being elucidated at atomic resolutions. Generally, when a steric structure of a target protein is available, one of the most effective methods to predict the strengths of the binding of ligands is a "docking simulation", i.e. a computational method to estimate stable binding modes of ligands to the protein and then to estimate the strengths of the binding based on the most stable binding modes.

We have developed a program "ADAM" originally as a tool for the docking simulation. ADAM enables accurate and automated prediction of the most stable binding mode between a protein and a ligand through an exhaustive search of the ligand conformation and relative positions between the protein and the ligand. Furthermore, in order to predict strength of the binding between a protein and a ligand quantitatively, we are developing another program "GenB" to predict free energy of binding based on the most stable binding mode obtained by ADAM. We have been using these programs in projects of drug design based on target protein structures, and succeeded in discovering and/or designing novel active compounds.

In order to assess applicability of our methods to the ED research, we have tried docking simulations using the estrogen receptor (ER), which is a typical target receptor of EDs, as a target protein. Assuming the application to structure-activity relationship (SAR) analysis of EDs, we tried to predict the strengths of ER binding for EDs with known experimental values (i.e. RBA relative to estradiol). In this trial, we could obtain a good correlation between the predicted and the experimental binding strengths, with a correlation coefficient being more than 0.8. Assuming the application of discovering novel ED candidates, we performed a trial as follows. 1. Put a small number of compounds capable of binding to ER ("active compounds") into a larger pool of arbitrary compounds; 2. Perform docking simulations for all compounds in the pool; 3. Rank all the compounds based on the predicted strengths of binding to the ER. In this trial, most of the active compounds were highly ranked among all the compounds. This implies the applicability of our methods for searching novel ED candidates.

Advantage of the docking simulation is that we do not need to have samples of proteins and ligands to be studied. We can evaluate compounds that are unavailable or that do not yet exist. We expect that the docking simulation will become an important tool in the ED research, as well as in the drug design.

Efficacy of Highthroughput Pre-Screening Procedure Based on Reporter Gene Assay

Masahiro Takeyoshi

Chemicals Evaluation and Research Institute, Japan (CERI-Japan)

The reporter gene assay technique has been used as a tool for investigating gene function, especially for testing enhancer or promoter activities of regulatory sequences of various genes. Thus the reporter gene assay technique may be suitable for detecting the hormonal activity of chemicals because it can detect enhancers, such as estrogen responsive elements (ERE), which mediate magnification of transcriptional activity with hormone receptors. CERI have been trying to establish the reporter gene assay systems to detect hormonal activity and to prioritize the chemicals to be tested for endocrine disrupting potentials on the joint development project with the Ministry of Economy, Trade and Industry (METI) and the Ministry of Health, Labour and Welfare (MHLW), Japan.

We have already established several kinds of reporter gene assay system to detect hormonal activity of chemicals. Currently, more than 500 chemicals containing aliphatic compounds, benzene derivatives, polycyclic aromatic compounds, condensed polycyclic compounds, phthalates and the other types of chemicals were examined with human ER alpha mediated transcriptional activation with stable transfected cell line and the results were compared with the results of receptor binding assay. In the Comparative study of the results of rat ER alpha mediated reporter gene assay with transient transfection technique and those of the uterotrophic assay revealed that positive chemicals obtained by each assay are well corresponding. Moreover we confirmed the efficacy of the reporter gene assay by the results obtained from the series of predictive tests, the receptor binding assay, reporter gene assay and immature rat uterotrophic assay with all *trans* retinoic acid.

Consequently, the reporter gene assay may be a promising prescreening procedure because it can be adopted in the high throughput screening process for thousands of chemicals and it requires no use of experimental animals.

A New Approach to Functional Genomics

Bruce Blumberg, Bridget Riggs, Gaurav Sharma, Felix Grim

University of California

The mapping and sequencing phases of the human and rodent genome projects are rapidly nearing completion. This extraordinary amount of information poses a new challenge for the future - How does one determine the functions of these genes? This growing new field of research is often generally referred to as "Functional Genomics". One would like to understand the molecular interactions of the gene products and the biological consequences of those interactions to make maximum use of genomic information. This requires a new generation of flexible, high throughput tests of molecular interactions and biological responses.

Many new and useful approaches are currently available to test the response of cells or organisms to varying experimental conditions. Such experiments provide important information about the cellular response programs but say little about the functions of genes identified. Other approaches have been developed to detect interactions between proteins. Despite significant advances in technology, all such approaches suffer from a critical limitation. This is that it is impossible to detect interactions between proteins and macromolecular complexes in a high throughput format capable of performing genome-wide screens. This is a troublesome limitation because virtually all cellular proteins function as members of large complexes.

We have recently developed a high-throughput screening technology that is capable of detecting interactions between single proteins and macromolecular complexes of arbitrary size and composition. A significant advantage of our approach over others is that the target can be protein, protein complexes, nucleic acids, protein :nucleic acid complexes, small molecule drugs, or even carbohydrates. This flexibility allows the rapid identification of interacting proteins and facilitates the elucidation of signaling pathways.

Combinatorial Phage Library Screening for Estrogen Receptor Interacting Peptides: A Tool for Studying Xenoestrogen Biology and Pharmacology

Julianne M. Hall

National Institute of Environmental Health Sciences

The biological actions of estrogen are mediated through two genetically distinct estrogen receptors (ERα and ER/β) that function as ligand-dependent transcription factors in target cells. Hormone activated ERα and ER/β bind with high affinity to specific DNA enhancer sequences, estrogen response elements (EREs), located within the regulatory regions of target genes. The DNA-bound receptors recruit coactivators that enhance the transcriptional activity of the receptors and facilitate contact with the general transcription apparatus. ERα and ERβ both contain an activation function-2 (AF-2) domain, which includes a highly conserved amphipathic α-helix (H12) that is essential for agonist-dependent transcriptional activity and interaction with members of the steroid receptor coactivator (SRC) family of coregulators. Specifically, agonist binding to the receptor induces a conformational change in AF-2, resulting in the formation of a hydrophobic coactivator-binding pocket. This event enables the receptor to interact with the LXXLL motifs (NR box) contained within the receptor interaction domains of many of the validated coactivators. Conversely, the conformational changes induced in ER upon antagonist binding do not permit recruitment of LXXLL motifs.

Previous studies in our laboratory and others have demonstrated that different ER ligands (estrogens, antiestrogens and xenoestrogens) induce unique global structural changes in ERα and ERβ that correlate with their differential biological activities on the receptors. However, until recently there were no tools available to examine the effect of different ligands on the structure of the AF-2 coactivator-binding pocket. Furthermore, the lack of ER subtype specific antagonists has hindered an evaluation of the roles of ERα and ERβ in different estrogen responsive cells, and the impact of xenoestrogens on the activity of each receptor subtype. Here, we have screened combinatorial phage libraries, expressing peptides in the format $X_7LXXLLX_7$, for peptides that interact with the coactivator-binding pockets of ERα and/or ERβ. Using this approach, a series of highly specific, potent peptide antagonists have been identified that completely block estrogen signaling when introduced into target cells by competitively inhibiting the association of ERα and ERβ with required coactivators. We have also used these peptides as AF-2 conformational probes to detect ligand-specific structural changes in the ER coactivator-binding pocket. Using a panel of estrogens, antiestrogens and xenoestrogens we determined that the nature of the ligand dictates the specificity of LXXLL motif binding. Furthermore, LXXLL peptide binding efficacy correlated with the ability of different estrogens and xenoestrogens to activate ERα and ERβ-mediated transcription. Thus, phage display provides a novel functional screen to access the estrogenicity of different environmental compounds and known xenoestrogens and tools to antagonize their activities on the receptors.

More recent studies have also demonstrated that xenoestrogens do not regulate all estrogen-responsive genes in an equivalent manner and that the sequence of the ERE within ER target promoters can influence receptor activity. Specifically, measuring ER activity on the vitellogenin A2, complement 3 gene, pS2, and lactoferrin EREs, revealed that the activities of estrogen and xenoestrogen ligands through ERα and ERβ are

significantly influenced by both the nature of the ligand and the response element. Using the series of ER α and ER β Interacting peptides identified in phage library screening, showed that the type of ERE with which the receptor associates regulates the structure of the coactivator pocket on ER and that there is a combinatorial effect of ERE and xenoestrogen ligand on ER AF-2 structure. Interestingly, we found that these different conformational states of ER α and Er β are functionally relevant, as they dictate receptor coactivator binding preferences by the estrogen and xenoestrogen-bound receptors.

The identification of ER α and ER β high affinity peptides has enabled the development of conformational probes to evaluate the agonist and antagonist properties of estrogens, antiestrogens and xenoestrogens and has provided tools to dissect their molecular mechanisms of action. Current studies are focused on the creation of modified version of these peptide antagonists that will make them suitable for studies in whole animals. We believe this will enable us to better define the roles of ER α and ER β in estrogen responsive tissues and study the effects of endocrine disrupting chemicals on estrogen signaling *in vivo.*