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REVIEW

Alternatives to animal experimentation for hormonal compounds research

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Abstract Alternatives to animal testing and the identification of reliable methods that may decrease the need for animals are currently the subject of intense investigation worldwide. Alternative testing procedures are particularly important for synthetic and natural chemicals that exert their biological actions through binding nuclear receptors, called nuclear receptors-interacting compounds (NR-ICs), for which research is increasingly emphasizing the limits of several models in the accurate estimation of the physiological consequences of exposure to these compounds. In particular, estrogen receptor interacting compounds (ER-ICs) have a great impact on human health from the therapeutic, nutritional, and toxicological point of view due to the highly permissive nature of the estrogen receptors towards a large number of natural and synthetic compounds. Similar to in vitro systems, recently generated animal models (e.g., animal models generated for the study of estrogen receptor ligands) may fulfill the 3R principles: refine, reduce, and replace. If used correctly, NR-regulated models, such as reporter mice, xenopus, or zebrafish, and models obtained by somatic gene transfer in reporter systems, combined with imaging technologies, may contribute to strongly decreasing the overall number of animals required for NR-IC testing and research. With these models, flexible and highly standardized parameters and reporter marker quantification can be obtained. Here, we highlight the need for the substitution of currently used testing models with more appropriate ones that can reproduce the features and reactivity of specific mammalian target tissue/organs. We consider the promotion of this advancement a research priority bearing scientific, economic, social, and ethical relevance.

Keywords 3Rs · Alternatives to animal experimentation · Nuclear receptors · Pharmaco-toxicology · Reporter models

Council directive of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products (76/768/EEC) (OJ L 262, 27.9.1976, p. 169).

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Introduction

The nuclear hormone receptor (NR) family and their ligands are important for the maintenance of vital processes in humans. The NRs are ligand inducible transcription factors with the ability to bind to specific DNA enhancer elements located in the vicinity of target genes. Upon ligand binding, the receptors modulate gene expression and regulate the cellular concentration of specific target proteins [10]. Receptor function/dysfunction has been linked to numerous diseases, including osteoporosis, immune diseases, cancer, depression, and health problems linked to metabolism such as metabolic syndrome and correlated

diseases (i.e. cardiovascular disease) [17, 24, 40, 58]. Thus, NRs are important pharmacological targets of several pharmaceutical molecules that act as NR-ligands. Humans and animals are also exposed to a variety of compounds from both food (natural non-nutritional hormones) and the environment (industrial compounds of very different structures) that act through NR binding, affecting the endocrine system and health [3, 22, 28, 54]). The great impact on health exerted by the interaction of the wide variety of existing natural and synthetic chemicals with the NRs requires a careful pharmaco-toxicological analysis of these compounds.

Need of model systems for NR-ICs

To accurately accomplish a careful analysis, the development of new products, including pharmaceuticals, chemicals, cosmetics, and foods, require fast and economic models that can provide predictive data on the actions of these products on the target systems and functions. In this regard, the nuclear receptors-interacting compounds (NR-ICs) and, in particular, the estrogen receptor interacting compounds (ER-ICs), also called selective estrogen receptor modulators (SERMs) when referring to pharmaceuticals and endocrine interferents (EIs) or endocrine disruptors (EDs) when referring to food components and environmental pollutants, are of great interest and impact human health, both therapeutically and toxicologically. Thus, the requirements for improved NR-IC testing methods are increasing for several reasons:

1. Many of the drugs under development by the pharmaceutical industry are targeted towards women's health. A significant proportion (80%) of the drugs marketed for women includes birth control pills and anticancer and hormone replacement therapeutics [21, 34, 37, 41, 44], although the outcomes of major clinical investigations have led to a dramatic restriction in the use of hormone replacement among post-menopausal women. The systemic use of endocrine active drugs is mainly restricted to the peri-menopausal phase. Several new molecules in this class of drugs are currently under intensive investigation in order to understand their endocrine mechanisms.

The earliest ER-ICs in the clinic were clomiphene and tamoxifen, used for the induction of ovulation and as antiestrogens for secondary prevention of breast cancer, respectively [25]. Other common chemicals are toremifene, a chloroderivative of tamoxifen with closely related properties but fewer side effects in the liver, and raloxifene, a chemical extensively used for the prevention of osteoporosis and recently approved as a drug for the treatment of breast cancer. New alternative compounds are being developed to alleviate symptoms and degenerative processes associated with the loss of estrogen production after menopause. For example, bazedoxifene [31], which is developed for osteoporosis, ospemifene [43], which has a unique profile by being active (i.e. having an estrogen-like effect) on the vaginal epithelium, and lasofoxifene [29], which is also a new ER-IC being developed for the prevention of osteoporosis.

2. In addition to pharmaceutical products, there is an increasing number of hormonally active xenocompounds that originate as side products from the industrial production of several classes of chemicals. Accumulation of these chemicals in the environment is a great concern for the health of both men and women as they potentially can disrupt normal endocrine functions and impair development, reproductive functions, and increase the risks of hormonally regulated malignant diseases (e.g., EDs such as bisphenol A, 4-octylphenol, 4-nonylphenol, etc.) [6, 7, 9].
3. Numerous natural food components and nutraceutical formulas commercialized by the food industry have been selected basing on their biological activity as hormone mimics and proposed exertion of beneficial effects on human health (i.e. isoflavones, stilbenes, lignans, etc.) [1, 27, 46, 49].

"State of the art" and major drawbacks of existing in vitro models for NR-IC testing

The tissue- and organ-specific in vitro models generated thus far present several serious limitations:

(a) Most of the available cell lines of mammalian origin are derived from tumors or have a transformed phenotype. The functional and structural features of the cells do not mirror the original tissue, resulting in an altered response to various endogenous and exogenous factors with respect to the in vivo situation [53].
(b) When "non-transformed" mammalian-derived in vitro models are available, they merely consist of primary cell cultures or isolated tissue slices. The in vitro survival of such models is limited, thus time-course and dose-response studies are more difficult and subject to larger inter-individual variation. This difficulty results in increased complexity when comparing large datasets from primary culture models over long periods of time. Moreover, when cells are of human origin, they have the drawback of

depending on regular supplies from available clinical sources.

(c) In tissues, cellular architecture is always 3D. Two-dimensional culture conditions may not be optimal for tissue-like organization and all cellular functions. For example, polarized cells of the parenchymal tissue, which normally require complex cellular interactions, may not behave physiologically when adhering to solid substrates, as in the case of conventional culture conditions.

(d) Conventional cell cultures often do not express suitable, easy-to-assay quantifiable markers, or they require transfection procedures that increase result variability among experiments.

(e) Most cell cultures originate mainly from female tissues (i.e. endocrine responsive cancers) and, therefore, may be biased towards female-specific effects. The recent findings of the presence of high concentrations of estrogen receptors in male tissues [4, 52, 57] make these biological materials obsolete for some aspects. All new systems originating from male tissues should be compared to the responses in female tissues.

(f) The systems used for the in vitro and ex vivo analysis of NR-ICs (mainly estrogens and androgens) are generally composed of cells derived from reproductive tissues. Recent knowledge of the widespread distribution of NRs, in particular steroid receptors, in all tissues of an organism and their involvement in several diseases [2, 18, 30, 50, 55] make the available systems inadequate for assessing the effects of NR-ICs on the whole physiology. Moreover, the tissue levels of several NRs change with age (i.e. PPARγ); thus, test systems should take age-related responses into consideration. In fact, the available models do not easily provide information on the effects of compounds at different developmental stages (i.e. embryonic and fetal stages, breast feeding, pubertal period, fertile age, and post fertile age).

(g) Finally, the expression profile of tissue-specific NR co-regulators should be known in the adopted systems because the combination of these factors is a determinant of the specific cell response to receptor ligands [38, 39, 45].

Research for alternatives to animal testing in Europe

The EU member states have agreed to reduce the number of laboratory animals in the cases where existing and valid alternatives are established. The Cosmetic Directive, Council Directive 93/35EEC, amending Directive 76/768/EEC and the seventh Amendment to the European Cosmetic Directive (27 February 2003) (http://ec.europa.eu/enterprise/cosmetics/html/consolidated_dir.htm), prohibits the marketing of cosmetic ingredients and products tested on animals from the year 2013 onward. Several countries already comply with this directive. For instance, Holland has reported no animals being used for cosmetics testing since 1994. In 1992, 18.4% of all scientific procedures conducted on live animals were performed for regulatory approved toxicological purposes, and a smaller percentage for efficacy/potency testing. Yet, we cannot ignore that basic research is still responsible for 80% of the total number of animals used, although this has already begun to gradually decline, and that the current rodent test systems are likely to remain in use for pharmacokinetic (i.e. dosage, formulation, administration, half-life), toxicological (systemic and organ-specific), and biological evaluations of candidate therapeutic compounds and toxic xenocompounds for years to come.

Several EU programs (FP5, FP6, and FP7) have been started since the 1990s by the Directorate General for Research of the European Commission to support research with the aim of improving human health and quality of life. In the field of life sciences, researchers were asked to make a consistent effort at relevantly decreasing animal use in both basic and applied research, as well as for pharmaco-toxicological applications (framework of the European Environmental and Health Strategy [com 2003] 338, http://ftp.cordis.europa.eu/pub/fp7/docs/guidelines-annex5ict.pdf). One of the actions was to dedicate specific calls and funds to networks of scientists and industries with the specific aims of finding new alternatives to animal experimentation. Panels of experts from different fields, nominated to periodically analyze research needs in the EU, have recently focused their attention on the pharmaco-toxicological testing of endocrine active compounds, both from pharmacological and industrial/environmental sources. During these technical working groups (TWGs), scientists strongly emphasized the need for more informative in vitro systems as alternatives to animal testing (http://www.environmentandhealth.org). In particular, weaknesses were pointed out in the technical approaches for pharmaco-toxicological testing of NRs, including the following:

(a) A lack of possibility of easily addressing tissue-specific actions.

(b) Scarce availability of systems sensitive enough to provide a description of the activity of low-potency compounds at realistic exposure doses, particularly for EDs.

(c) A lack of systems to provide data on the cumulative effects over time for low potency compounds.

In addition, the experts in more recent TWGs advised that these recommendations should be integrated into

fundamental research programs as a part of method and model development and improvement. At a recent meeting involving several EU project coordinators (http://www.altaweb.eu/exera) in the area of alternatives to animal experimentation, research needs were further evaluated. Participants discussed a few basic points that should be taken into consideration by scientists and the EU Commission during the development of the current FP7 Program. The conclusions were as follows:

(a) The use of animals for testing should be avoided whenever possible.
(b) Alternative in vitro tests should always be considered when applying for EU funding, specifically in those fields of life science where the use of animal models is generally advised (i.e. basic research, applied research, pharmacology, toxicology, etc.).
(c) New research and technical opportunities, such as new cell types, mechanisms, new biomarkers, technologies for detection and analysis, and in silico systems, should be systematically explored for their ability to decrease the use of animal models.
(d) Emerging technologies that may improve in vitro–in vivo correlations should be standardized.
(e) New animal models, including transgenic animals, should be explored if their use contributes to new understanding and a sensible decrease in the total number of experimental animals.
(f) Knowledge and expertise acquired by EU-supported research in the area of the 3Rs (reduce, refine, replace) should be consolidated beyond the lifetime of time-defined projects.
(g) Ethical aspects of animal use in research and pharmaco-toxicology should allow for the creation of a research priority in FP7.
(h) Alternative methods should be suitable for applications in the context of the REACH program [Regulation (EC) No 1907/2006, Directive 67/548/EEC, Directive 2006/121/EC]. The new EU Regulation on the registration, evaluation, and authorization and restriction of chemical substances and their safe use entered into force on 1 June 2007. Alternative tests in this area should offer the opportunity to save a substantial number of animals currently required for in vivo and ex vivo assays. This objective is one of the seven that need to be considered within the overall framework of sustainable program development (http://ec.europa.eu/environment/chemicals/reach/pdf/2007_02_reach_in_brief.pdf).
(i) The correspondence between the available in vitro tests and test strategies used by the pharmaceutical industry should be analyzed.
(j) Financial support dedicated to these targeted actions is required.
(k) Regulators should meet with scientists to clarify requirements for the regulatory acceptance of test methods, and scientists should be trained for familiarity with pre-validation and validation processes.

International collaboration should also be supported by the establishment of structured networks joining EU and non-EU supported projects addressing or including the 3Rs in their research activities. The main objective of such networks should be the exploitation of acquired knowledge and expertise by exchanging progress, achievements, and problems. Workshops with participants from academia, manufacturers of in vitro tests, and pharmaceutical industry and legal authorities should be conducted to facilitate links among the stakeholders and discuss crucial issues on the availability of recently developed in vitro methods ("The World Congress on Alternatives", an international forum for worldwide confrontation is in its 7th edition. Rome, September 2009). Last, but not least, the parallels between priorities in EU work programs and those of the US Environmental Protection Agency (EPA) reinforce the needs for integrated approaches. Consultations and collaborations have been established between ECVAM and US agencies on these topics. Connections could be further facilitated by promoting a broader participation of international players in FP7 projects. Such interactions would facilitate worldwide acceptance of the emerging alternatives.

Testing the endocrine potential of NR-ICs

Ongoing EU projects in the area of in vitro testing seek to overcome the limitations of conventional in vitro approaches for risk assessments of active endocrine compounds (ftp://ftp.cordis.europa.eu/pub/fp7/docs/alternative-test-strat_en.pdf). In addition to scientific and regulatory advances, these projects taking place from 2006 to 2010 will promote technological innovations by including research on the applicability of novel cellular and molecular-based methods and novel end-points in the assessment of the biological actions of NR-ICs. The investigation of tissue-specific regulatory pathways and hormone-dependent physiological processes are major research tasks. The achievements are expected to fulfill the need for new, practical, and easily standardized end-points for all target organs while limiting the number of animals required. New in vitro tissue/cell technologies have been proposed as suitable alternatives for investigating the role of receptor specificity in hormone action, gender-specificity, age-dependency, endocrine pharmaco-toxicity at embryonic stages, and the

development of high throughput genomics-based tests for NR-ICs.

The improved systems described in recently funded EU research projects propose suitable tools for the characterization of newly synthesized drugs that interact with nuclear receptors and for the risk assessment of industrial NR-ICs that may contaminate food and the environment. The aim of these projects is to translate risk assessment data into regulatory issues and political actions, as well as consistently reduce and replace animal use.

Extend the concept of the 3Rs to animal models

Similar to in vitro systems, advanced animal models may also fulfill two of the 3Rs: refine and reduce. If correctly used, NR-regulated models like reporter mice or zebrafish [8, 12, 16, 23, 26] and models obtained by somatic gene transfer of reporter systems [51] may strongly decrease the overall number of animals required for testing and NR-IC research. With these models, flexible and highly standardized parameters and marker quantification may be assayed.

Reporter animals for hormone action

The first mouse models that may optimize the use of in vivo systems for pharmaco-toxicology, providing both new information and allowing decreased animal use, are the transgenic mouse models of hormone action. These mice are generated by the insertion of DNA elements that provide the template for recognition by NRs. Hormone responsive elements (HRE) are placed upstream of minimal promoter sequences, such as those containing the TATA box, or the minimal regulatory sequences of the thymidine kinase promoter. The best HRE arrangements may consist of two or three palindromic sites correctly spaced at optimal distance from the minimal promoter [12]. In some cases, to limit the position effects and gradual extinction of reporter expression [56], the generation of constructs in which the transgene was flanked by either the insulator matrix attachment region (MAR) [48] or HS4 (globin hypersensitive site 4) [11] proved to be the best functioning element, and they were estrogen inducible with limited basal activity. On these reporter elements, the transcription complex formed by the ligand-activated receptor and co-regulators, modulates a downstream gene that generally encodes for an enzymatic activity (firefly or renilla luciferase, GFP, β-Galactosidase, etc.) in a hormone dependent fashion. Different reporter enzymes have proved to be suitable and sensitive markers for easy detection, although enzymes with higher turnover rates are significantly better for providing pharmacokinetic and pharmacodynamic profiles (i.e. wild-type firefly luciferase) [12, 19]. The insertion of these constructs into the mouse genome through different available technologies may lead to ubiquitous and hormone-regulated expression of the reporter. Moreover, modern imaging technologies in conjunction with animals expressing luminescent or fluorescent markers provide the opportunity to generate a notable amount of information without the need for animal sacrifice [14, 19, 42, 47, 55].

These reporter systems, and other similar models, developed during the past 7–8 years have provided major insights into ER physiology [13, 32], and their initial use for toxicological purposes show that estrogen reporter mice represent suitable models for:

- Identifying food and environments where estrogenic compounds are present.
- Providing a complete view of the body regions in which these contaminants are acting.
- Assessing the potential hazard of acute or chronic exposure to estrogenic compounds.
- Producing reliable and informative data on physiological changes without animal sacrifice, thus fulfilling two of the three principles (refine, reduce).
- Enabling the generation of tissue-specific cell lines for the high-throughput screening of estrogenic compounds.

Once the necessary reproducibility, reliability, specificity, and sensitivity have been achieved, these reporter systems may provide new approaches for studying the pharmacodynamics and kinetics of hormones. Reports from our and other laboratories have identified the consistency and validity of reporter mouse methodology, demonstrating the direct relationship between the administered dose of the estrogenic compound and the intensity of photon emission measured in different body areas [13, 32]. It has also been shown that luciferase activity measured ex vivo generally reproduces and expands on what is observed in vivo, thus demonstrating the robustness of in vivo imaging with regard to the identification of the body areas targeted by the receptor ligands [20].

For classical pharmaco-toxicological studies, markers of internal dose are often used as a direct measure of the bioavailability of dietary or food-contaminating compounds and their related metabolites at the systemic level (body fluids). More useful, but often difficult to obtain, are biomarkers able to model the biological activity of the same compounds at the organism level. To this aim, the analysis of the ability of NR ligands to modulate receptor activity in vivo, using reporter systems as surrogate biomarkers, is of great interest. At the same time, the analysis limits the preliminary studies needed to identify relevant time- and dose-dependent points of activity. Subsequently, analytical data can be correlated to functional data (dose, function) to determine optimal treatments. Moreover,

ligand effects on NR signaling can be evaluated after acute and chronic exposure ex vivo (in the target tissues) and in living mice [5, 12, 13, 15, 16, 33], thus allowing for longitudinal studies that provide whole body data.

These systems are under continuous improvement in order to allow the collection of an increasing amount of data with reduced cost and time. In this respect, multiple transgenics, such as animals responsive to different contemporaneous signals, may represent further advancement.

Derivation of in vitro systems from pathway-specific transgenic animals

The possibility of deriving in vitro systems for tissue-specific evaluation of the same reporters/markers expressed in reporter animals may increase the efficiency of in vitro/in vivo correlations (covering one R, replace). Reporter mice represent a unique source for the generation of cell-specific reporter systems. Derivation of these systems may allow the analysis of the same end-point in different tissues (quantitative evaluation) in vivo, ex vivo, in primary culture, and in immortalized cell lines derived from the same animal.

Ongoing studies within the EXERA network are showing that both primary cells and fresh tissue, as well as immortalized cells, can be cultured through 3D technologies like the Rotating Wall Vessel Bioreactors (RWV), which provide investigation tools that may generate data more similar to whole tissue [36]. By reproducing specific tissue-like structures that mimic the functions and responses of real tissues in a way that is more physiologically relevant than what can be achieved through traditional 2D cell monolayers, 3D cell cultures also represent a potential bridge for covering the gap between animal and human studies. The coupling between new animal models and 3D cell cultures adds a further possibility for the application of these technologies to pharmaco-toxicology and research. Furthermore, applying reversible immortalization techniques to primary cells (i.e. the improved tet on/tet-off system [pRITA]) [35] may allow researchers to get closer to a more physiological response compared to constitutively transformed cells, thus furnishing a system in which the regulation of cell parameters is not related to cancer.

To conclude, the generation of innovative in vitro and in vivo models for the characterization of NR-ICs will directly contribute to the goal formulated by the European Community in its sixth and seventh Environmental Action programs: "*a high level of the quality of life and social well-being of citizens by providing an environment where levels of pollution, food safety, pharmacological and industrial production do not increase harmful effects on human health and environment*" (CEC 2001).

(These topics were extensively discussed during the EXERA workshop held in Genoa on the 5th September 2008).

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