REVIEW

Molecular Imaging, an Innovative Methodology for Whole-Body Profiling of Endocrine Disrupter Action

Diego Di Lorenzo,* Gianpaolo Rando,† Paolo Ciana,† and Adriana Maggi†,1

*Laboratory of Biotechnology, Civic Hospital of Brescia, 25123 Brescia, Italy; and †Centre of Excellence on Neurodegenerative Diseases, University of Milan, 20133 Milan, Italy

Received July 7, 2008; accepted September 4, 2008

Endocrine disrupters (EDs) are environment and food contaminants known to alter metabolic functions of mammals by interfering with specific endocrine pathways. Many EDs act on steroid hormone target cells by interacting with intracellular receptors (IRs) like estrogen receptors, androgen receptors, and thyroid hormone receptors; other receptors may be engaged. IRs are ligand-operated transcription factors acting in concert with general or cell-specific coregulators. The newly acquired awareness on the panoply of IR functions has increased the concern on potential, unsought, harmful effects of EDs on human health and has questioned the capability of currently available methodologies to identify and study EDs in the environment and in the food chain. Indeed, current in vivo and in vitro methodologies restrict the analysis to very specific organs or cell systems, with obvious limitations in predicting the systemic metabolic consequences of ED exposure. The emphasis recently laid by Regulatory Authorities, including European Center for the Validation of Alternative Methods, on the generation of in vitro model systems for toxicological analyses discouraged the development of models suitable to envision the whole spectrum of ED body actions required when studying compounds acting through IRs. Molecular imaging now provides the opportunity to quantify ED effects in living organisms enabling, for the first time, to acquire a full comprehension of the systemic effects of acute and prolonged exposure to EDs, solving the issue of the potential harm due to repeated low-dose exposure. The systems here reviewed are of unquestionable toxicological relevance and need to be taken into consideration to improve the methodology currently available and in use.

Key Words: reporter animals; optical imaging; endocrine disrupters; dietary estrogens; estrogen receptors; animal testing.

The effects of exogenous substances polluting the environment and contaminating the food chain represent a hazard for human health to be seriously addressed in view of the fact that the number of potentially toxic synthetic compounds is steadily increasing. A study conducted by the World Wildlife Fund (Strucinski et al., 2006) has recently shown that blood from three generations of European adults contains traces of more than 70 exogenous substances that have reasonably passed into the organisms through the food chain. As for many of them a real evaluation of risks and effects on human health is lacking, the problem gains further relevance.

Several environmental and food contaminants, with the common denomination of endocrine disrupters (EDs), owe their undesired effects to their ability to interact with hormone receptors, such as thyroid hormone (THRs), estrogen (ERs), and androgen receptors (ARs). Thus, by interfering with endocrine signaling, EDs impair development and metabolic functions in mature animals and humans (Degen and Bolt, 2000; Di Lorenzo et al., 2002; Hoyer, 2001; Palanza et al., 2001; Sonnenschein and Soto, 1998).

THR, ER, and AR all belong to the superfamily of intracellular receptors (IRs) that has been object of a major research interest for the last twenty years (Baniahmad and Tsai, 1993; Dahlman-Wright et al., 2006; Gottlicher et al., 1998; Jensen, 1996; Parker, 1986; Zhang and Lazar, 2000). IR signaling mechanism is well known at the molecular level. In fact, IRs are hormone-regulated transcription factors controlling in trans the activity of selected target promoters in concert with general or cell-specific coregulators. The coregulators have an important role in the IR activity and contribute to the tissue-specificity of numerous synthetic ligands acting on these receptors (Katzenellenbogen et al., 1996; Lonard and O’Malley, 2007; McDonnell and Norris, 2002; McKenna and O’Malley, 2000; Shiao et al., 1998). Each IR ligand induces a unique conformation in the cognate receptor (Brzozowski et al., 1997): this is crucial to the ultimate cellular response, because the recruitment of appropriate coregulators to the ligand-receptor complex is strictly conformation-dependent (Nettles and Greene, 2005; Shiao et al., 1998). As the coregulator complement confers promoter and cell specificity of action, it can be understood why several synthetic IR ligands...
were found to possess even opposite actions (e.g., agonist or antagonist) depending on the promoter or the cell targeted.

Furthermore, like other toxic pollutants EDs may be present in the environment or food at concentrations that are not sufficient to cause any measurable effect after acute exposure, but, because of their lipophilic nature, may accumulate in fat tissue and with time reach concentrations perturbing physiological metabolism (Herron and Fagan, 2002; Imbeault et al., 2002; Jaga and Dharmani, 2003; Lordo et al., 1996; McFarland and Clarke, 1989; Safe, 1993). Thus, the methodology for the study of EDs should allow the measurement of their effects after prolonged exposure even at low concentration.

The emphasis laid by European Authorities on the generation of model systems for the in vitro identification of toxic compounds and the assessment of their activity has discouraged the development of models suited to provide a view on the full spectrum of body actions required when dealing with EDs. However, the potential differential activity of ligands on each target tissue demands for model systems where the entire spectrum of ED actions can be investigated. Modern imaging techniques applied to animal engineering give novel opportunities to create innovative model systems enabling to quantify, spatio-temporally, the receptor activity on synthetic reporter genes with noninvasive technologies (Gossen et al., 1989; Sacco et al., 1997; Wirth et al., 2002; Zhang et al., 2003). Molecular imaging makes it possible, for the first time, to measure the activity of a given compound in all the organs of a living mammal without distress or prior sacrifice of the laboratory animal itself. These models are of particular relevance for the study of transcription factors such as IRs, because genes encoding reporter proteins under the control of hormone-IR responsive promoters can be integrated into the genome of small laboratory animals. In these transgenic models, the presence of ligands for specific IRs can be easily detected by measuring the amount of synthesized reporter protein. The first paradigmatic transgenic reporter mouse, the ERE-Luc reporter mouse, was specifically engineered to obtain an ER-regulated expression of the reporter gene luciferase in each cell of the organism (Ciana et al., 2001). This reporter system and other similar models provided major insights into ER physiology (Ciana et al., 2003; Lemmen et al., 2004a) and their initial use for toxicological purposes show that the ERE-Luc mouse represents a suitable model to:

- identify food and environments where estrogenic compounds are present;
- provide a complete view of the body districts in which these contaminants are acting;
- assess the potential hazard of acute or chronic exposure to estrogenic compounds;
- produce reliable and informative data on physiological changes without animal sacrifice;
- enable the generation of tissue-specific cell lines for high-throughput screening of estrogenic compounds.

On the basis of the results obtained with the ERE-Luc reporter system here summarized, we propose to revisit the use of animal models as a most suitable system for the analysis of ED toxic effects and for the respect of the 3R’s principle.

**BIOLUMINESCENCE-BASED IMAGING FOR QUANTITATIVE AND DYNAMIC ANALYSIS OF ESTROGENIC COMPOUNDS IN REPORTER MICE**

Luciferase reporter mice provide a systemic view of the activity of any estrogenic compound by combining the quantitative analysis of in vivo imaging and of ex vivo luciferase enzymatic activity. The in vivo analysis is carried out by injecting ip the substrate luciferin to the anesthetized mouse and by measuring photon emission from the whole animal by CCD camera exposure. Preventive in depth analysis of D-luciferin distribution provided a clear view of the distribution of the substrate in the different organs and defined the dose sufficient to fully activate the luciferase produced in different tissues (Biserni et al., 2008). Studies carried out in two different luciferase reporter mice anesthetized with either Ketamine-Xilazine-H2O or isofluorane proved that luciferine, when administered ip, readily distributes and reaches concentrations sufficient to saturate the enzyme in all organs, but the brain (Biserni et al., 2008). No significant difference in time and dosage was observed in mice of different strains (Ciana et al., unpublished observation). The range of linearity of photon measurements (characteristic of each given CCD camera) is determined by the use of bioluminescent standards (recombinant luciferase or stable gas tritium light sources); these standards are also important for the calibration of different measuring equipment and thus for the normalization of data obtained in experiments carried out in different laboratories at very different time.

Once set up the basis for in vivo measurements, the dynamics of the systemic activity of a given estrogenic compound on ER is measured in vivo by a time-course study where luciferase activity is estimated by in vivo imaging at different times after administration of the estrogenic compound; to this aim, photon emission in selected body areas may be analyzed quantitatively by measuring total counts in regions of interest (ROIs) selected manually by a specific grid (Fig. 1A) or by the use of appropriate algorithms.

As an example of such a study, Figure 1 shows the effect of sc administration of 17β-estradiol at a dose leading to plasmatic concentration of the endogenously synthesized hormone (5.5 μg/kg). The effect of the hormone was analyzed by bioluminescence imaging in selected body areas of living animals and by enzymatic assay in selected tissues. Both in vivo (Fig. 1B) and ex vivo (Fig. 1C) measurement of luciferase activity indicated that maximal accumulation of luciferase occurred at about 6 h; 24 h after estradiol administration, luciferase activity had decreased to time 0 values. Several
reports using imaging (Lemmen et al., 2004b; Nagel et al., 2001) or studying endogenous genes support the fact that injection of the natural hormone has its maximal effect 6–8 h after administration. Furthermore, it is well known that 17β-estradiol is readily catabolized and this explains why luciferase activity was back to time 0 levels 16 h after treatment. It is important to underline that, to be able to obtain an accurate assessment of the dynamics of the activity of the estrogenic compound, the reporter used must have a very short turnover time (firefly luciferase half-time is about 2 h in mammals); reporter animals generated with long half-life reporters failed to faithfully report the decrease in ER activity shown by the ERE-Luc mouse 16 h after estradiol administration (Nagel et al., 2001). It is worth to underline that on the basis of previous experiments carried out in our (Biserni et al., 2008; Ciana et al., 2005) and other laboratories (Penza et al., 2007), photon emission measured in tail and head is considered to be due to ER activation in bone, whereas photon emission in chest is largely reflecting ER activity in liver. The study shown in Figure 1 showed an inter-assay variability below 15%: the consistency of the results obtained with different animals shows the validity of the in vivo imaging procedure when compared with the classic enzymatic assay. In addition, this study highlights how in vivo imaging allows for a factorial reduction in the use of experimental animals, proportional to the number of observations at different time points: in fact the study in Figure 1B was carried out on eight animals, whereas 32 animals (8 × 4) were used for the study in Figure 1C.

Once the time of maximal activity of the compound under study was established, its relative potency in each organ was investigated in a dose-response study. Figure 2 and several reports from our and other laboratories have shown 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 (Ciana et al., 2003; Lemmen et al., 2004a). Present technology for bioluminescence-based in vivo imaging can be carried out in reporter mice only in two dimensions, thus the definition of the organ/tissue contributing to the photon emission as measured in vivo imaging is limited; furthermore, signaling from the most inner organs is significantly reduced by photon scattering and absorption by the tissues crossed by the photons. Thus, until a satisfactory whole-body 3D-bioluminescence tomography is developed, it will be possible to achieve the complete knowledge of the effects of the estrogenic compounds in each tissue only by combining the in vivo study to the ex vivo analysis of luciferase activity in dissected organs. Yet, as shown in Figures 1 and 2, luciferase activity as measured ex vivo generally reproduces and extends what observed in vivo, this demonstrating the robustness of in vivo imaging with regard to the identification of the body areas targeted by the compound and to the comprehension of the potency of the effects.

**FIG. 1.** *In vivo* and *ex vivo* quantitative analysis of luciferase expression in ERE-Luc mice. Adult male ERE-Luc mice in groups of a minimum of seven animals each were treated with 17β-estradiol (5.5 μg/kg, ip) and exposed to a CCD camera at different times after injection or sacrificed for luciferase analysis in tissue extracts. (A) Definition of the body areas for the study of photon emission. (B) Quantitative analysis of photon emission in living, anaesthetized ERE-Luc mice as expressed in counts/unit of time in selected body areas. Data represent the mean ± SD of groups of seven animals. (C) Quantitative analysis of luciferase enzymatic activity as measured in tissue extracts of organs dissected from mice euthanized 0, 3, 6, and 24 h after treatment. ERE-Luc mice were treated with estradiol. Photon emission was measured after exposure to a charged couplet device camera 0, 3, 6, and 24 h after treatment. Bars represent the average ± SEM of groups of seven animals each. *p < 0.05 versus controls as determined by ANOVA followed by Bonferroni post hoc analysis.
studying its response to the fluctuations of sex hormones disorders. For instance, in the case of estrogenic compounds, obtain a global view of ER state of activity in males and doses may be overridden. In fact with reporter mice we can the use of reporter animals, the issue of the sensitivity to low debate in toxicological analysis. We firmly believe that, with unbiased view of the state of ER activation.

ERE-tK ensure the maximal specificity, providing an carried out to compare the effects of estrogen treatments in receptors. This indeed appears to be the case in several studies of a physiological picture has to be considered suitable for toxicological applications.

PREDICTABILITY AND SENSITIVITY OF LUCIFERASE REPORTER SYSTEM IN THE ERE-LUC MOUSE

Two major questions need to be addressed before proposing the introduction of reporter animals for molecular imaging in the routine of toxicological studies: (1) the former relates to the significance of the use of a synthetic reporter for the understanding of the potential metabolic harm of EDs; (2) the latter addresses the sensitivity of the reporter system. The data obtained so far show that the synthetic transgene faithfully reports on the state of ER activity (e.g., in terms of timing and tissue of activation) on endogenous targets such as progesterone receptor (Ciana et al., 2003) and CYP17 (Montani et al., 2008); conceptually, data obtained with natural, by definition complex, promoters are very difficult to interpret, because the transcriptional regulation of each of them is modulated by a multitude of factors which vary spatio-temporally. In each tissue, depending on the metabolic state of the animal, each natural target gene may respond to various stimuli, not necessarily estrogenic, in very different manners, thus limiting the possibilities to obtain a clear picture of the exact state of the activation of hormone receptors. This indeed appears to be the case in several studies carried out to compare the effects of estrogen treatments in different tissues. Conversely, we find that synthetic reporters like ERE-tK ensure the maximal specificity, providing an unbiased view of the state of ER activation.

The issue of the sensitivity has been the object of a lengthy debate in toxicological analysis. We firmly believe that, with the use of reporter animals, the issue of the sensitivity to low doses may be overridden. In fact with reporter mice we can obtain a global view of ER state of activity in males and females during all stages of life and in selected pathological disorders. For instance, in the case of estrogenic compounds, the validity of the reporter model to be used can be assessed by studying its response to the fluctuations of sex hormones (Ciana et al., 2003), and currently we are completing such ER activation pattern for all the stages of mouse life, from development to aging. Once set up the physiological picture, the identification of compounds perturbing what is known as “physiological” is facilitated: thus any compound interfering with the established pattern of ER activity can be identified. This also solves the issue of sensitivity: once established that a method discriminates among the different physiological events regulated by the endogenous hormone, this same method will be endowed with the necessary sensitivity to detect ED effects after single as well as repeated exposure administration even at low doses. Of course, the ERE-Luc system cannot give any hint on the physiological impact of this altered ER activity: however, it is of fundamental relevance to identify an agonist/antagonist activity in specific tissues where then to carry out more specific analyses to unravel the pathophysiological consequences of ER misactivation. Any model that faithfully reproduces alterations of a physiological picture has to be considered suitable for toxicological applications.

ERE-LUC MODEL IN TOXICOLOGICAL ANALYSIS

The applicability of the ERE-Luc model to the study of in vivo effects of endogenous, dietary, and environmental estrogens has been shown in several studies (Ciana et al., 2003, 2005; Di Lorenzo et al., 2002; Lemmen et al., 2004b; Montani et al., 2008; Massi et al., 2005; Nagel et al., 2001; Penza et al., 2004, 2007; Villa et al., 2004). Considering that most tissues in mammals express ERs and that each synthetic chemical may have an unpredictable tissue-specific action on ERs, the ERE-Luc mouse model represents at present the most appropriate model to obtain a realistic, systemic view of the effects of endogenous or exogenous estrogens. Dose-dependency studies have clearly shown the usefulness of the model to determine the potency of the effect of estrogenic compounds and time-course analyses have proven the persistence of their effects. Indeed, the studies carried out so far have clearly shown that, not unexpectedly, each contaminant of food components has its own pattern of activity on ERs in terms of time of action or of target tissue.

Estrogenic Activities in Complex Mixtures

The applicability of reporter mice to the identification of estrogenic compounds in complex mixtures was tested by investigating the effect of the administration of natural foods such as raspberry juice and wine supposed and alleged to exert estrogenic activity because of the presence of resveratrol. In a first experiment raspberry juice was substituted to drinking water for 4 days and animals were allowed to drink ad libitum; at the end of the fourth day, the juice was replaced with water. Luciferase activity was monitored during the entire experiment by means of in vivo imaging. Figure 3 shows photon emission from the different body areas of mice during the 7 days of treatment. No significant increase in the activity of luciferase was detected in the

FIG. 2. In liver of ERE-Luc mouse, luciferase synthesis is directly proportional to the dose of 17β-estradiol administered. Groups of eight animals were treated with 17β-estradiol sc and euthanized 6 h after treatment. Luciferase content was measured in vivo by CCD camera in terms of photon emission (cts/s) and in liver extracts (RLU).
whole animal, with the exception of chest (liver), where we observed an increase of luciferase activity after 2 days of treatment; withdrawal of the juice resulted in a decrease of luciferase activity back to the levels measured at day 0. In the second experiment, animals were treated daily for 7 days by gavage with either an extract (equivalent to 0.12 ml: 5 ml wine/kg) of red wine resuspended in water or with 250 mg/kg of resveratrol in water or in water containing 13% ethanol (Fig. 4) and on the seventh day animals were exposed to the CCD camera and euthanized to study luciferase activity in selected organs. Similarly to what observed with the raspberry juice treatment, no effect of resveratrol or wine extract was observed in these animals, however also in this case the liver was an exception because both in vivo and in vitro analyses showed a significant increase of luciferase activity after administration of resveratrol dissolved in the ethanol containing solution. The wine extract failed to modulate luciferase activity suggesting that, at moderate dosages, red wine does not possess estrogenic compounds at levels sufficient to be active on ERs.

These results demonstrate the usefulness and efficiency of reporter systems to evaluate the presence of estrogenic compounds in the components of our diet and to precisely determine the extent of their activity on the endogenous ERs in each potential target organ.

Effects of Long-Term Exposure to Estrogenic Compounds

The major advantage in the use of in vivo imaging is linked to the possibility to follow in time the effects of long-term exposure. Preliminary experiments were carried out by administering daily low doses of isoflavones (5 mg/kg/day by gavage). Photon emission was measured each day in selected body areas as illustrated in Figure 5. For the first experimental week, luciferase

FIG. 3. Luciferase expression in ERE-Luc mice after prolonged assumption of raspberry juice. On day 1 drinking water was substituted with raspberry juice (commercial juice from Zuegg) and animals had free access to it. On day 4 the juice was replaced with water. During treatment, ERE-Luc mice were exposed daily to a CCD camera for measurement of photon emission at 3:00 P.M. Bars represent the average ± SEM of groups of six animals each. *p < 0.05 versus controls as determined by ANOVA followed by Bonferroni post hoc analysis.

FIG. 4. Luciferase expression in ERE-Luc mice after 7-day administration of red wine extract or of resveratrol. Mice were treated (at 10:00 A.M.) by 200 µl of oral gavage of water, ethanol (13%) in water, lyophilized red wine (equivalent to 5 ml wine/kg) reconstituted in water, resveratrol (250 mg/kg/day) dissolved in water, and resveratrol (250 mg/kg/day) dissolved in water plus 13% ethanol. The imaging session was performed on the seventh day at 16:00 P.M. (6 h after the last treatment). Immediately after imaging, animals were sacrificed and brain, liver, intestine and bone (femur) were collected for luciferase enzymatic assay. Bars represent the average ± SEM of groups of six animals each. *p < 0.05 versus controls as determined by ANOVA followed by Bonferroni post hoc analysis.
activity was comparable in control and in genistein-treated mice. Starting from day 7, in chest, but not in limbs and reproductive tissues, luciferase activity was found to be significantly elevated in animals treated with the isolavone. No effect of the treatment was observed in areas other than chest in ERE-Luc mice up to the end of the experiment at day 20 (Fig. 5).

This study shows that, with a limited number of animals, it is possible to verify the effects of any given compound in time and to identify its potential targets.

Reproducibility

The issue of reproducibility was addressed by evaluating the variability of the reporter activity within each experimental group and among different experiments carried out in different labs in the frame of the EDERA Program of the European Community. Most interestingly, several unpredicted problems were encountered during the study. The most important of them was represented by the diet and by the fact that eating by itself constitutes a trigger for ER activity, particularly in liver (Ciana et al., 2005; Paolo Ciana, unpublished results). Measures had to be taken to minimize the dietary effects and a specific experimental diet (AIN93-M) has been experimentally identified to minimize ER activation. The use of standardized procedures has decreased the intragroup variability from 50% to about 15%; thus, to provide significant results, each experimental group must be composed of a minimum of eight animals, although the use of homozygous mice can improve significantly the intragroup variability. The results obtained with specific compounds in two laboratories, with two different CCD-cameras, were superimposable.

FUTURE DEVELOPMENTS

Selectivity: Generation of models able to discriminate among compounds acting on ERα or ERβ

It is becoming quite apparent that the two ER subtypes have quite diversified spectrums of physiological activities, therefore it would be useful if we could differentiate between compounds acting preferentially on either of the two receptors. Models for the identification of compounds selective for ERα or ERβ were obtained by breeding homozygous ERE-Luc mice with the ERβ−/− mice (BERKO) or ERα−/−. Preliminary studies in these animals are providing a first insight on the prevalent activity of each receptor in the different mammalian organs. These models will be instrumental for the identification of compounds more likely to be harmful for reproductive (compounds acting through ERα) or nonreproductive (compounds targeting ERβ) functions.

High Throughput

A major limitation to the use of in vivo imaging for the screening of toxicological compounds resides in the limited throughput of the methodology, the two major bottlenecks being the CCD camera exposure time and the analysis of the data. Both issues promise to be solved with the use of gas anesthesia and the modification of the dark box to host an increased number of animals: we believe that the dark rooms, the optical apparatus, and the apparatus for gas anesthesia can be easily modified to accommodate at least 10 animals at a time: this would significantly increase the output of the analyses. In addition, the development of appropriate algorithms should speed up data analysis by providing measurements of photon emission in restricted areas of the animal body.

Finally, it should be mentioned that reporter mice might represent a unique source for the preparation of tissue-specific reporter cells. At present time, the EU EXERA Consortium is addressing the issue of the systematic generation of 2D and 3D cell cultures from the ERE-Luc mouse.

Multimodality

A drawback of the presently available imaging technologies is that each imaging methodology has its own limitations and only the combined application of bioluminescence, fluorescence, radiisotope, and magnetic resonance imaging provides a perfect analysis of the activity of a given reporter in response to environmental cues. This limitation can be overcome with the use of multimodality reporters hosting an array of genes suitable for the application of the necessary imaging modality (Ottobrini et al., 2008; Stell et al., 2007).

Looking at More Than one Molecular Event at the Time

The availability of numerous bioluminescence and fluorescence reporters (Contag and Bachmann, 2002; Matz et al., 2002), together with the rapid progress in the bioluminescence technology, will soon enable to develop mice engineered to be responsive to more than one stimulus at a time: for instance, mice responsive to EDs acting through ERs, ARs, and THR. These mice would be extremely useful for a rapid identification of potential EDs.
The 3R’s Issue: Replace, Reduce, and Refine

The data obtained so far with the application of the ERE-Luc model clearly showed the significant advantages of these models enabling for the first time to obtain an insight on the systemic activity of any given compound when administered acutely of repeatedly (Russell and Burch, 1959). Most interestingly, the exposure to the potentially toxic compound might completely mimic what naturally occurring because the compound may be administered orally or the animal can be raised in a contaminated environment. The most interesting feature of the reporter animal models, however, is that they are excellent candidates to Reduce the number of animals to be used in the in vivo tests because (1) animal sacrifice is factorially reduced and (2) the possibility to follow the endocrine effects in time in the same animal reduces the need of large number of animals in each experimental group to obtain significant data and also reduces the need for control groups (the effect of a treatment is evaluated versus the baseline state of activity of the receptor in the same animal); the technology will Refine current methods by providing, for the first time, the possibility to study the effects of EDs systematically and after long-term exposure even to low doses and to mixtures, furthermore the technology will abolish the pain for the test animals and the necessity of animal sacrifice; finally, in view of the peculiarity of ED action which demands the use of whole organisms to obtain a reliable picture of their action, we feel that the application of molecular imaging techniques should be considered as a very valid Replacement alternative.

ACKNOWLEDGMENTS

We are indebted to Maria Letizia Penza and Silvia Belcredito for their critical comments, helpful in the preparation of this manuscript.

REFERENCES


OPTICAL IMAGING AND ENDOCRINE DISRUPTERS


