Discovery of novel nuclear receptor modulating ligands: an integral role for peptide interaction profiling

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There is currently a marketed drug for nearly every nuclear receptor for which the natural ligand has been identified. However, because of the complexity of signal transduction by this class of ligand-regulated transcription factors, few of these drugs have been optimized for pharmaceutical effectiveness. Over the past several years, structural and biochemical work has shed light on some of the ligand-induced features of nuclear receptors that enable them to trigger signal transduction cascades. This review will highlight the use of peptide interactions to cluster different classes of ligands and to identify novel nuclear receptor-modulating ligands as potential drug candidates. Phage display and a multiplexed peptide interaction assay are two of the technologies that are key to this approach. When used as part of a drug discovery platform, this type of biochemical characterization can bridge the gap between high-throughput chemical synthesis and disease model testing. Furthermore, the development of these methodologies is timely because there is a significant medical need for new and improved nuclear receptor drugs that retain beneficial effects but do not have undesired side effect activities.

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▼ Most nuclear receptors (NRs) act as complex molecular switches that are triggered by the binding of small-molecule ligands. Typically, upon activation by a small lipophilic hormone, NRs act as transcription factors that can specifically regulate the expression of target genes. The products of these NR-controlled genes help to maintain a variety of essential physiological processes, including reproduction, metabolism, development and differentiation. To date, of the genes characterized from the sequencing of the human genome, 48 human gene products have been categorized as a NR and this class of receptors is referred to as the NR superfamily.

Based on sequence alignment, receptors within the NR superfamily have been subdivided into six general groups [1]. Historically, the most studied and the most widely recognized subclass of the NRs is the steroid hormone receptor family, which comprises receptors for the steroids, glucocorticoid (GR), estrogen (ER), androgen (AR), mineralocorticoid (MR) and progesterone (PR) [2]. In addition, other well-studied ligand-activated receptors within the superfamily include thyroid hormone receptor (TR), vitamin D receptor (VDR) and retinoid receptors (RXRs). There is a more recently studied set of receptors that is rapidly gaining attention as important drug targets and includes the liver X receptor (LXR), farnesoid X receptor (FXR) and the peroxisome proliferator-activated receptors (PPARs); these receptors are commonly referred to as orphan receptors because the majority were cloned before ligand discovery.

Many NRs are highly validated and disease-associated, which make them attractive targets for drug discovery. Although compounds that target NRs represent only ~2% of all 'safe and efficacious' drugs [3], data from 2002 shows that eight of the top 100 prescription drugs target an NR, which accounts for sales of over US$9 billion dollars (http://www.pharmalive.com/special_reports/index.cfm?start=41#nav). However, one major concern is that many of these drugs are accompanied by potentially serious side effects that limit their utility and safety (Table 1). Presently, there are programs throughout the pharmaceutical industry to find more-selective modulator ligands for NRs. These types of synthetic molecules, which in some cases are drugs, that do not have all the

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Table 1. Currently marketed drugs that target nuclear receptors and their associated efficacies and side effects

Receptor	Ligand	Efficacy and/or beneficial effects	Side effects	Refs
Estrogen	Estrogens	Protection against osteoporosis Prevention of 'hot flushes' Increase in HDL-C	Uterine stimulation Increase in triglycerides	[59,60]
	Tamoxifen	Protection against osteoporosis Treatment for breast cancer	Increased risk of uterine cancer Increased risk of 'hot flushes'	[59,60]
	Raloxifene	Protection against osteoporosis Reduces triglycerides Does not stimulate uterine tissue	Does not increase HDL-C Increased risk of 'hot flushes'	[59,60]
Glucocorticoid	Dexamethasone	Anti-inflammatory Rheumatoid arthritis treatment Adrenal insufficiency treatment Chemotherapeutic agent Immune system suppressant Reduces transplant organ rejection	Diabetes mellitus Peptic ulcer Cushing's syndrome Osteoporosis Skin atrophy Psychosis and/or depression Glaucoma	[31,61]
Mineralocorticoid	Spironolactone	Prevention of cardiac fibrosis	Renal fibrosis Thrombosis	[62]
	Eplerenone	Prevention of cardiac fibrosis Heart failure prevention Hypertension treatment	Hyperkalemia Increased cholesterol	[62]
Peroxisome proliferator-activated-γ	Thiazolidinediones	Lowers blood glucose in diabetes Improves lipid profile Lowers blood pressure Anti-inflammatory	Increased body weight Increased fluid retention	[63,64]

Abbreviation: HDL-C, high-density lipoprotein cholesterol.

activities of the natural ligand are typically referred to as 'selective receptor modulators' (SRMs), or more specifically as 'selective estrogen receptor modulators' (SERMs), 'selective androgen receptor modulators' (SARMs) or 'selective glucocorticoid receptor modulators' (SGRMs). The ideal NR-modulating drug would be able to retain beneficial effects in specific target tissues without displaying undesired side effect activities.

Although all the NRs within the larger superfamily share a similar domain organization and significant sequence homology, some of the 48 receptors appear to be constitutively active and have as yet not been shown to be regulated by a small molecule [4]. For the most part, the receptors that have been linked to either a natural or synthetic ligand are currently the subject of intense drug discovery pursuits. This review focuses primarily on biochemical methods that use the current knowledge of ligand-regulated NR mechanisms to further these investigations.

Domain organization and structural features of the nuclear receptor ligand-binding domain

NRs are modular proteins that consist of three major functional domains (Figure 1a). Although there is undoubtedly interplay between the domains, each domain has a separate crucial signal transduction function. The domain at the N-terminus, which is referred to as the activation-function-1 (AF-1) domain, varies in length between NRs and generally has a role in constitutive transcriptional activity [5]. The

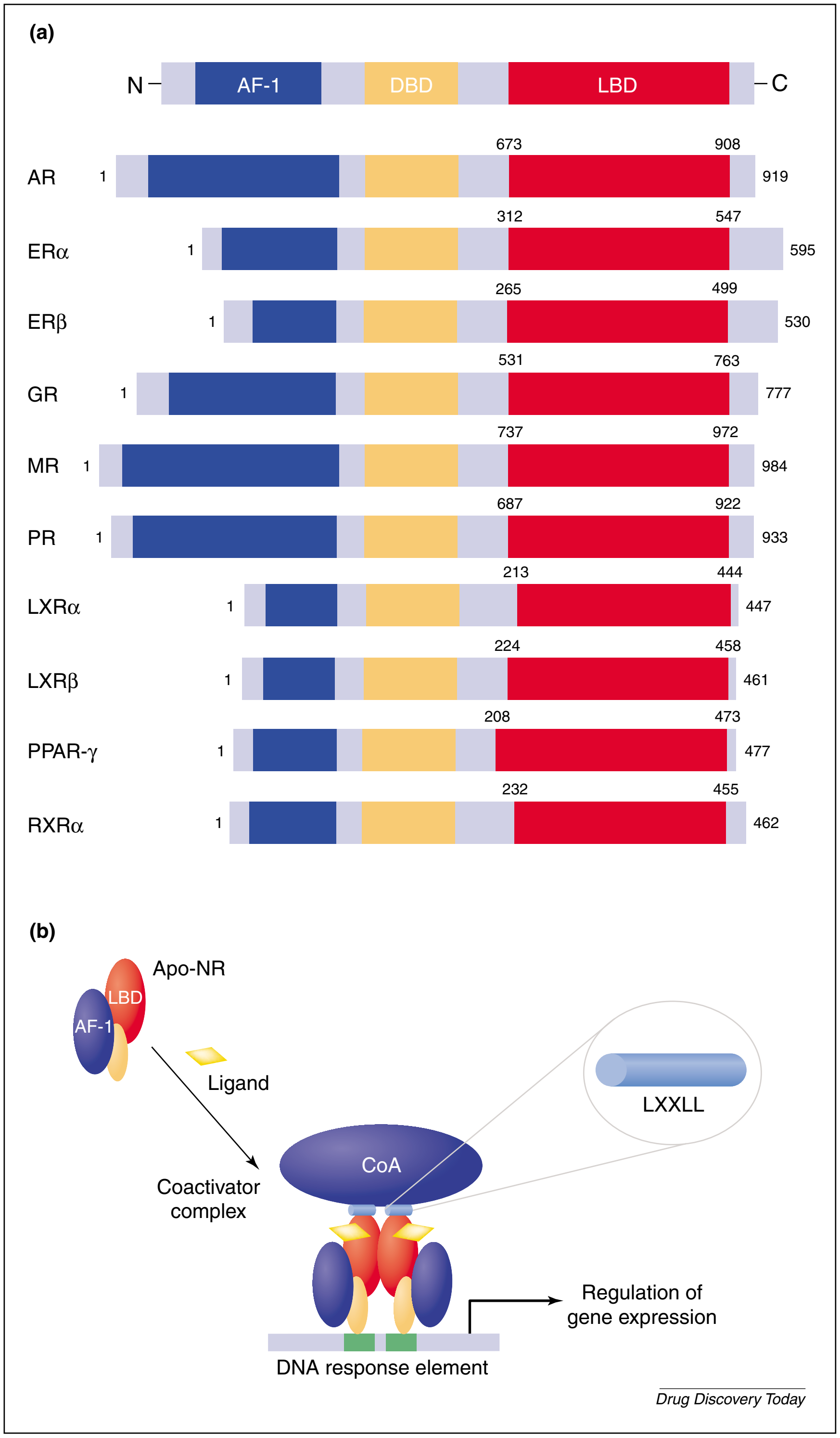

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Figure 1. General features of nuclear receptors. **(a)** Schematic linear representation of various NRs that shows the varying lengths of the functional domains. The AF-1 domain tends to vary in length (and sequence) among NR superfamily members. The DBD is the most conserved region of the receptors and the domain size is relatively constant. The LBD is typically ~250 amino acids in length. Sequence identity within this region varies from subclass to subclass. For example, sequence identity among the steroid receptors is between 54% and 61%. **(b)** Simplified cartoon representation of NR activation by an 'agonist' ligand. Ligand binding typically triggers heat shock protein release and receptor translocation to the nucleus, where coactivators can associate and the complex binds to an appropriate DNA response element. This complex, along with basal transcription factors, initiates gene transcription. Coactivators dock onto activated nuclear receptors via short helical LXXLL motifs or 'NR boxes'. Abbreviations: AF-1, activation function-1; AR, androgen receptor; CoA, coactivator; DBD, DNA-binding domain; ER, estrogen receptor; GR, glucocorticoid receptor; LBD, ligand-binding domain; LXR, liver X receptor; MR, mineralocorticoid receptor; NR, nuclear receptor; PPAR-γ, peroxisome proliferator-activated receptor-γ; PR, progesterone receptor; RXR-α, retinoid receptor-α.

DNA-binding domain (DBD), which is typically located in the mid-section of NRs, is the most highly conserved region among the receptors. Although the precise mechanism of DNA interaction varies from receptor to receptor, for example, receptors can bind as monomers, homodimers or heterodimers, a common theme is that direct contact of the NR to DNA is made through two consecutive zinc fingers. Zinc fingers are DNA-binding motifs that contain the key amino acids necessary for the association of the NR to the major groove of an appropriate DNA response element that is located within the promoter segment of a target gene (Figure b) [6,7].

Currently, the final domain at the C-terminus is by far the most important to the drug discovery efforts of the pharmaceutical industry. This region, called the ligand-binding domain (LBD), contains all the binding determinants of the small molecule hormone or synthetic ligand. Crystal structures of this domain have been solved for the majority of the 48 NRs [8–11]. Overall, these structures reveal a highly conserved 3D fold that consists predominantly of three major α-helical layers (Figure 2). When positioned in the standard orientation, the front face of the LBD consists of helices 1–3. Helix 4/5 and helices 8 and 9 occupy space in the upper section of the centre of the domain. The lower half of the middle of the domain is void of protein and thus there is space for the binding of a ligand. The back sheet contains helix 7

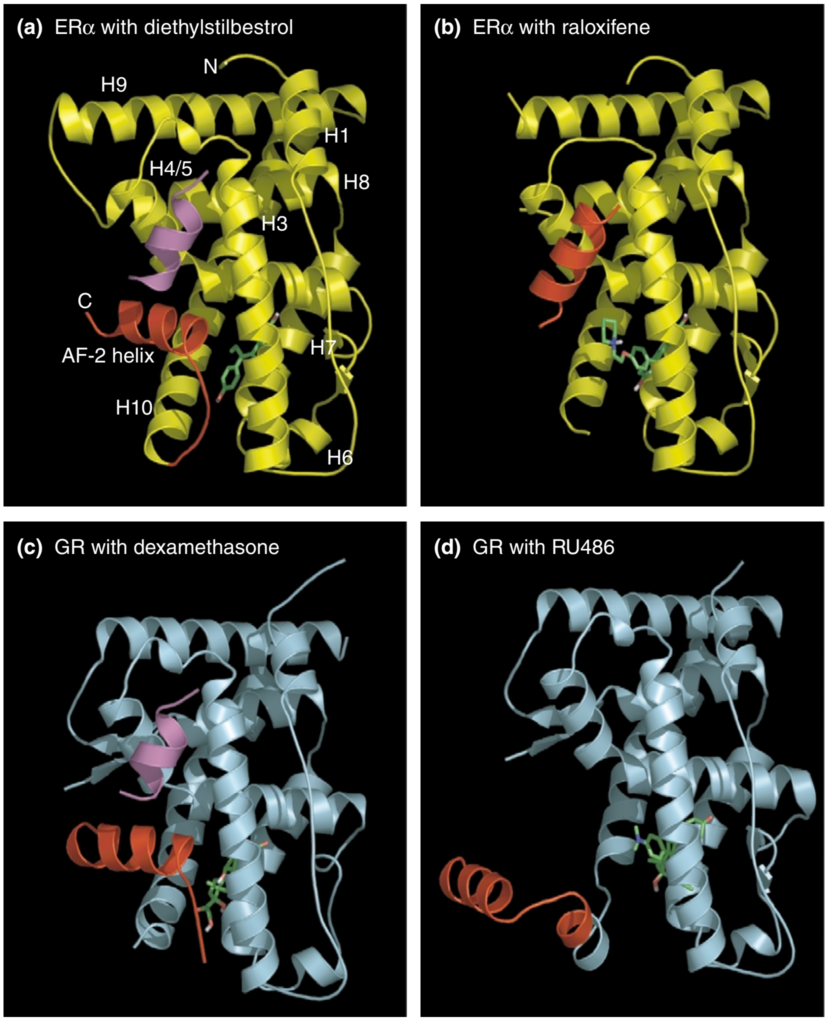

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Figure 2. Key nuclear receptor ligand-binding domain crystal structures. These ribbon diagrams show classic examples of NR LBD–ligand complexes to demonstrate the ligand-stabilized conformational differences, particularly with respect to the AF-helix (red).The crystal structures shown are **(a)** ERα (yellow) complexed with DES [15], **(b)** ERα in complex with raloxifene [14], **(c)** GR (cyan) bound to DEX [11] and **(d)** GR complexed with RU486 [19]. Note that some of the loops between helices have not been well-resolved by crystallography. The two agonist bound structures (ERα–DES and GR–DEX) both show bound coactivator peptide [magenta (both peptides originate from the coactivator TIF2)]. Respective ligands are shown in green stick representation with oxygen atoms represented in red and nitrogen atoms represented in blue. Figures were made using PyMOL (DeLano Scientific; http://www.delanoscientific.com). Abbreviations: DES, diethylstilbestrol; DEX, dexamethasone; ER, estrogen receptor; GR, glucocorticoid receptor; LBD, ligand-binding domain; NR, nuclear receptor; TIF2, transcriptional intermediary factor-2.

and the domain-spanning helix 10. The final helix, which is commonly referred to as the AF-helix, is a crucial structural element that can adopt multiple positions depending on the nature of the bound ligand.

When bound with 'agonist' ligands, such as the natural hormones estradiol for ER, cortisol for GR or progestin for PR, the AF-helix of the respective receptor crosses helices 3, 4 and 10 to create an adjacent hydrophobic cavity. The slightly concave groove that is formed can accommodate an amphipathic helix that is presented by several cofactor proteins such as steroid receptor coactivator-1 (SRC-1), cAMP response element-binding protein (CBP) or transcriptional intermediary factor-2 (TIF2) (Figure 2a,c). The helical region presented by the cofactors that subsequently docks onto the NR contains three leucines (L), which are arranged as LXXLL, where X represents any amino acid [12]. These short helices, which are commonly referred to as NR boxes, present the leucine side-chains on one face of the helix. By attaching to the ligand-activated, DNA-bound NR, transcriptional-enhancing features of coactivators are colocalized to the promoter region (Figure 1b); the essential activities of these large proteins (~300 kDa) include histone acetylation and chromatin remodeling, as well as recruitment of additional transcription factors and other elements of the transcriptome complex [13].

Crystal structures of NR LBDs complexed with various synthetic ligands have revealed that the AF-helix can adopt an array of alternative positions [14–16]. One such case is the forcing of the AF-helix into the coactivator-binding groove. This conformation was first observed with the ERα LBD structure in complex with raloxifene [14] (Figure 2b). Here, the piperazine-ring headgroup of raloxifene protrudes from the ligand-binding pocket toward the loop that precedes the AF-helix; this steric interference causes the helix to reposition and form new intra-domain contacts. In other crystal structures, such as ERβ complexed with genestein [17], ERβ complexed with ICI164384 [18] and GR with RU486 (Figure 2d) [19], several slightly different ligand-induced positions of the AF-helix have been observed.

Although a two-state model for NR LBD activation (i.e. 'on' versus 'off') might at first seem intuitive, NR LBD activation is realistically much more complex. Synthetic chemistry efforts over the past decade, coupled more recently with detailed protein and peptide studies, have revealed that the LBD, as well as the entire receptor, is able to adopt a myriad of ligand-induced conformational states. This inherent conformational complexity is one of the topics of this area of research and drug discovery. This review focuses on the use of peptide interactions to characterize and to understand the features and the dynamics of this property of NRs.

Tissue specific effects of non-natural nuclear receptor ligands

The complexity of NRs extends from the conformational diversity of the receptor at the molecular level to the multitude of signal transduction functions at the cellular level. Although the role of the complex coregulator environment within a cell is far from being completely understood, this cellular environment has an important influence on the phenotypic outcome for a particular ligand [20]. Evidence for this stems from the observation that different NR ligands can produce unique cellular or physiological effects in different cell types. For example, tamoxifen, which was designed as an ERα antagonist for the treatment of ER-positive breast cancer, has long been known to possess agonist properties within specific tissue types, particularly within uterus and lumbar spine [21]. Although the mechanism of this agonist activity remains mostly unresolved, it has been proposed to result from either activation of alternative pathways [22] or cell type alteration of coregulator balance [23,24].

In addition to coactivators, cells contain proteins (called corepressors) that are able to downregulate or repress NR activity. Whereas over 200 coactivators have been identified to date, less than 20 corepressors have been found. The two most characterized are silencing mediator of retinoic acid (SMRT) and nuclear receptor corepressor (NCoR). Several reports have shown that some NR ligands, such as tamoxifen, raloxifene and GW7604 for ER [25–27], RU486 for PR [28] and RU486 for GR [29], enhance corepressor recruitment.

Many NRs have crosstalk activities with other signal transduction pathways, which further complicates the coregulator interaction network. For example, some NRs, such as ER, GR, AR, PR, RXR, and retinoic acid receptor (RAR), have the ability to either directly interact or otherwise interfere with the ability of the nuclear factor-κB (NF-κB) or activator protein-1 (AP-1) transcription factors to regulate gene expression [30–32]. This activity of NRs is generally referred to as transrepression, whereas direct DNA-mediated gene regulation is called transactivation. Additionally, NRs are involved in numerous kinase-driven pathways [33,34] and are also targets for post-translational modification via phosphorylation and sumoylation. Collectively, these types of NR activities are referred to as ‘nongenomic’ or ‘extranuclear’ signaling activities [35].

In spite of the complex network of NR functions, the effort to find ligands that display differential activities has led to the discovery of several molecules that appear to demonstrate novel and selective properties, such as exhibiting differential tissue effects or maintaining transrepression activity while minimizing transactivation. Most of this research has relied on cell-based assays such as transient transfection reporter assays, cytokine production assays or cell-proliferation assays that use immortalized cell-lines. For example, the ER ligand GW5638 was identified using both a response element reporter assay and a uterine cell-stimulation assay [36]. Recently, AL-438 and ZK216348 have been identified as new ligands for GR [37,38]. These two ligands appear to retain significant transrepression activity while minimizing the response element driven transactivation activity. Overall, these studies highlight that novel and desired properties can be obtained with new ligand templates that allosterically regulate the many activities of NRs by mechanisms that are distinct from those of previous ligands or the natural hormone.

Given the interwoven complexities of NR signal transduction, where a variety of coactivators, corepressors, transcription factors and kinases have a functional role, recognizing precisely how to select the desired properties of a NR ligand has proven to be a daunting task. For drug discovery efforts, wading through larger compound sets that fully explore NR ligand-binding pocket space is not possible with the difficult, yet informative, cellular assays or animal models that are currently available. Historically, there has been a need for a higher-throughput screening mechanism with some basis for ligand activity. The NR conformational change induced by ligand binding and the consequent array of protein–protein interactions play a crucial role in triggering and driving the multitude of signal transduction events. These ligand-induced protein–protein interactions ultimately affect the cellular outcomes of ligands. Advances made within the NR field using biochemical interaction technologies, such as phage display and fluorescence-based techniques, have simplified and expanded our throughput for analyzing novel, newly synthesized NR ligands.

Random peptide technologies for probing nuclear receptor conformation

The recent structural knowledge about ligand-driven NR conformation and the importance of small peptide motifs within coactivators has led several groups to seek unique small peptides and/or interacting proteins to sense ligand-induced NR conformations. The end result is that simple *in vitro* peptide

Table 2. Examples of nuclear receptor conformation-sensing peptides selected using random peptide phage display

Receptor	Peptide name	Peptide sequence	Compound used as target	Compound selectivity	Refs
Androgen	3-18	NTNAFSRLFYPS	Testosterone	Testosterone> DHT	[65]
	4-11	QPKHFTELYFKS	Testosterone	DHT> testosterone	[65]
	D-11	VESGSSRLMQLLMANDLLT	ERα and/or estradiol	DHT	[66]
	D-30	HPTHSSRLWELLMEATPTM	ERα and/or estradiol	DHT	[66]
Estrogen	GW5P2	EDGGSLFERVWLRELG	GW5638X	GW5638X	[27]
	I8P2	EDGGSWVEWVEEERRG	Idoxifene	Idoxifene	[27]
	R5P2	EDGGSRRFTFQYGSVG	Raloxifene	Raloxifene	[27]
	K7P2	EDGGSLLRWYLEHEFG	Estradiol	Estradiol, agonists	[27]
	7β-16	ASRHFLINQHLYKLLQDTDIVVSRL	GW5638X	GW5638X	[67]
	bT1	ELFDAFQLRQLILRGLQDDIPYH	Tamoxifen	Tamoxifen	[25]
	bI2	EMEWMKALRQHISGELRRNYTEE	ICI164384	ICI164384	[25]
	D47	HVYQHPLLSLLSSEHESG	Estradiol	Estradiol, agonists	[68]
	αII	SSLTSRDFGSWYASR	Estradiol	Non-selective	[41]
	α/βIII	SSWDMHQFFWEGVSR	Tamoxifen	Tamoxifen	[41]
	α/βV	SSPGSREWFKDMLSR	Tamoxifen	Tamoxifen	[41]

Abbreviation: DHT, dihydrotestosterone; ER, estrogen receptor.

interaction assays can be established to improve understanding of NR conformation at the receptor level.

Random peptide methods that were first developed in the 1980s, such as peptides-on-plasmids and phage display, have provided a rapid means to identify conformation-sensing peptide tools [39]. Northrup *et al.* [40] used peptides-on-plasmids to find a wide variety of LXXLL-containing peptides that are able to interact with TR and the ERs. Although this report showed that coactivator-like peptides could be easily found, it has become clear from other reports that the range of peptide tools identified varies with the different ligands that are used to alter the NR conformation. For example, Norris *et al.* [41,42] were the first to demonstrate that phage display could be used to find peptides that are much more compound discriminating. Although it was known that both ERα and ERβ, when complexed with the classic 'antagonist' ligand tamoxifen, were unable to bind LXXLL-containing coactivators, Norris *et al.* [41,42] showed that random peptide phage display could be used to find peptides that only bound to ERs in the presence of tamoxifen. These initial experiments were key in showing that various ligands can induce intermediate conformations that allow for differential coregulator recruitment [43]. Additionally, NR-binding peptides identified with random libraries have the potential to represent cellular interaction partners that have yet to be discovered. In this way, the random peptide approach is a powerful technique for the rapid isolation of peptides against specific NR–ligand complexes, where the novel peptides could represent a natural cellular interaction partner. A summary of some of the compound-selective peptides identified for NR drug targets is shown in Table 2.

Conformation-sensing peptides from random display libraries have provided valuable tools to differentiate NR ligands. However, there are other sources for identifying interaction partners. Conventional interaction cloning techniques, such as yeast two-hybrid or the T7 display system, can identify novel conformation-sensing partners [44,45]. In addition, antibody mimetics (i.e. 'monobodies') have been used to differentiate ligand-induced conformations of ER [46].

In vitro techniques for monitoring nuclear receptor–peptide interactions

Several technologies have been conventionally employed to investigate the *in vitro* effects of ligand on NR binding interactions with protein cofactors or their peptide surrogates [47]. Among these technologies is surface plasmon resonance, which is an optical detection technology where the binding or dissociation of macromolecules on a sensor chip is measured as a change in mass concentration [48,49]. Time-resolved fluorescence resonance energy transfer (TR FRET) assays have proven useful for the investigation of NR binding interactions and, compared with surface-plasmon resonance, offer throughput advantages for routine screening applications [50]. In TR FRET, the excitation energy of a donor fluorescent molecule (usually a chelate or cryptate of a lanthanide element) is transferred by a resonance mechanism to a neighboring acceptor fluorescent molecule. The acceptor molecule releases energy through fluorescent emission. The long-lived emission that is contributed by the donor molecule enables the measurement of the fluorescence of the acceptor molecule in a time-delayed method. The time-resolved measurement reduces fluorescent background by eliminating the detection of fluorescent emission from freely diffusing acceptor molecules.

Another recently developed bead-based molecular interaction measurement technology is the luminescent proximity assay or AlphaScreen™ (PerkinElmer; http://www.perkinelmer.com) [47]. On illumination, a photosensitizer in a donor bead produces singlet oxygen. If an acceptor bead is in close proximity (~200 µm), energy is transferred from the singlet oxygen to a thioxene derivative, which results in light production between 520 and 620 nm. The advantages of the proximity-based energy transfer approaches are sensitivity of detection, 384-well microtiter plate format and automation compatibility.

A multiplexed format for simultaneous nuclear receptor–peptide interaction measurements

Recently, a novel approach to profiling NR ligands has been described that simultaneously analyzes up to 100 multiplexed binding interactions in a single well of a 96-well microtiter plate (Figure 3) [51,52]. The assay uses fluorescently encoded microsphere populations (Luminex®; http://www.luminexcorp.com) and fluorochrome-coupled recombinantly expressed NR; the read-out platform is flow cytometry. Multiplexing of the peptides is accomplished by separately coupling each peptide interaction partner (cofactor binding motifs or peptide sequences identified by phage display) to a unique population of microspheres. The different peptide-coupled microspheres are then combined (i.e. multiplexed) within a single well. In addition, each well contains a fixed concentration of subsaturating fluorochrome-coupled NR and a receptor-saturating concentration of ligand. The microsphere suspensions are analyzed with a microsphere-dedicated

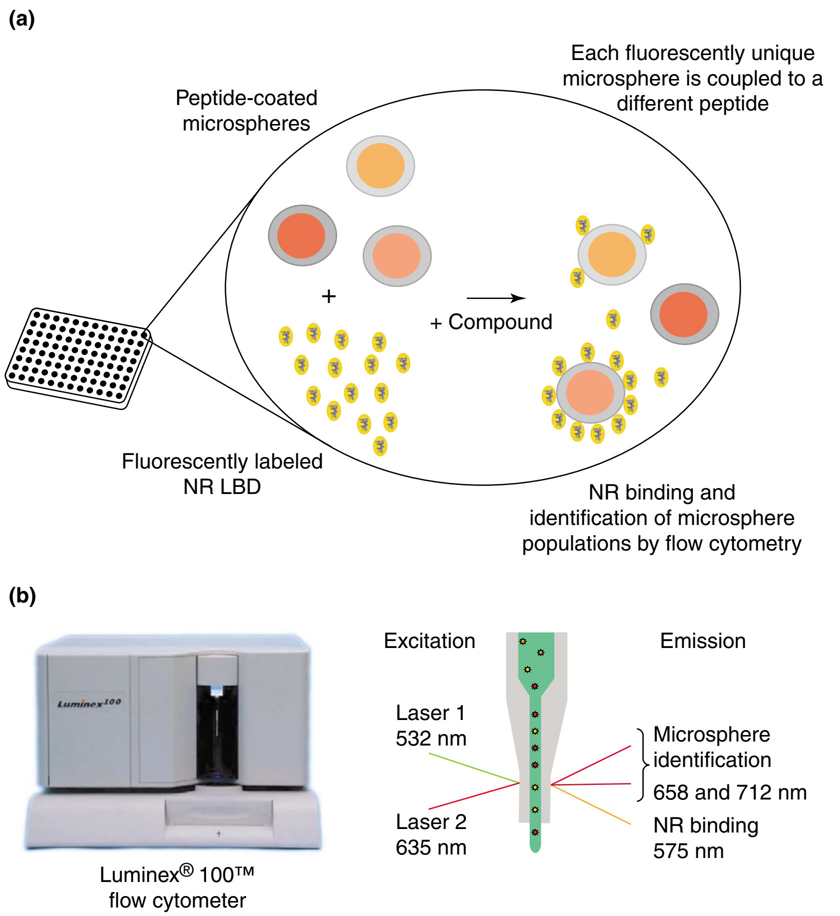

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Figure 3. Multiplexed microsphere-based analysis of nuclear receptor binding interactions by flow cytometry. **(a)** Nuclear receptor binding assays are conducted in a 96-well microtiter plate by combining up to 100 fluorescently encoded peptide-coupled microsphere populations and fluorochrome-coupled recombinant nuclear receptor in the presence or absence of a compound. Each well contains the same complement of peptide-coupled microspheres but a different compound. After incubation, the microspheres are analyzed by flow cytometry. **(b)** Analysis of nuclear receptor binding is conducted using a microsphere-dedicated flow cytometer [Luminex®100™ (Luminex; http://www.luminexcorp.com)]. The microsphere suspensions are drawn into the flow cytometer one well at a time using an XY autosampler. Once inside the flow cytometer, the microspheres move rapidly in single-file past two focused laser beams. At the point of interrogation, three separate fluorescent emissions are measured. From the red laser, far red and near infrared fluorescent emission are measured. These two colors identify the microsphere population and, by inference, the peptide coupled to its surface. From the green laser, microsphere-associated orange fluorescence is measured, which indicates the level of nuclear receptor binding.

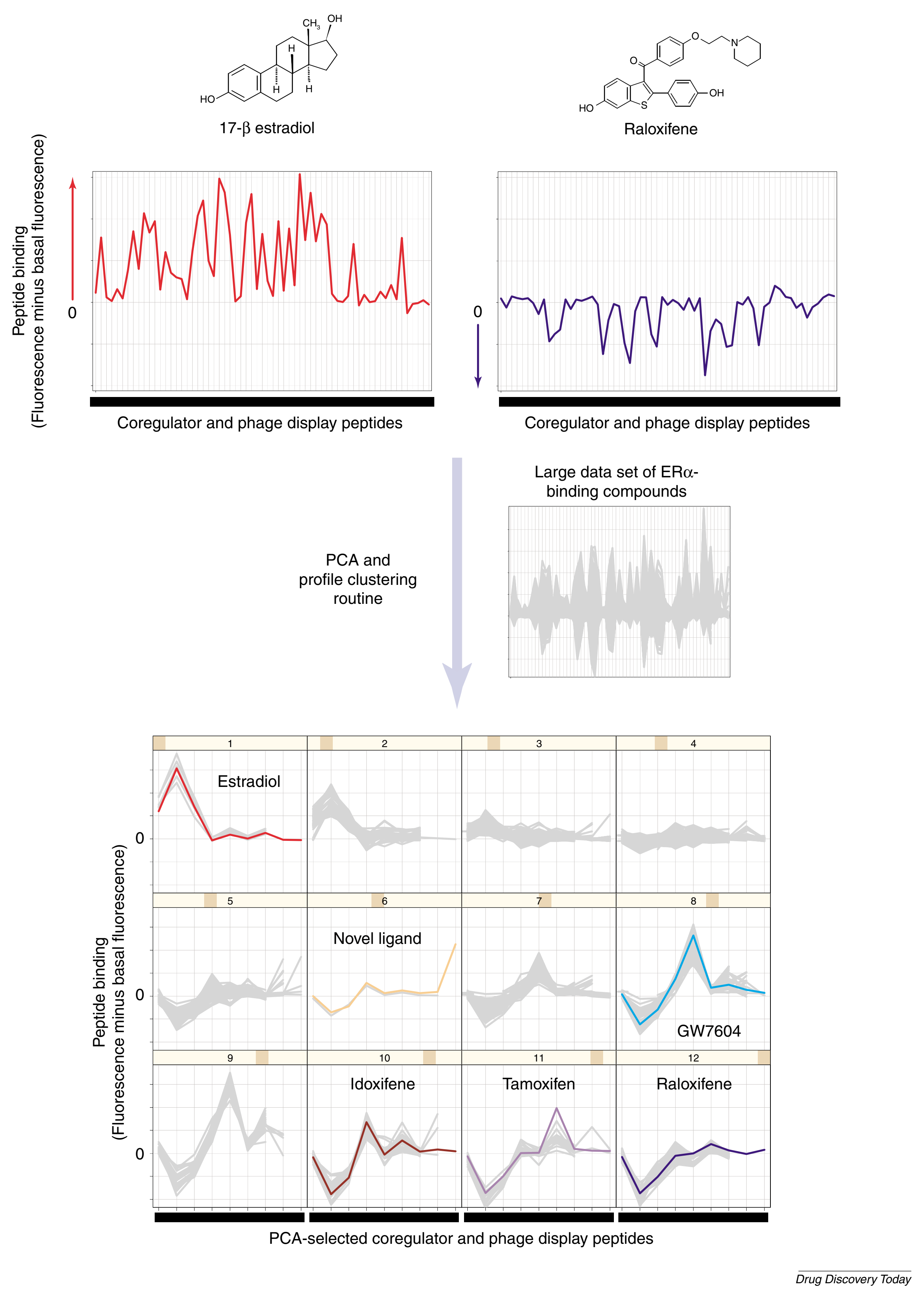

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Figure 4. Example of estrogen receptor peptide-profiling data and cluster analysis using data from a Luminex (http://www.luminexcorp.com) bead assay. Two example profile plots for 17-β estradiol and raloxifene are shown. Each plot represents binding data for a single concentration of fluorescently labeled ERα LBD under saturating ligand conditions. To show the effect of compound on peptide binding, the basal fluorescence is subtracted from the fluorescence in the presence of compound. A value of zero indicates no effect on peptide binding relative to the apo-receptor. Each vertical grey line shows data for a single peptide and the histogram data points are connected at the maximum or minimum to yield a profile plot. After acquiring data from a large set, PCA selects the most statistically relevant peptides and the profiles are then clustered based on overall profile shape. The bottom of the figure shows an example of a cluster analysis for ERα data, where a few key tool compounds are shown as colored profile plots. Each grey profile plot represents data for a single compound. Abbreviations: ER, estrogen receptor; LBD, ligand-binding domain; PCA, principle components analysis.

flow cytometer (Luminex®100™; Luminex). During flow cytometric analysis, the microspheres, which are encased in a fluid stream, move rapidly in single-file past two focused lasers. As each microsphere passes the laser light, the intensities of three fluorescent colors are measured. Far red and near infrared fluorescence are measured to identify the microsphere population (and therefore the peptide coupled to its surface) and orange fluorescence is measured to assess the level of NR binding. The assay is sensitive, compatible with automation, of medium-throughput and facilitates the rapid acquisition of a ‘profile’ of NR binding information for each ligand.

Analysis of *in vitro* peptide-profiling data to differentiate nuclear receptor-modulating ligands

To probe NRs in complex with various ligands thoroughly, it is desirable to have as many interaction partners as possible, whether they are identified from natural or random display sources. The relative ease with which large datasets of peptide binding information can be generated, particularly when using a multiplexing system, creates a need for uncomplicated methodologies for the observation of and analysis of peptide profiles. The software package Spotfire® DecisionSite™ (Spotfire; http://www.spotfire.com) has all but revolutionized the way that massive amounts of data can be visualized and analyzed (Figure 4). This program facilitates user flexibility in changing plot types (i.e. scatter, histogram and trellis profile plots) and axis contents. Additionally, the use of structure strings and a structure visualizer enable the user to observe compound structure and to analyze peptide or interaction partner-binding data simultaneously. This feature makes it relatively easy to identify SARs among large sets of ligands.

To enhance the differentiation of peptide-binding profiles, plots can be clustered according to overall profile shape. Spotfire contains several clustering routines that range from k-means to hierarchical clustering. An example of profile plot clustering for ERα data is shown in Figure 4. Visualization of the variety of compound-induced profile plots facilitates the rapid recognition of subtle ligand-induced conformational differences.

As part of an overall strategy to find NR modulators with differing activities, peptide profiling can be a valuable tool for the rapid determination of whether a newly synthesized compound produces an NR conformation that is like or unlike that of existing tool compounds. This conformational profiling step can expedite the front end of the search for novel NR modulators by analyzing large and diverse compound sets in medium- to high-throughput mode. In effect, this enables chemists to probe NR ligand-binding pocket space with the confidence that molecules can be analyzed, parsed and differentiated based on peptide interactions, which leads to rapid comparisons and timely decision-making to focus on compounds that are different from previous ligand tools. The overall goal is that the compounds that show unique peptide profiles could be approaching the ideal modulator; at minimum, it is probable that the compound will display a different set of cellular activities.

It is inherent in this type of up-front approach that it initially might not be known what the profile for a novel compound suggests or what activity that a particular ligand might have in a cellular environment. Simply, the peptide profile for a novel compound could indicate that the molecule allosterically regulates receptor conformation by a mechanism that is unlike previous tool compounds. However, in one example with the ER, a good correlation between peptide binding and the ability of sets of compounds to elicit a cellular response was observed [27]. Obviously, this relatively high-throughput peptide-profiling step should be followed by the more traditional, and typically more laborious, cellular or animal model work on compounds that represent unique peptide profile clusters.

Limitations of peptide profiling for compound selection

The limitations of profiling novel compounds with purified receptors using peptides either from random libraries or known interaction partners should also be discussed. For example, it has been shown that ligand-induced coactivator recruitment and consequent functional activity might be influenced by promoter context [53]. This level of detail could require cell-type and promoter-specific assays. Alternatively, it could prove advantageous to use gene expression fingerprinting [54,55]. The recent discovery that some nongenomic effects of steroids could be driven through novel non-NR membrane receptors is another emerging area of research [56,57]. In some cases, metabolites that alter the receptor-activation properties of the ligand, or even bind to non-NR targets, might result in cellular activities that could not be predicted using the purified receptor [58]. Finally, it is well accepted that the NR LBD drives much of the ligand-related features of signal transduction, but clearly the next step in this approach will be to extend the analysis to the full length receptor to include DNA binding, homodimerization, heterodimerization and other domain activities. In the end, there can be no substitute for appropriate cell-based and disease models, and ultimately clinical trials, for the adequate testing of the activities of a ligand that has been identified using an *in vitro* technique such as peptide profiling.

Conclusions and perspectives

All the currently available NR drugs have undesirable side effects and there is clearly a pharmaceutical opportunity for improvement. For example, a selective ER modulator that slows progression of osteoporosis and eliminates hot flush symptoms without having reproductive organ activities would be clinically valuable, as would a glucocorticoid receptor modulator that blocks inflammation without inducing osteoporosis. Despite many years of intensive study on NRs, a generalized approach to identifying modulators with the desired properties has not been straightforward or obvious. With developments over the past several years, peptide interaction profiling has found a niche in the NR drug discovery process and has helped to shorten the gap between chemical synthesis and disease model testing. However, many unanswered questions about peptide interaction profiling and NR modulation remain. What conformations or peptide interactions indicate desired versus undesired effects? How many repertoires of ligand-induced NR conformations are possible? Does each set of conformations yield unique biology? The strength of the receptor-based peptide profiling approach is that it enables the classification of available compounds as being either similar to or different from preceding drugs or tool compounds at an early stage in the drug discovery process. In effect, this approach helps to alleviate, but does not resolve, the 'needle-in-a-haystack' problem and quickly identifies novel NR modulators that might have unique physiological properties relative to earlier ligands. Additionally, the correlative information that is gained should help to improve understanding of the relationship between ligand-induced protein–protein interactions, signal transduction and cellular phenotype. Overall, the use of these peptide interaction technologies should play a valuable role in the discovery of new classes of modulators that have distinct and pharmaceutically desired biological effects.

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