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Panning for SNuRMs: using cofactor profiling for the rational discovery of selective nuclear receptor modulators

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Drugs that target nuclear receptors are clinically, as well as commercially, successful. Their widespread use, however, is limited by an inherent propensity of nuclear receptors to trigger beneficial, as well as adverse, pharmacological effects upon drug activation. Hence, selective drugs that display reduced adverse effects, such as the selective estrogen receptor modulator (SERM) Raloxifene, have been developed by guidance through classical cell culture assays and animal trials. Full agonist and selective modulator nuclear receptor drugs, in general, differ by their ability to recruit certain cofactors to the receptor protein. Hence, systematic cofactor profiling is advancing into an approach for the rationally guided identification of selective NR modulators (SNuRMs) with improved therapeutic ratio.

Introduction

Nuclear receptors (NRs) are a family of transcription factors that constitute an important and successful class of drug targets (reviewed in references [1–3]). Many of them are regulated by the binding of low-molecular weight ligands, such as steroid hormones or cholesterol metabolites, and for all nuclear receptors where a natural ligand is known, synthetic compounds that modulate the receptor's activity have been identified. However, the pleiotropic effects of NR activity make NR-based drugs, in general, sharp but double-edged swords. Examples of NR targets whose drugs typically exhibit adverse effects that limit their use are the glucocorticoid (NR3C1) and the estrogen (NR3A1, NR3A2) nuclear receptors. The potent anti-inflammatory activity of glucocorticoids is limited to severe applications because of side effects such as Cushing Syndrome, hyperglycaemia and bone loss [4]. Postmenopausal hormone replacement therapy with natural estrogens that target the estrogen receptors (ERs) is limited by their proliferative activity in breast and endometrial tissue, leading to increased risk for reproductive cancers. Current efforts, therefore, aim to develop drugs that modulate nuclear receptors in a tissue and/or gene-specific way. The first hint that this was possible came from selective estrogen receptor modulators (SERMs) such as raloxifene, which, unlike other SERMs and natural ER ligands, do not cause the problematic endometrial activation of ER [5,6]. This example of tissue-selective activation of an NR has instigated the quest for selective nuclear receptor modulators.

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How are nuclear receptors activated to yield a transcriptional response?

To understand how SNuRMs might work, one needs to consider how nuclear receptors exert their function on a molecular level. The general layout of the nuclear receptor protein family is conserved: An N-terminal domain, which varies widely amongst family members, is followed by the well-conserved DNA binding domain (DBD), which uses two highly conserved zinc fingers to recognise NR-response DNA elements [7,8]. A less well-conserved hinge region connects the DBD to the ligand-binding domain (LBD), a globular alpha-helical three-layered 'sandwich' that harbours the cavity for the natural or synthetic ligand [9–12] reviewed in reference [13].

Activation of transcription involves modification of chromatin proteins, alterations in chromatin structure and recruitment of the transcriptional machinery. NRs drive chromatin modifications by serving as 'landing platforms' for numerous proteins or protein

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complexes with the diverse chromatin modifying enzymatic functions, collectively termed cofactors (Figure 1a; reviewed in references [14,15]). To date, more than 300 different cofactors for nuclear receptors have been identified (currently catalogued in the NURSA initiative www.nursa.org). Binding of these cofactor complexes to NRs occurs in a certain temporal and spatial order and is a cyclic process, allowing termination of transcriptional induction at the end of each cycle ([16,17] reviewed in references [18,66]) (Figures 2–4).

The primary consequence of ligand binding to an NR is a conformational change induced in the helical architecture of the ligand-binding pocket, primarily in the outmost C-terminal region, the so-called Activation Function-2 (AF-2). In some cases the conformation of the DNA binding and AF-1 domains of a receptor are affected as well. It is straightforward to conclude that differences in the pharmacological effects of NR ligands are a direct consequence of different receptor conformations induced by these ligands. Such a view is most supported for full agonists and antagonists: Crystal structures have shown that the differential positioning of helix 12 in the ligand-binding domain of the receptor is different for agonists and antagonists. In agonist-bound receptors, helix 12 is packed tightly against the LBD, such that a binding surface is formed for short alpha helical peptide containing a leucine-rich motif of the general sequence LXXLL (L, leucine or isoleucine, x, any amino acid). This surface constitutes the activation function 2 (AF-2). A great number of cofactors bind to the AF-2 via LXXLL-type motives. In unliganded receptors, or receptors that are complexed to co-repressors, helix 12 is turned away from the LXXLL-binding surface, which allows the preferential binding of a similar leucine-rich motive present on the co-repressors NCoR1 or SMRT (Figure 1b and [12,11,19,20]). In addition to the well-characterised interaction of cofactors via leucine-rich motives in the LBD, some cofactors bind to the receptors by additional contacts in the AF-1 domain of the receptor [67,68], or by contacts with the hinge region or the DNA-binding domain [12,11,19,20,69]). *In vitro* assays that measure the binding of activating and repressing cofactors to NRs can, therefore, be successfully used to predict the activating or repressive effect of a ligand *in vivo*.

Selective modulators confer different receptor shapes leading to differential cofactor binding and ultimately to a tissue or gene selective transcriptional response

If differential recruitment of cofactors constitutes the molecular basis for the differential effects of agonistic and antagonistic ligands of NRs, could the cofactor interaction pattern (the 'cofactor profile') of NR–ligand complexes be used to explain, and perhaps predict, gene induction patterns or even *in vivo* pharmacology of SNuRMs? Supportive evidence for this idea comes from a study comparing the effects of estradiol to the SERMs tamoxifen and raloxifene, which showed that differences in selective recruitment of one particular cofactor, SRC1, directly translate into gene-selective and tissue-selective effects [21].

In general terms, the idea of cofactor profiles as an explanation of SNuRM effects is based on the following assumptions:

1. NR ligands induce ligand-specific receptor conformations.
2. This leads to ligand-specific interaction of the liganded receptor with cofactors.
3. Differences in the expression and post-translational modification of cofactors in different tissues lead to tissue-specific effects.
4. Differences in the promoter setup and cell type specific chromatin structure of individual genes are the basis for differential cofactor requirement for activation or repression of transcription. Since the ability of the ligand-bound receptor to recruit these cofactors is ligand-specific, this leads to gene-specific effects.

In the simplest representation of this idea, NR conformations can be ordered on a one-dimensional scale between the two extreme poles of full agonism and full antagonism. SNuRMs would be found at intermediate position, with intermediate effects on co-activators and co-repressors. The implication that the relative effect of SNuRMs depends on the ratio of co-activators and co-repressors in the cell has been confirmed in several reports [22–29]. However, the situation is likely to be more complex: Firstly, compounds can be found that induce the binding of co-activators to a comparable level but differ with respect to their recruitment of co-repressors [30,31]. Other types of NR compounds induce the release of co-repressors but do not promote binding of co-activators [32]. Finally, NR compounds can have gene-specific effects even within the same cell type, where the ratio of co-activators and co-repressors is a constant [33]. Lastly, examples of drug-like compounds are described in the literature that are selective in the recruitment of individual cofactor proteins over others without preferring a clear pattern of co-repressors versus co-activators [34–44]. In addition to the cofactor-mediated DNA and response element dependent or independent genomic actions of NRs, non-genomic activities have been described for NR ligands (e.g. for Estradiol and the ERs) promoting fast changes in the activity of kinase pathways (like Srck, PI3K, AKT, PKA, ERK1/2, STAT, MAPK) in a cell type dependent manner. Such non-genomic actions could explain some of the selective behaviour of certain ligands [72].

How can these concepts be reduced to practice in drug discovery? In an ideal situation, the cofactor recruitment 'fingerprint' of a given NR ligand should allow prediction of the selectivity of pharmacodynamic effects of this compound or – in other words – its therapeutic ratio in *in vivo* studies. However, *in vivo* pharmacology may add many additional layers of complexity to the behaviour of SNuRMs (see discussion of limitations at the end of this article) that might blur the prototypical SNuRM pattern that one wants to elaborate. So, we propose to establish a correlation between cofactor recruitment, gene expression and physiological cell culture assay patterns first, and then look for an extension of this model towards *in vivo* results.

As a first step, reliable and practical methods are needed to measure these interaction patterns. We will refer to all approaches used to compare the protein–protein interactions induced by the various SNuRMs as 'cofactor profiling' as opposed to gene expression or pharmacodynamic readout analysis.

Peptide-based cofactor profiling

A great number of co-activators have been shown to interact with nuclear receptors through a canonical 'LXXLL'-type motif [45,46], while co-repressors bind via a slightly longer variation of the motif (LXXI/HIXXXI/L) that accommodates the altered position of helix 12 on the receptors [47–49]. These motifs vary between cofactors,

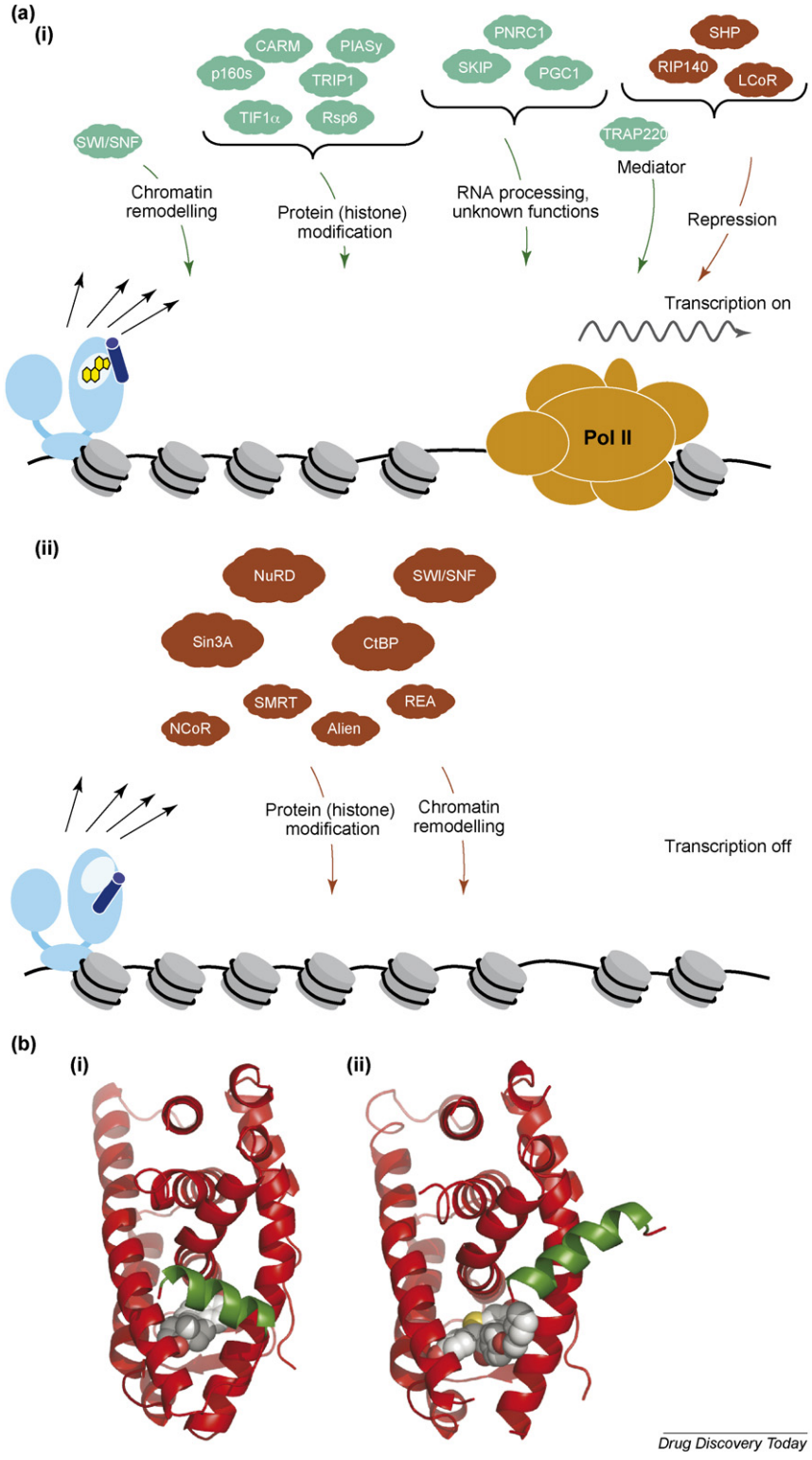

FIGURE 1

(a) The protein interaction repertoire of NRs in transcriptional regulation. Examples of proteins binding to NRs are given. (i) Agonist-dependent interactions, leading to activation of transcription. Upon binding of an agonist, the chromatin is changed to an activated state. This involves the ordered recruitment of a large number of cofactor proteins, including chromatin-remodelling complexes (such as the SWI/SNF complex), protein modifications, including protein acetyl transferases (exemplified by the p160s), protein arginine transferases (such as CARM1), kinases (such as TIF1alpha), E3-ligases for SUMO and ubiquitin (such as PIASy and Rsp6), proteasomal ATPases (such as TRIP1), proteins with functions in RNA processing such as PGC1, proteins that mediate the interaction with the basic transcriptional machinery, such as TRAP220, as well as proteins with largely unknown functions such as PNRC1. Agonists also lead to the recruitment of proteins such as RIP140, SHP and LCoR, which exert a repressive influence on transcription through associated enzymatic activities such as histone de-acetylases and histone methyl transferases. Many of these interactions involve LxxLL-type motifs on the co-activators and the activation function 2 region on the NR, depicted as the dark blue rod. (ii) In the absence of ligands, or in the presence of antagonists, the altered shape of the activation function 2 region leads to preferential interaction of NRs with the co-repressors NCoR and SMRT via an extended leucine-rich motif, and others such as Alien and REA. These co-repressor

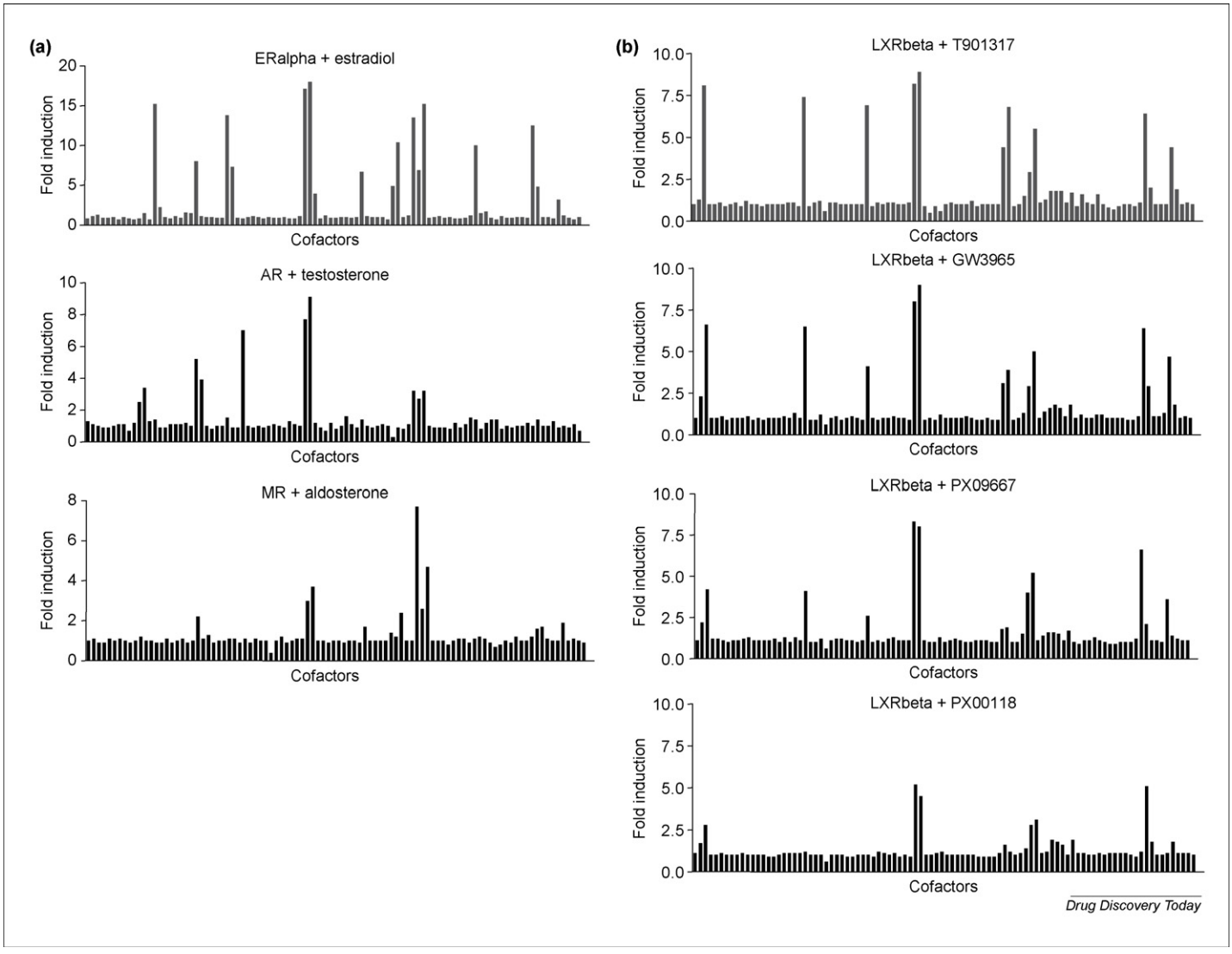

FIGURE 2

Cofactor profiling using the yeast two-hybrid system. (a) Cofactor profiles are nuclear receptor specific. Members of the steroid receptor superfamily estrogen receptor alpha (ERα), androgen receptor (AR, NR3C4) and mineralocorticoid receptor (MR, NR3C2) were tested for ligand-dependent cofactor recruitment. Each bar represents a nuclear receptor–cofactor interaction signal in the presence of the natural ligand of the respective receptor as fold induction over the non-ligand bound state. (b) Cofactor profiles are ligand specific. Four synthetic liver X receptor (LXRα and β, NR1H3 and NR1H2) ligands (T901317, GW3965, PX009667 and PX00118) induce differential cofactor recruitment patterns. Each bar represents an LXRbeta–cofactor interaction signal as fold induction of the liganded over the unliganded state.

both in the core sequence, as well as in amino-terminal and carboxy-terminal sequences surrounding the LXXLL interaction box [50]. Peptides representing these binding motifs for nuclear receptors have been used to identify differences in the cofactor interaction profile of nuclear receptors using standard biochemical binding assays, such as HTR-FRET [30,51,43], fluorescence polarisation [52] or bead-based multiplexed measurements coupled to flow cytometry [53,54]. In addition to peptides derived from natural NR-binding sequences, artificial peptides selected from phage display libraries that bind to ligand-free or ligand-bound NRs have been used in such experiments [55].

The advantages of these cell-free cofactor profiles are their high throughput and the fact that they are based on fully defined reaction conditions. This allows titration of the peptide in the binding reaction at fixed ligand concentrations, as opposed to titration of the ligand concentration done in usual dose response experiments [30]. The resulting EC_{50} value is a direct measure of the cofactor peptide's affinity in dependence of the ligand-induced

proteins recruit protein complexes of overlapping composition, such as the NuRD and Sin3A complexes, to induce transcriptional repression. In many cases, these complexes contain enzymes such as histone de-acetylases that counteract the activities of the co-activating complexes. Together, these activities lead to repression of transcription. (b) Full Agonists and SERMs differ in the relative position of the LBD's Helix 12. (i) Crystal structure of the human ER(-LBD in complex with 17-(-estradiol; (ii) ER(-LBD in complex with Raloxifene (data from pdb structures 1ERE and 1ERR after [12]). The natural (full agonist) ligand 17-(-estradiol induces a conformation that 'locks' the helix 12 (in green) in a position that results in the formation of a co-activator recruitment patch (termed AF-2), together with other structural elements of the LBD. Raloxifene, a selective ER modulator (SERM) protudes from the binding pocket towards the solvent and hence displaces the Helix 12 (in green) from its fully agonistic and co-activator recruiting position.

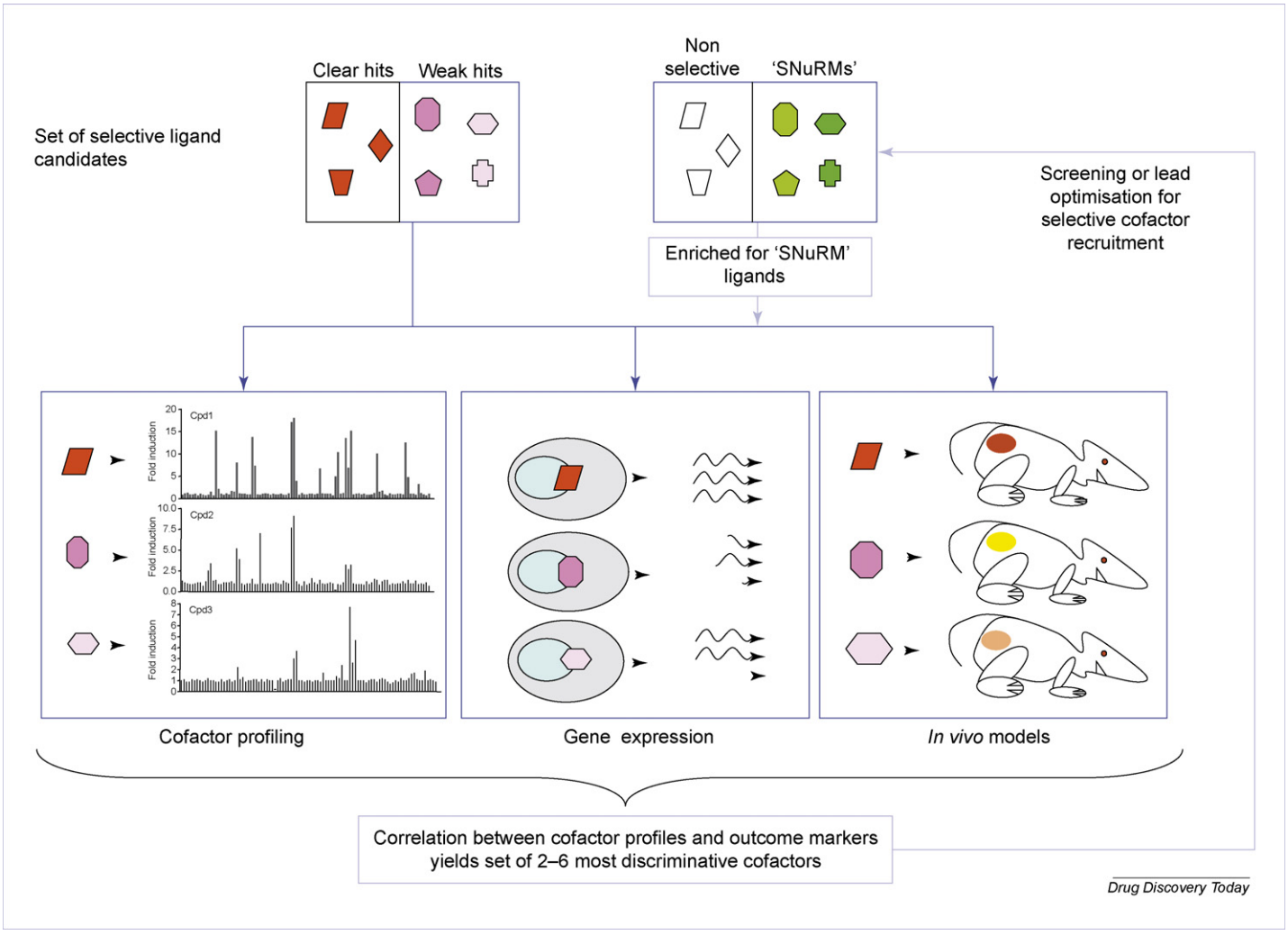

FIGURE 3

Assay path towards the identification of and panning for SNuRMs. Classical screening assays for nuclear receptors tend to yield potent and highly efficacious agonists. SNuRMs have a diminished efficiency compared with full agonists in cofactor recruitment based assays such as FRET or Alphascreen. Ligands for selectivity testing should encompass the full spectrum of full and partial agonists to encompass potential SNuRMs as well. The correlation of cofactor profiling, gene expression and animal model data results in the identification of 'predictive SNuRM markers' in the form of cofactors or peptides. Those cofactor(s) peptides can be used to tune an existing recruitment assay into a predictive SNuRM assay. Subsequent rounds of screening and lead optimisation should enrich for NR ligands with these specific SNuRM properties.

conformation and can thus be directly used to compare different sets of SNuRMs.

Limitations of peptide-based cofactor profiling are their reductionist conditions: Cell-free cofactor profiling methods using recombinant proteins have been limited, to date, to NR fragments, encompassing the ligand-binding domain only, and short peptides. Thus, the influence of receptor homodimerisation or heterodimerisation, its binding to DNA or to third cofactors cannot be tested. The impact of post-translational modifications of nuclear receptor, as well as cofactors, cannot easily be mimicked in such assays, which is a severe drawback, given the emerging evidence for their importance. Furthermore, the contribution of the AF-1, hinge and DNA binding domains to cofactor binding are neglected in peptide-based cofactor profiling since all of these peptides bind to the AF-2. This may be especially important for steroid receptors such as the estrogen receptor where the AF-1 domain is responsible for the agonistic activity of partial agonists in certain cell types [70,71].

Two-hybrid-based methods

To overcome the limitations of the peptide approach and look at NR–cofactor interaction patterns, using full-length proteins, yeast and mammalian two-hybrid methods is a useful approach. These methods use fusions of the two interacting proteins (i.e. the NR and the cofactor) to a sequence-specific DNA-binding domain, such as that of the yeast transcription factor GAL4, and to a transcriptional activation domain. Interaction of the two hybrid proteins generates a transcriptionally active protein complex at a GAL4-responsive promoter, which can be read out by conventional reporter genes [56]. An advantage of the use of yeast cells for this method is their lack of nuclear receptors, which alleviates some of the complications arising from competitive interactions with endogenous proteins in mammalian cells. In many cases, two-hybrid-based methods allow the use of full-length receptors. Such systems have been applied in our laboratory to look at interaction profiles of numerous receptors, including ERα and β (NR3A1, NR3A2), AR (NR3C4), MR (NR3C2), PR (NR3C3), LXRα

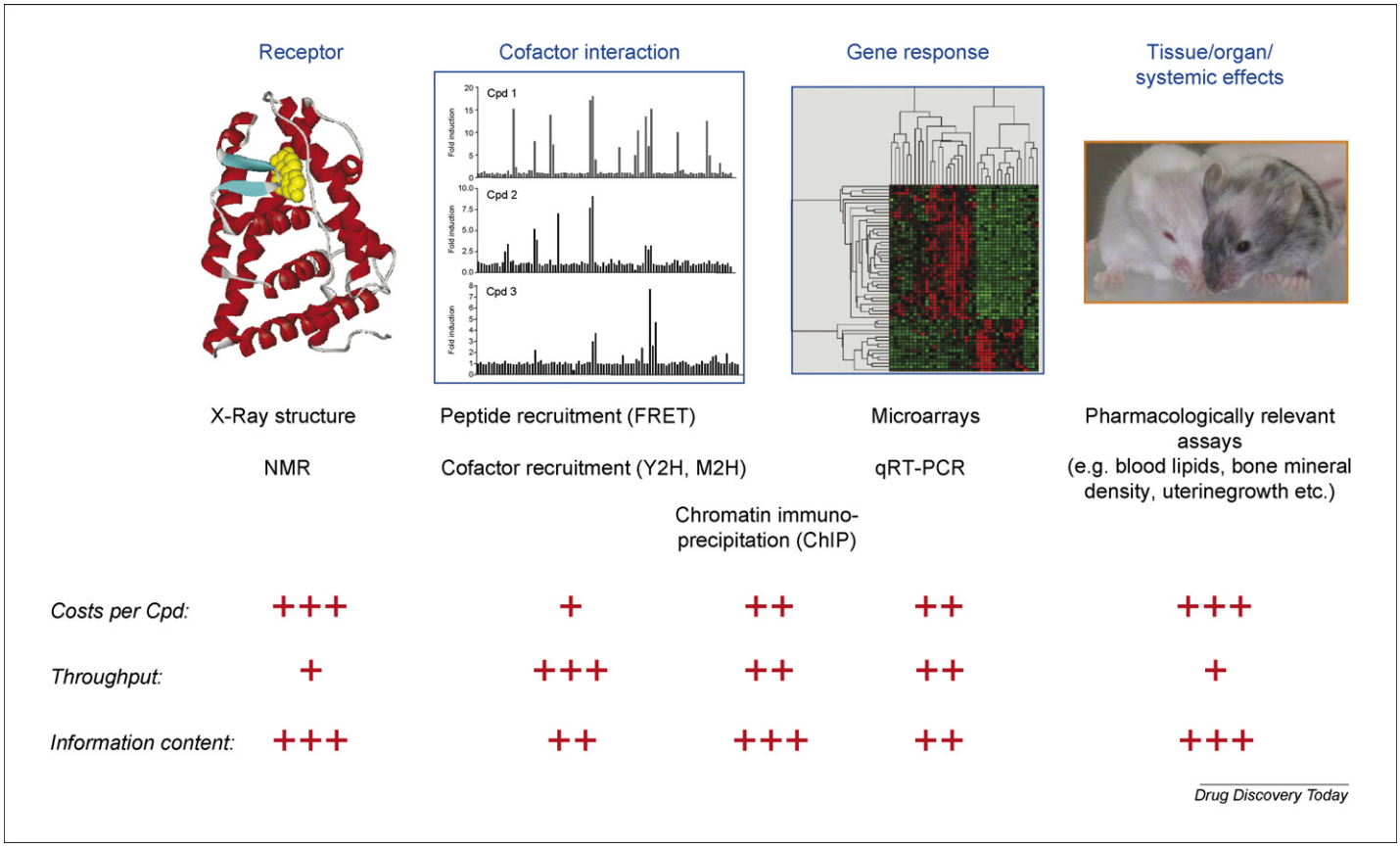

FIGURE 4

Different techniques to assess and analyse selective effects of SNuRMs. Techniques for the analysis of NR ligands in general and for the assessment of SNuRM-like activity range from protein crystallography up to animal studies. They differ in their informational content but high costs and low throughput, as well as ethical concerns, limit the extensive use of animal models. Animal models might be substituted by the concerted use of other 'indicative' techniques, such as cofactor profiling, chromatin IPs and gene expression analysis.

and β (NR1H3, NR1H2), PXR (NR1I2), VDR (NR1I1), PPARα, β/δ and γ (NR1C1-3), RXRα, β and γ (NR2B1-3) as well as RARα, β and γ (NR1B1-3) [30,57,58]. Like peptide-based interaction assays, the yeast two-hybrid system can be automated and performed at a scale of several thousand data points per day. This allows comparison of interactions for one given NR with hundreds of different cofactor constructs using dozens of SNuRM candidates in a reasonable timeframe at affordable costs. A key advantage over the peptide-profiling approach is that interaction constructs can be used that do not represent canonical cofactors with an LXXLL-containing NR-box, such as forkhead-like transcription factors, gelsolin or CaM Kinase II.

An unusual alternative to the use of cell-based two-hybrid methods is the application of protein microarrays with either prefabricated nuclear receptors or cofactors that can be probed by the respective other interaction partner in the presence of different ligands [59].

Chromatin immunoprecipitation

Since the conformation of an NR is influenced not only by ligand but also by the receptor's interaction with other proteins and with DNA, the ideal method for cofactor profiling will look at the interaction repertoire of the NR when bound to chromatin. Currently, the only method that can accomplish this is chromatin immunoprecipitation (ChIP). ChIP can determine the amount of cofactor bound to a specific NR-binding site on chromatin *in vivo*. ChIPs can be done in a quantitative manner [16], but one major limitation is the availability of antibodies against NRs and a broad panel of cofactors. Also, ChIP-based cofactor profiling requires a hypothesis as to which cofactors and which genes are relevant for ligand-specific differences.

Gene expression analysis

Differential cofactor profiles caused by SNuRMs are expected to translate into differential modulation of regulated genes, which, in turn, is the basis for the different physiological effects [60,61]. Many robust techniques are available for a cost-efficient and time-efficient determination of changes in mRNA expression patterns, such as microarray hybridisation or quantitative real time-PCR (qRT-PCR) of individual target mRNAs. When used for the classification of SNuRMs, both methods have the key disadvantage that one still needs to run a time course and dose response analysis. In the case of microarrays, this can become a very costly undertaking. In addition, it is difficult to determine whether changes in expression of relevant genes are due to direct regulation by the NR of interest of whether those are indirect effects.

Nevertheless, gene expression analysis is at least 'one step closer' to the observed pharmacological effect of a compound, since it already integrates the complex interplay between NRs, ligands, cofactors and other components for a given cell type or tissue.

Therefore, it is very conceivable to assess the selectivity of SNuRMs first in assays that are either predictive of the receptor's physiology, or, by expression analysis of genes, believed to be relevant for the beneficial and liability effects.

ChIP on Chip and combination with aforementioned techniques

This is an approach intended to overcome the limitations of both, gene expression profiling and individual promoter-specific ChIPs. In contrast to single ChIPs, ChIP on Chip does not use a single PCR primer set to specifically amplify a promoter sequence of interest, but it employs parallel amplification of promoter regions that were captured by cross-linking NRs or cofactors with the DNA sequences they were bound to at the moment of cell lysis and fixation. The resulting amplified fragments are probed against known promoter-containing genomic sequences, commonly termed 'tiling arrays', which can be made of DNA stretches from dozens to hundreds of basepairs long. Depending on the size and complexity of such an array it is possible to make a genome-wide scan for promoter occupancy with a certain NR or cofactor in dependence of ligands. A broad ChIP on Chip approach that would include systematic SNuRM comparisons on a dose response basis is certainly not a 'cheap' approach but can yield a powerful overview on the molecular actions of ligand-activated NRs. The combination of gene expression analysis and ChIP on Chip should yield the desired information about which genes in a given cell or tissue are directly regulated by a certain NR–ligand combination and which ones are indirectly regulated. In practice, promoter occupancy by a certain NR does not necessarily have an impact on gene transcription, and there are now examples known where, in direct comparisons between the two techniques, many more occupation sites than genes regulated have been found [62]. Combined with a systems biology view on the pathways involved, the combination of ChIP on Chip with gene expression analysis should ultimately allow scientists to establish the link between differential conformation of the NR involved to the differential gene expression evoked and the differential physiology or pharmacology it is translated into [63].

Assuming that the cofactor hypothesis is true and a significant part of SNuRM-selective pharmacodynamics is due to the different conformational changes they induce within the receptor, then we may ask: How can one identify those cofactor interactions or gene signatures that are the most relevant ones to describe the selective pharmacology of a certain type of SNuRM? Additionally, how can this be exploited for rational guidance of high-throughput screening and lead optimisation towards identification of selective NR modulators over classical agonists?

The existence of prototype compounds that exert the type of selective behaviour (or very similar to) that one would like to select is a major prerequisite for a rational assay path towards the discovery and optimisation of SNuRMs. For the identification of such prototype SNuRMs out of existing NR ligands one has to define the intended selective behaviour first. Selectivity should be represented by a ratio of simple endpoints for desirable and undesirable pharmacological effects that are thought to be indicative for actual *in vivo* selectivity. Such pragmatic endpoints are typically cell-based assays that monitor effects thought to be associated with clinical efficacy or side effects. Examples of these are breast or uterine cell proliferation assays that anticipate the mammary gland and uterine growth promoting effects of ER compounds. More recently, direct monitoring of gene expression from cell-based assays as predictors has also been utilised such as in the quest for selective PPARγ (NR1C3) modulators [64]. Here, differential expression of target genes such as aP2 is regarded as an 'undesirable' marker for adipocyte differentiation, and induction of Glut-4 is regarded as a marker for the beneficial insulin-sensitising effects.

In a very pragmatic and affordable approach, one can then compare and cluster different sets of modulators and non-selective ligands using one or more of the aforementioned cofactor profiling techniques. If the number of compounds, but not the cofactors or peptides, is limiting, a two-step route is conceivable: In a first experiment, a complete set of cofactors or peptides is tested with only few compounds at a limited number of concentrations. On the basis of these data, the cofactors that appear to be the most discriminative are selected for a subsequent detailed analysis of differences in recruitment of these selected cofactors. At first instance, this approach yields information by which the ligands tested can be 'binned' into groups of the different induced receptor conformations. This can already reduce the number of compounds that have to undergo extensive animal testing to the most effective and representative members of each compound 'conformational' class.

In order to tune screening assays such as FRET, Alpha screen or mammalian two-hybrid reporter assays towards selective modulators, one has to use recruitment of the most discriminative (cofactor) peptides as a readout in an 'informed' screen, as opposed to standard co-activators. The same set of 'SNuRM-indicator' cofactor (peptides) can also be used for guidance in subsequent rounds of lead optimisation. The use of such 'high content' or 'informed' assays should enrich for the type of SNuRM intended. If the desired compound properties are not achieved, it may either require further rounds of profiling and 'panning' for SNuRMs, or the chemical nature of the prototype SNuRM compound(s) makes it hard to be mimicked by other chemotypes.

Iannone has extended this approach towards a real broad systematic and statistical correlation analysis using 405 ER agonists and modulators and data from the interaction with 52 peptides as an input [53]. They provided a clear path for the application of statistical methods for clustering of SERMs by means of their cofactor profile. The principle is the same as with the analysis of data from gene expression profiling: Peptide interaction profiles were regarded as independent parameters to describe the similarity of compounds. Ligand-induced cofactor interactions were monitored by a flow cytometric multiplexed binding assay. This yielded one quantitative data point for each pair of compound–peptide interaction. A principal component analysis (PCA) was used to identify systematically those cofactors or peptides that were the most discriminative between these sets of ligands. The PCA yielded 10 peptides that were sufficient to account for the majority of differences between the compounds. The data from these 10 peptides were used in a hierarchical clustering that resulted in quantitative measures for plotting the similarity of individual compounds in a dendrogram. Finally, a correlation analysis between these similarity measures against proliferation data from Ishikawa cells treated with the ER ligands was employed to draw relationships between cofactor recruitment and pharmacological outcome patterns. In a continuation of this approach,

Hoekstra *et al*. [65] used these principles to 'filter' novel quinoline-type estrogen receptor ligands with regard to an appropriate cofactor pattern and came up with compounds of reduced Ishikawa cell proliferative activity. Similarly, Folkertsma *et al*. [51] clustered the natural ligand 9-*cis* retinoic acid and 10 further RXR compounds using cofactor peptide profiling and correlated these profiles to the predicted ligand-binding modes and AF-2 conformations of RXR.

Limitations of the approach and outlook on future

One major limitation to the prediction of SNuRM behaviour, based on prototypical cofactor recruitment 'fingerprints', is the fact that *in vivo* pharmacology is far different from a compound's behaviour in cell lines or biochemical assays. Immediate correlation between simple *in vitro* assays and pharmacological outcome *in vivo* might be hampered by the many complexities that may override the nuclear receptor's selective activation. Effects that can easily disturb the extension of *in vitro* selectivity towards animal models or humans are

- Differences in the pharmacokinetic properties of the compounds, in particular in tissue distribution.
- Metabolism of the parent drugs, leading to compounds with a different degree of agonism.
- Off-target activities, in particular if the compounds represent different chemotypes.
- And finally, indirect actions of nuclear receptors such as transrepression of other transcription factors, competition for interacting proteins or other non-genomic actions such as cross-talking to signal transduction pathways add a further layer of complexity that cannot be addressed by cofactor profiling. Nevertheless, when using mammalian two-hybrid techniques, gene expression analysis or ChIP on Chip, differential behaviour of SNuRMs – even with regard to non-transactivation effects – might be reflected in the outcome. It may just be even more difficult to pin down the molecular roots of their differential effects.

Besides the limitations that are given by these incalculable factors it should be noted that cofactor profiling as it was used so far regards differentially recruited cofactors or peptides as surrogate markers that have to be correlated to *in vivo* pharmacology or indicative cell-based assays. In other words, Determining cofactor fingerprints of SNuRMs means to gauge the SNuRM-induced receptor conformations and to correlate them to the pharmacological outcomes, leaving apart the molecular and cellular mechanisms in between.

It would require a different level of efforts to try to understand the 'real' cofactor dynamics and cellular pathways that are underlying the differential pharmacological effects of SNuRMs *in vivo*. The ChIP and ChIP on Chip techniques in conjunction with gene expression profiling might pave the way towards an understanding of the conformational, transcriptional and cell biological differences in different cell types or tissues that together form the basis for the pharmacologies observed.

From a pharmaceutical company's point of view it might be questionable whether such a fundamental approach really pays off. The surrogate marker approach as it was demonstrated by Iannone *et al*. and Hoekstra *et al*. is certainly a much more practicable route that already yielded an enrichment of desired properties of SNuRMs. This has to be confronted with the conservative approach that requires testing all candidates in relevant animal models without offering the opportunity to filter or pan for desired properties early on in the drug discovery process by, for example cofactor-biased screening assays. If the discovery and development of SNuRMs is a strategic direction of the company, a systematic approach that aims in understanding the specific mechanism of action of modulators or uses cofactors as surrogate markers at least will probably provide greater return on investment.

The use of cofactor profiling and the other techniques used to assess differential SNuRM effects will probably change from an accompanying approach to a driving force in the future when data from cofactor profiling, gene expression and *in vivo* pharmacology could become the basis for a growing SNuRM knowledge base that will allow more precise predictions of selective pharmacology of SNuRMs. In this respect the exciting efforts to establish a general open access Nuclear Receptor Signaling Atlas (see: www.nursa.org) that will certainly contribute to the achievement of this goal have to be mentioned. The exceptional potential of nuclear receptor drugs, which are currently limited by the high level of adverse effects, deserves further endeavours in this direction.

Disclosure

CK, MA and UD are employees of Phenex AG, a company that commercially offers cofactor profiling on a fee for service basis.

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