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Review Article

Reverse endocrinology as an approach to drug discovery

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Summary

This manuscript is intended to provide a summary of a relatively new approach to drug discovery that has been termed “reverse endocrinology”. Traditionally, hormones were identified on the basis of their physiological effects, *i.e.*, they were purified, characterized chemically and their physiological effects determined. Identification of their specific receptor(s) followed through ligand-receptor interaction studies utilizing radioactive ligands. Finally, the gene and its chromosomal location were characterized. In reverse endocrinology, molecular biological approaches permit the identification of orphan receptors first and, subsequently, their specific ligands. In this manner, new synthetic ligands can be tested in reporter gene activation assays to eventually open up possibilities and suggest selective ligands as new drugs in endocrine and related diseases.

Introduction and background to orphan receptors

A strategy to detect and eventually identify new orphan receptors is based on the ability of their mRNAs to hybridize to labeled-oligonucleotides that represent canonical sequences common to various members of the same superfamily. Thus, clones that expressed an orphan receptor eventually characterized as the human retinoic acid receptor (hRARα), were initially hybridized with a 24 nucleotide probe engineered as a consensus sequence for the DNA-binding domain (C-region) of the steroid-thyroid hormone receptor superfamily (1, 2). The next hurdle in this approach was to identify candidate ligands upon transcriptional activation of target genes after expression of the receptor in cultured cells. The main problem with this approach for RARs was that there were no known target genes whose promoter could be used for the reporter gene construct (1, 2).

The problem was solved by engineering chimeric receptor constructs as initially done by Green and Chambon for the human estrogen receptor (hER) (3). These authors replaced the DNA-binding domain (DBD) 66-amino-acid C-region of the hER with that of the human glucocorticoid receptor (hGR) to yield a chimeric ER containing the GR DBD, “cassette” (ER-GR.CAS). When coexpressed with reporter constructs containing a glucocorticoid response element (GRE) in target cells, the chimeric construct enabled the authors to measure estrogen responsiveness on a GRE built into the ER molecule. The sequence of steps can be summarized as follows: the ligand, estrogen, binds to the estrogen binding domain of the ER and this binding induces a conformational change that permits the subsequent binding of the exposed GR-derived DBD to the GRE on the reporter construct with consequent transcription of the reporter gene.

The use of chimeric receptors has provided the key to the identification of the nuclear receptors for retinoic acid (RARs). Since no targets were known for hRARα, the DBD of the ER was inserted in the candidate orphan receptor, hRARα, in place of the orphan receptor DBD sequence, yielding hRAR-ER.CAS. The ability of the chimeric receptor to activate gene expression was then tested using the vitellogenin thymidine kinase-cholesterol, acetyltransferase (vit-tk-CAT) reporter gene cotransfected with the chimeric receptor construct in HeLa cells. Petkovich *et al.* (1) found that CAT activity depended on the presence of retinoic acid (10^{-8} M) but

was unaffected by estradiol or retinol at concentrations of 10^{-8} M. This approach, therefore, waved the necessity to use downstream retinoic acid-responsive genes and utilized an ER DBD and its response element to identify the orphan receptor as a RARα (1).

Competition binding assays in lysed HeLa cells after transfection with the hRARα cDNA also showed specificity of binding for retinoic acid. Similar results were obtained by Giguere *et al.* (2) in a similar independent approach, thereby establishing that a nuclear receptor RARα exists for retinoic acid.

The ensemble of these techniques has been called "reverse endocrinology" (4) because the assignment of the receptor to its ligand occurs after the cloning of the receptor itself. This is obviously the reverse of the traditional approach of first characterizing the ligand and then the receptor through biochemical binding studies and final relevant studies in physiology, *etc*.

Thanks to these molecular engineering approaches, it has been possible to identify orphan receptor ligands and to develop new synthetic and receptor-selective compounds (5, 6). These have been summarized elsewhere for the retinoids (7-12).

The RXR ligand Targretin as a chemopreventive agent against breast cancer

As a promising prototype drug for this category of compounds, we will discuss Targretin, a synthetic rexinoid receptor (RXR)-selective ligand also called LGD-1069 (Table I). Targretin binds tightly to the 3 RXRs (α, β, γ), but has little affinity for the RARs and does not transactivate retinoic acid-responsive genes (13, 14). Because 9-*cis* retinoic acid (agonist for all 6 retinoid receptors, *i.e.*, for both RARs and RXRs) (Table I) prevented mammary carcinogenesis (15, 16), Targretin was tested and shown to prevent nitrosomethylurea (NMU)-induced mammary carcinogenesis in rats, interestingly, without the classic pattern of retinoid toxicity (*e.g.*, hypertriglyceridemia, mucocutaneous toxicity and headaches) observed with other retinoids (13). Additionally, the maximum inhibition in tumor incidence and multiplicity with Targretin was similar to that achieved with tamoxifen (13). Although Targretin was not shown to alter estrogen, progesterone or prolactin levels in this model, it did inhibit both estrogen- and tamoxifen-induced growth of the uterus (13).

The mechanism responsible for the inhibition of NMU-induced tumors by Targretin is unknown. Gottardis *et al.* (13) hypothesized that Targretin may suppress the formation of breast tumors by several possible mechanisms which may involve a direct activation of RXR-specific pathways or an effect on the endogenous retinoids that activate the RARs. Targretin also induced complete regression in 72% of NMU-induced rat primary tumors (17). Tamoxifen, at the highest dose studied, completely inhibited growth in only one-third of tumors. A moderately effective dose of Targretin (10 mg/kg) in combination with a low dose of tamoxifen (150 μg/kg) induced tumor regression in 26% of the tumors (17). Apparently, this finding provided greater than additive efficacy compared to similar doses of either tamoxifen (5.6%) or Targretin (10.5%) alone.

Retinoid receptors and their synthetic and natural ligands (covered elsewhere) and the most recent ligands characterized for orphan receptor binding are shown in Table I. They include ligands of the peroxisome proliferator-activated receptors (PPARα, βδ and γ), the ROR-RXRs, and the pregnane X (PXR), liver X (LXR), constitutive androstane (CAR) and farnesoid X (FXR) receptors.

PPARα ligands as anticholesterol and PPARγ ligands as antidiabetes drugs

PPARs are fundamentally involved in the regulation of energy balance (4). Though this family of receptors comprises at least 3 members, only ligands for PPARα and PPARγ are known. Chemicals that increase the number of peroxisomes when given to rodents are typical ligands for PPARα (18, 19) and because of this, the family received its name. Liver, kidney, heart and muscle express PPARα while PPARγ is mostly expressed in fat cells, the large intestine and monocytes. PPARβδ has an ubiquitous pattern of expression (20).

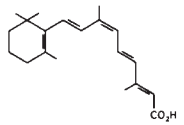
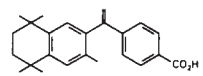
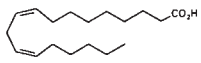
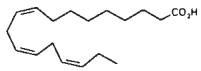
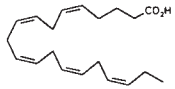
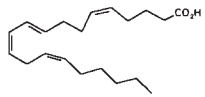
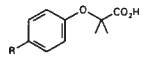
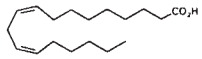
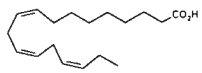
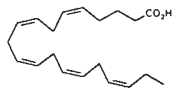
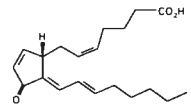
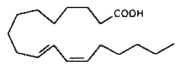
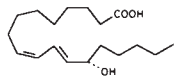
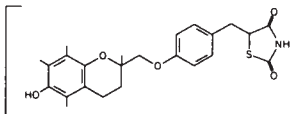
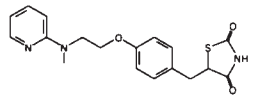
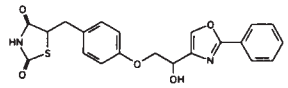
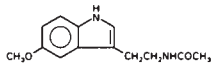
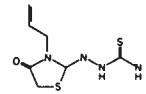
Panagonists for these receptors are the linoleic, linolenic and eicosapentaenoic acids (18, 21-27) (Table I). However, selective synthetic ligands are important as prodrugs and some of them are already utilized as drugs. The fibrates (Table I) are ligands for PPARα (18-20) and powerful agents for lowering blood triglycerides, one of the important risk factors for coronary heart disease (19). Fibrates binding to PPARα regulate the release of fatty acids from triglycerides and their metabolism as well as that of lipoproteins (20). PPARα deficiency in rodents leads to obesity resulting from inadequate metabolism of triglycerides and cholesterol (4).

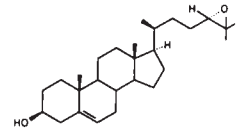
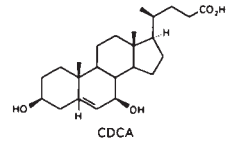
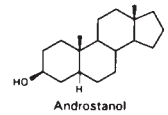
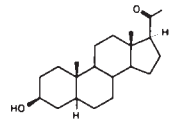
The PPARγ orphan receptor has been found to be the molecular target for the antidiabetic drugs thiazolidinediones (TZDs). This family comprises the drug troglitazone which is now used in the clinic (28) (Table I). Effects of the TZDs include lowering blood glucose levels and decreasing circulatory fatty acids. This receptor is also highly expressed in adipocytes (29) which also depend on it for proper differentiation (30), thereby providing a connection between the differentiation of fat cells and the control of blood glucose levels by the same receptors.

PPARγ ligands in arteriosclerosis

Very recent studies have made important progress in the understanding of atherogenesis and the involvement of the orphan receptor PPARγ in this process. The arterial wall is covered with the initial lesion called fatty streak made up of cholesterol-laden macrophages or the so-called "foam cells". Brown and Goldstein have

Table I: Receptors and ligands identified by reverse endocrinology.

RECEPTORS	LIGANDS: ENDOGENOUS — PAN AGONISTS	LIGANDS: ENDOGENOUS — SELECTIVES	SYNTHETIC DRUGS
RXR	 9-*cis* RA		Anti-Cancer:  LGD1069
PPARα	 Linoleic Acid;  Linolenic Acid;  Eicosapentaenoic Acid	 8(S)-HETE	Anti-Cholesterol:  Fibrates
PPARγ	 Linoleic Acid;  Linolenic Acid;  Eicosapentaenoic Acid	 15d-PGJ$_2$;  9(S) HODE;  13(S) HODE	Anti-Diabetes/Anti-Lipemic:  CS-045 Troglitazone;  BRL 49653;  AD-5075
RZR/RORα	 Melatonin		Anti-Arthritic:  CGP 52608

RECEPTORS	ENDOGENOUS LIGANDS	SYNTHETIC DRUGS
LXR	 24(S), 25-Epoxycholesterol	
FXR	 CDCA	
CAR	 Androstanol	
PXR	 5β-Pregnane-3, 20-dione	Xenobiotics, Stimulators of CYP3A4

established that "scavenger receptors" are responsible for the uptake of cholesterol-rich, low-density-lipoproteins (LDLs) (31, 32). However, LDLs are taken up only after they have undergone a series of chemical and/or enzymatic modifications which form oxidized LDLs or ox-LDL (33, 34). Apparently, these modifications can be carried out in every and each arterial cell type including endothelial cells, smooth muscle cells or macrophage cells (34). The formation of fatty streaks is in part the result of the recruiting action of ox-LDL on monocytes and on the ability of ox-LDL to reduce motility of resident macrophages. Ox-LDLs also transcriptionally activate gene expression of several cytokines, including TNFα, IL-1α, IL-1β, IL-6 and PDGF.

Since PPARγ had been linked to adipocyte differentiation (30, 35) and the control of cellular lipid uptake, Tontonoz *et al.* (36) investigated PPARγ expression and found it to be high in foam cells of atherosclerotic lesions. In fact, the authors observed induction of PPARγ expression in monocytes exposed to ox-LDL. They also showed that ox-LDL induced monocytic differentiation in myelomonocytic cells. The ox-LDL induced its own uptake through increased transcription of the cell surface scavenger receptor CD36. These studies suggested that PPARγ ligands are important regulators of atherogenesis.

In an accompanying paper (27), the same group of investigators reported the identification of two of the oxidized fatty acid components of ox-LDL as 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE) (Table I) as potent endogenous ligands and activators of PPARγ. Therefore, these reports have defined a signaling pathway of apparent major importance in the atherogenic response. The resulting circuit is switched on by ox-LDLs, which are then internalized by the CD36 cell surface receptor. Once internalized, ox-LDLs liberate 9-HODE and 13-HODE from their inactive cholesterol esters. 9-HODE and 13-HODE then activate PPARγ, which, in turn, increases transcription of the CD36 cell surface receptor for ox-LDL. This vicious cycle is suggested to be, at least in part, responsible for the atherogenic process, since it occurs simultaneously with macrophage differentiation (27). Monocytes are the important players in this cycle of events. The ox-LDL uptake process has at least two important actions: on the one hand, it induces differentiation of monocytes in macrophages, while on the other, it induces the synthesis of the cell surface scavenger receptor CD36. Both actions are apparently the result of PPARγ activation by 9-HODE and 13-HODE.

RZR/RORα ligands as antiarthritis drugs

In research aimed at prevention and/or therapy of rheumatoid arthritis, a rat adjuvant arthritis model is utilized because it is a chronic T-cell-dependent autoimmune disease similar to rheumatoid arthritis. Using this model, Missbach *et al.* (37) were able to identify a class of TDZs as highly potent antiinflammatory agents and inhibitors of the destruction of joints. CGP-52608 (Table I) was found to be the lead compound for this new class of TDZs with antiarthritic activity at concentrations as low as 0.01 mg/kg. The authors then searched for a molecular target and discovered that CGP-52608 specifically bound and activated the orphan receptor retinoid Z receptor/retinoid acid receptor-related orphan receptorα, RZR/RORα. The same group had suggested that melatonin is the endogenous ligand for this receptor (38). A recent paper has described the interesting finding that two structurally different TDZs, BRL-49653 and CGP-52608, specifically activate PPARγ and RZR/RORα, respectively (Table I), resulting in different pharmacological effects consistent with the different functions of the receptors (39). BRL-49653 (Table I) specifically inhibited leptin production in differentiated adipocytes, antagonized weight loss and blood glucose and triglycerides in insulin-resistant rats. In contrast, and with remarkable specificity, CGP-52608 exhibited potent antiarthritic activity in a rat adjuvant antiarthritis model; it also had steroid-like effects on triglyceride levels and on body weight in insulin-resistant rats. The authors suggest that both compounds may be prototype drugs for novel therapeutic agents against noninsulin-dependent diabetes mellitus and rheumatoid arthritis.

Orphan receptors and their natural ligands

For a recent discussion of these receptors, the reader is referred to recent excellent reviews (4, 40). Here, we highlight a few major characteristics that make them important for future insights into drug development.

Liver X receptor

The liver X receptor (LXR) is abundantly expressed in liver tissue (41). Like the other orphan receptors discussed here, it also forms heterodimers with RXR thereby controlling the expression of the cytochrome P450 (CYP)7a gene via binding to its promoter (42). This cytochrome is the rate-limiting key enzyme for the conversion of cholesterol to bile acids and disposes of excess cholesterol. Interestingly, oxisterols, such as 24(*S*),25-epoxycholesterol and 24(*S*)-hydroxycholesterol (42-44), rather than cholesterol itself, are the physiological LXR ligands (Table I). LXRα-deficient mice accumulate excessive amounts of cholesterol esters in their livers, eventually resulting in impaired liver function when fed diets high in cholesterol (45).

LXR also forms heterodimers with RXR and induces transcription of a key drug metabolizing monooxygenase, CYP3A4, upon ligand binding and interaction with the xenobiotic response element (XRE) in the promoter of this gene (43).

Pregnane X receptor

Interestingly, ligands for pregnane X receptor (PXR) are the same drugs that stimulate CYP3A4 gene transcription (46). PXR is expressed mainly in liver and

intestine. Drug interactions can be predicted on the basis of reporter construct activation with the cotransfected PXR and the XRE on CYP3A4 controlling the reporter gene. Natural endogenous ligands for PXR are the pregnanes, *e.g.*, the progesterone metabolite 5β-pregnane-3,20-dione, among others (47-50).

Constitutive androstane receptor

The constitutive androstane receptor (CAR) is highly expressed in liver tissue (51) and apparently regulates the transcription of the steroid hydroxylase CYP2B gene (52). It binds testosterone metabolites with inhibitory rather than stimulatory effects (53) with high constitutive transcriptional activity in the absence of a known ligand (4, 51). This inhibitory activity may be the result of an antagonistic action of the androstane against an unknown endogenous ligand produced by the cells (4). However, since hormone binding also causes the dissociation of the activator protein complexes from CAR, it is likely that CAR is constitutively activated in the absence of a physiological ligand and that hormone binding simply changes the active, in this case unliganded, conformation to an inactive structure which also is incompatible with activator protein complex formation (4).

Farnesoid X receptor

Farnesoid X receptor (FXR) was so named because rat FXR was weakly activated by the C-15-product (farnesol) of the mevalonate pathway responsible for steroid and polyisoprenoid biosynthesis (54). However, neither the mouse nor human orthologs were activated by farnesol (55). Instead, a natural ligand for this orphan receptor has been characterized in chenodeoxycholic acid (CDCA) (Table I) (40, 56, 57). This binding regulates several genes involved in bile acid homeostasis. Since FXR is expressed in tissues that synthesize bile acids such as the liver, intestine and kidney, (40, 58), FXR may indeed function as a nuclear receptor for bile acids.

Conclusions

This brief review has highlighted the importance of reverse endocrinology (4) for the discovery of useful drugs. Some of the listed synthetic ligands such as the PPARγ-selective antidiabetic TDZ, troglitazone, are presently used in the clinic. The RXR-selective ligand, Targretin, and the PPARγ-selective TDZ, CGP-52608, offer hope for the control of diseases as life-threatening and pervasive as breast cancer and arthritis. The knowledge that fatty acid derivatives such as 9-HODE and 13-HODE are endogenous ligands for PPARγ and activators of macrophage differentiation and monocyte signal transduction, may eventually suggest novel approaches to fight arterial disease. Moreover, for other receptors such as LXR, FXR and CAR, the opportunity is there for synthetic ligands yet to be discovered but likely to impact diseases of drug metabolism deficiency and others. Finally, a practical application of the basic research in the PXR family is that drug interactions can be studied in the lab using this receptor coexpressed with a reporter gene under the control of the CYP3A4 promoter to study the pattern of drug interactions in screening approaches to drug development.

All this is the result of basic research approaches and the phenomenal advancements in discovery of novel orphan receptors and their ligands. From this brief review, it is obvious that, once again, basic approaches to biological problems have offered insight into medical applications for the development of new drugs.

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